# Albumin Nanospheres as Carriers for Passive Drug Targeting: An Optimized Manufacturing Technique

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***Purpose.*** The purpose of this study was to develop a new method to produce albumin particles in the sub-200-nanometer range with a narrow size distribution and in a controlled and reproducible manner.

***Methods.*** A new emulsion crosslinking method was developed using ultrasound and static mixing as homogenization steps and a central composite design was used to evaluate the influence of different process parameters on particle size, polydispersity and yield.

***Results.*** Response surface analysis allowed the location of the most important factors. Of all the factors investigated, only the albumin concentration and the aqueous phase volume showed a significant influence on response parameters. Albumin nanospheres with sizes below 200 nm in diameter and very narrow size distributions were obtained in high yields (>80%).

***Conclusions.*** This study describes a new preparation method for albumin nanoparticles which are suitable for future drug targeting studies.

KEY WORDS: albumin; nanospheres; manufacturing; glutaraldehyde; cross-linking; central composite design.

## INTRODUCTION

Since the first reports on the preparation of uniformly sized albumin microspheres in the early 70's, these biodegradable, biocompatible particles have found various applications. Initially conceived as diagnostic tool, albumin particles have been utilized as drug carrier systems (1). More than 100 therapeutic or diagnostic agents were incorporated into albumin microspheres and not only intravenous but also intraarterial, intramuscular and intraarticular administrations have been investigated. Albumin microspheres are well suited for drug targeting and drug delivery due to their biodegradability, lack of toxicity and antigenicity. Compared to other colloidal carrier systems, such as liposomes, their stability and shelf-life, controllable drug-release properties, and a higher loading capacity for hydrophilic molecules due to drug-binding properties of native albumin are clear advantages.

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With the development of genetically engineered proteins, such as interferons, interleukins and colony stimulating factors, the need for site-specific drug carriers or targeting systems increased considerably, because the new therapeutic agents tend to cause severe adverse drug reactions after systemic application. It would be clearly desirable to deliver these agents in a site-specific manner to target tissues, where they exert their beneficial therapeutic effects. Therefore, targeting of these proteins to the hematopoietic tissues such as the bone marrow, is an attractive proposition.

The biodistribution of intravenously injected carriers is mainly influenced by their physicochemical properties, e.g., particle size and surface characteristics (2). Larger particles are more rapidly removed by the liver and spleen than smaller particles. Reducing the particle size of colloidal carriers below a threshold of 100 to 200 nm introduces the possibility of escaping the vascular system via fenestrations or cavities in the lining of blood vessels (3). Recent studies using albumin nanospheres with poly(ethylene)glycol covalently attached to their surface resulted in a reduced phagocytic uptake under *in vitro* conditions and showed a promising tendency to overcome one of the major obstacles against site-specific drug delivery (4).

Albumin particles can be obtained by different methods (5). In this study a method based on emulsion crosslinking was used since with it water-soluble therapeutic or diagnostic agents can be efficiently incorporated into albumin microspheres. However albumin particles below 500 nm are difficult to obtain by the emulsion crosslinking methods (6). Although it has been demonstrated, that many process variables can influence particle size and size distribution, no comprehensive studies investigating the relevant factors for the preparation of albumin nanospheres are available. Mostly series of single-factor experiments were reported, lacking information on the potential interaction of two or more process variables. In the present study, a central composite design has been employed to evaluate the combined effect of five important process variables on particle size, polydispersity and yield, to optimize the preparation technique of sub-200 nm particles.

## MATERIALS AND METHODS

### Chemicals

Bovine serum albumin, aqueous glutaraldehyde solution (50% w/w), methylene chloride, methanol and acetone were purchased from Fluka AG (Buchs, CH). Fluorescein Isothiocyanat-Dextran (Mr = 40000) was obtained from Sigma AG (Buchs, CH) and Hydroxypropyl cellulose type Klucel GF® (Mr = 370000) was purchased from Aqualon Company (Wilmington, Delaware, USA).

### Equipment

A new apparatus was constructed for the preparation of albumin nanospheres on a laboratory scale, allowing however transfer to larger scales. The experimental set-up consisted of a double-walled thermostated reaction chamber, connected to a refrigerating thermostat (Frigomix 1495,

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Braun AG, Melsungen, Germany) as shown schematically in Fig. 1. A glass tubing with an integrated peristaltic pump allowed the emulsion to circulate during homogenization through 10 joined pieces of a static mixing device (SMXE DN4, Sulzer AG, Winterthur, Switzerland) at a rate of 500 ml/min. The aqueous albumin solution was added with a syringe to the circulating organic phase. Homogenization of the O/W-emulsion was carried out using a microtip sonic horn (Branson Sonifier Cell Disruptor B-30, Branson Sonic Power, U.S.A.). The emulsion was homogenized in a pulsed mode (0.5 s in 1 s period) with 65 W energy output at 20 kHz.

### Preparation of Microspheres

1.3 g of bovine serum albumin and 65 mg FITC-Dextran were dissolved in 6.5 ml distilled water. The solution was injected with a syringe into an organic polymer solution, consisting of 0.5% (w/v) hydroxypropyl-cellulose in methylene chloride/methanol (9:1), circulating in the previously described apparatus. The total volume of the external, organic phase was 93.5 ml. The emulsion was sonicated for 15 minutes, maintaining the temperature constant at 22° ± 0.5°C. After 15 min the final emulsion was transferred to a glass beaker equipped with a 4-bladed impeller and the cross-linking was initiated by adding glutaraldehyde saturated methylene chloride (6.6 mmol). The suspension was stirred for 100 min (2200 rpm) at room temperature. After dilution with 100 ml methanol the particles were isolated by centrifugation (47800 g, 30 min, 10°C). The pellet was washed several times with methanol and finally with n-hexane. The resulting nanospheres were obtained as yellow-brownish powder, dried under vacuum at room temperature for 24 h and stored in a desiccator at 4°C.

### Characterization

Electronmicrographs were taken with a scanning electron microscope (Stereoscan 180, Cambridge Instruments, GB- Cambridge) or a Field Emission electron microscope (JEOL 6300F, Kontron Electronics, D-München).

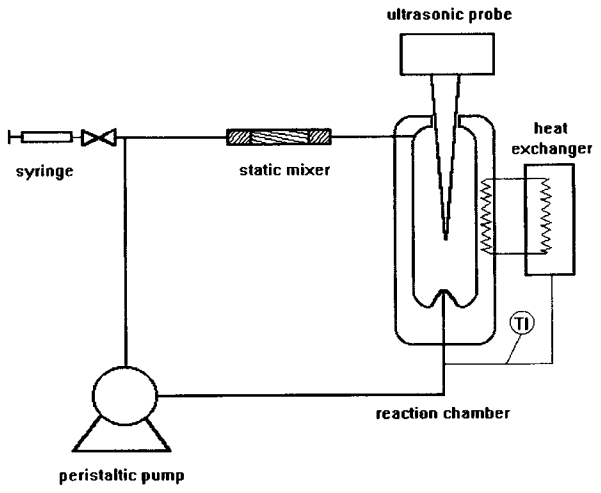


Fig. 1.

*Particle Size and Polydispersity Index*

The particle sizes of albumin nanoparticles were determined by photon correlation spectroscopy using a Malvern Zetasizer III (Malvern Instruments, UK) equipped with a helium-neon laser operating at 633 nm and 5 mW power output. The nanospheres were suspended in distilled water, briefly sonicated and measured at 20°C at a scattering angle of 90°C.

All measurements were carried out quadruplicate. Sizing results were analyzed by the method of cumulants using the application software supplied by Malvern Instruments. Particle sizes are z-average mean diameters and the polydispersity index (PI) is a measure of the width of the size distribution (7).

*Yield and Drug Loading*

Yield was calculated as the weight of the dried microspheres recovered from each batch in relation to the sum of the starting material. To separate free from encapsulated dextran, the particles were washed several times by resuspending them in water and subsequent centrifugation at 47800g, 30 min, 10°C. The amount of FITC-dextran incorporated into microspheres was determined by the method of Tomlinson and Burger (8).

### Data Analysis

A five-factor orthogonal central composite design with a confounded interaction E = ABCD was chosen. The center point was replicated a total of three times resulting in $(2^{5-1} + (2*5+1)+2)$ observations and an $\alpha$ of 1.664 (9). The evaluated factors and their respective levels in the central composite design are shown in Table Ia). Based on the observed data points, polynomial equations of the form $y = b_0 + b_1x_1 + b_2x_2 + \ldots + b_5x_5 + b_{11}x_1^2 + \ldots + b_{55}x_5^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + \ldots b_{45}x_4x_5$ were generated to establish the correlation between the independent variables (i.e. albumin concentration, aqueous phase volume, duration of emulsifi-

Table I. a) Factors and Levels Investigated in the Preparation of Albumin Nanospheres and b) Maximum $Y^{\Delta}$ and Minimum $Y_{\Delta}$ Acceptable Values for the Response Variables

| | Level | | | | |
|---|---|---|---|---|---|
| Factor | −1.664 | −1 | 0 | +1 | −1.664 |
| a) | | | | | |
| Albumin (% w/v) | 5 | 11 | 20 | 29 | 35 |
| Aqueous phase volume ratio (% v/v) | 1.3 | 3.4 | 6.5 | 9.6 | 11.7 |
| Duration of emulsification (min) | 5 | 9 | 15 | 21 | 25 |
| Glutaraldehyde (mmol) | 0.2 | 2.8 | 6.6 | 10.5 | 13 |
| Drug (mg) | 0 | 10 | 25 | 40 | 50 |
| b) | | | | | |
| Response variables | $Y^{\Delta}$ | $Y_{\Delta}$ | | | |
| Particle size (nm) | 500 | 0 | | | |
| Polydispersity index | 0.2 | 0 | | | |
| Yield (% w/w) | 100 | 50 | | | |

cation, glutaraldehyde concentration, amount of drug) and the dependent variables (i.e. particle size, polydispersity index, yield, overall desirability function). Multiple regression procedure was used to determine the model parameters for the dependent variables and analysis of variance was applied to perform the test of fit and to determine which effects are significant (10,11). Using a desirability function several responses can be evaluated simultaneously (12). Assignment of desirability function values from zero to one simplifies comparisons between experiments. A desirability value $d_i$ for each dependent variable was obtained as described using the following relationship (12):

$$d_i = \frac{Y_i - Y_\Delta}{Y^\Delta - Y_\Delta} \text{ if } Y_\Delta \leq Y_i \leq Y^\Delta$$

and

$$d_i = 0 \text{ if } Y_i \leq Y_\Delta$$

$$d_i = 1 \text{ if } Y_i \geq Y^\Delta$$

$Y_i$ = value of dependent variable

$Y_\Delta$ = minimum acceptable value of $Y_i$

$Y^\Delta$ = maximum acceptable value of $Y_i$

For variables that are desired to be minimized such as particle size and polydispersity index, $d_i$ can be calculated as:

$$d_{i\,min} = \frac{Y^\Delta - Y_i}{Y^\Delta - Y_\Delta}$$

Table Ib) shows maximum and minimum acceptable values for the observed variables.

An overall desirability function DF was calculated to determine the optimum conditions for the preparation of the albumin nanospheres. DF is the geometric mean of different desirability values according to the following equation:

$$DF = \left[\prod_{i=1}^{n} di\right]^{/n}$$

The optimum formulation conditions are represented by a combination of different levels of independent variables which generate highest values of DF. Response surface methodology was used to correlate responses to the values of the studied factors. Surface response graphs were then generated for the responses as functions of the significantly contributing factors as determined by regression analysis.

## RESULTS

Table II summarizes the observed values obtained from the 29 batches prepared according to the central composite experimental design. In every batch spherical particles were

**Table II.** Values of the Response Variables Following the Preparation of Albumin Nanospheres

| Level | Yield (% w/w) | Particle size (nm) | Polydispersity index | Overall desirability factor |
|---|---|---|---|---|
| −1 −1 −1 −1 +1 | 78 | 222 | 0.077 | 0.45 |
| +1 −1 −1 −1 −1 | 78 | 187 | 0.083 | 0.5 |
| −1 +1 −1 −1 −1 | 12 | 1186 | 0.586 | 0 |
| +1 +1 −1 −1 +1 | 89 | 286 | 0.274 | 0 |
| −1 −1 +1 −1 −1 | 83 | 323 | 0.232 | 0 |
| +1 −1 +1 −1 +1 | 80 | 182 | 0.112 | 0.47 |
| −1 +1 +1 −1 +1 | 8 | 1106 | 0.884 | 0 |
| +1 +1 +1 +1 −1 | 93 | 321 | 0.313 | 0 |
| −1 −1 −1 +1 −1 | 91 | 277 | 0.178 | 0.3 |
| +1 −1 −1 +1 +1 | 69 | 263 | 0.273 | 0 |
| −1 +1 −1 +1 +1 | 15 | 1781 | 0.878 | 0 |
| +1 +1 −1 +1 −1 | 81 | 296 | 0.294 | 0 |
| −1 −1 +1 +1 +1 | 89 | 324 | 0.267 | 0 |
| +1 −1 +1 +1 −1 | 70 | 197 | 0.11 | 0.38 |
| −1 +1 +1 +1 −1 | 75 | 228 | 0.089 | 0.43 |
| +1 +1 +1 +1 +1 | 11 | 708 | 0.664 | 0 |
| −α 0 0 0 0 | 90 | 330 | 0.35 | 0 |
| +α 0 0 0 0 | 93 | 372 | 0.394 | 0 |
| 0 −α 0 0 0 | 76 | 162 | 0.107 | 0.45 |
| 0 +α 0 0 0 | 7 | 1486 | 0.718 | 0 |
| 0 0 −α 0 0 | 83 | 2024 | 0.498 | 0 |
| 0 0 +α 0 0 | 99 | 381 | 0.394 | 0 |
| 0 0 0 −α 0 | 68 | 234 | 0.093 | 0.36 |
| 0 0 0 +α 0 | 81 | 215 | 0.039 | 0.56 |
| 0 0 0 0 −α | 73 | 220 | 0.126 | 0.37 |
| 0 0 0 0 +α | 84 | 220 | 0.093 | 0.5 |
| 0 0 0 0 0 | 97 | 218 | 0.101 | 0.56 |
| 0 0 0 0 0 | 82 | 221 | 0.073 | 0.52 |
| 0 0 0 0 0 | 74 | 217 | 0.083 | 0.44 |

obtained with a particle size ranging from 180 to 2020 nm and a polydispersity index varying from 0.04 to 0.88. Total nanosphere yields ranged from 7 to 97% (w/w).

At the center point of the experimental design four batches of albumin nanospheres were prepared to verify the reproducibility of the manufacturing procedure. The results are shown in Table III, demonstrating, that excellent reproducibility is achieved with this method, yielding ca 200 nm particles with narrow polydispersity. The albumin nanoparticles were spherical in shape and showed a smooth surface as determined by scanning electron microscopy of representative sample (Fig. 2). The integration of a static mixer into the flow-through system showed a pronounced effect on the homogenization process by reducing the particle size at the center point of the design from 270 to 220 nm and the polydispersity index from 0.157 to 0.085.

Among the independent variables investigated, (i.e. albumin concentration, aqueous phase volume, duration of emulsification, glutaraldehyde concentration, amount of drug) only the albumin concentration and aqueous phase volume showed a significant influence ($p < 0.05$) on the response factors. Figures 3 and 4 show the response surface curves which describe the relationships between particle size, polydispersity index, yield and overall desirability as a function of the albumin concentration and the aqueous phase volume. The smallest particle sizes were obtained at an albumin concentration of 5 to 20% (w/v) and an aqueous phase volume ratio of 4.5 to 9% (v/v) as shown in Fig. 3A). An increase in particle size was observed by using higher albumin concentrations and/or smaller aqueous phase volume ratio. No particles with sizes below 300 nm could be obtained with albumin concentrations higher than 20% (w/v) and aqueous phase volume ratios >9% (v/v). The surface response graph of the polydispersity index as a function of albumin concentration and aqueous phase volume ratio shows similar shape. The optimal region was found with albumin concentrations ranging from 5 to 22% (w/v) and aqueous phase volume ratios varying from 3 to 9% (Fig. 3B). Yields >60% (m/m) could be obtained independent of aqueous phase volume with albumin concentrations up to 20% (w/v) (Fig. 4A). A higher amount of albumin led to depositions of protein on the glass walls of the reaction chamber and therefore reduced the yield. Drug concentrations in the aqueous phase up to 30 mg/ml and glutaraldehyde ranging from 0.01 to 1 mg/mg albumin showed no statistically significant influence ($p < 0.05$) neither on particle size and polydispersity nor on particle yield. Drug loading efficiency was in all batches between 80 and 100% (w/w). The duration of emulsification was not found to have a marked effect ($p < 0.05$) on the response variables. The response surface graph of the overall desirability function shows a plateau at an albumin concentration of 12 to 20% (w/v) and at an aqueous phase volume ratio of 4.5 to 7% (v/v) (Fig. 4B).

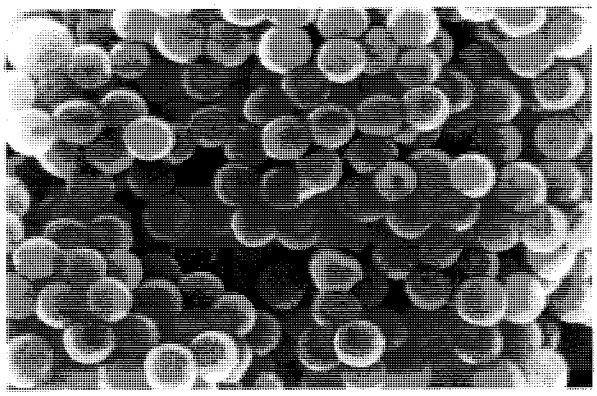

Fig. 2.

## DISCUSSION

In view of the fact that albumin nanospheres are intended for use as site-specific drug carrier systems, it is desirable that these particles have a size below 200 nm and narrow size distribution. Emulsion and suspension technique were used for the manufacture of albumin particles and therefore the relevant variables are numerous and difficult to characterize (5). A statistical central composite design was used, which does not assume linear relationship between factors and measured responses and allows the identification of optimum regions by using the surface response model (11). In addition to information about how the experimental variables operate in relative isolation, the experimenter can predict what will happen when two or more variables are used in combination (9). Preparation of albumin particles by emulsion crosslinking involves the formation of small droplets of aqueous albumin in an immiscible liquid, hardening these droplets by covalent crosslinking and recovery of the resulting particles.

The key element determining the size distribution of the droplets is the uniformity of the energy distribution throughout the emulsion system. In general, the more uniform the homogenization process, the more uniform the size of the droplets (5). By designing a new apparatus, which would not only allow a powerful homogenization, but also a continuous mixing during the emulsification, small particle sizes with

Table III. Reproducibility of the Preparation of Albumin Nanospheres

| Experiment: | Level: | Size (nm): | Polydispersity | Yield (% w/w) |
|---|---|---|---|---|
| a | 0 0 0 0 0 | 224 | 0.085 | 76 |
| b | 0 0 0 0 0 | 228 | 0.089 | 75 |
| c | 0 0 0 0 0 | 217 | 0.083 | 74 |
| d | 0 0 0 0 0 | 222 | 0.077 | 78 |
| Average | | 223 nm | 0.084 | 76% |
| Standarddev.: | | 4.6 nm | 0.005 | 1.7% |
| rel. Standarddev. | | 2% | 6% | 2% |

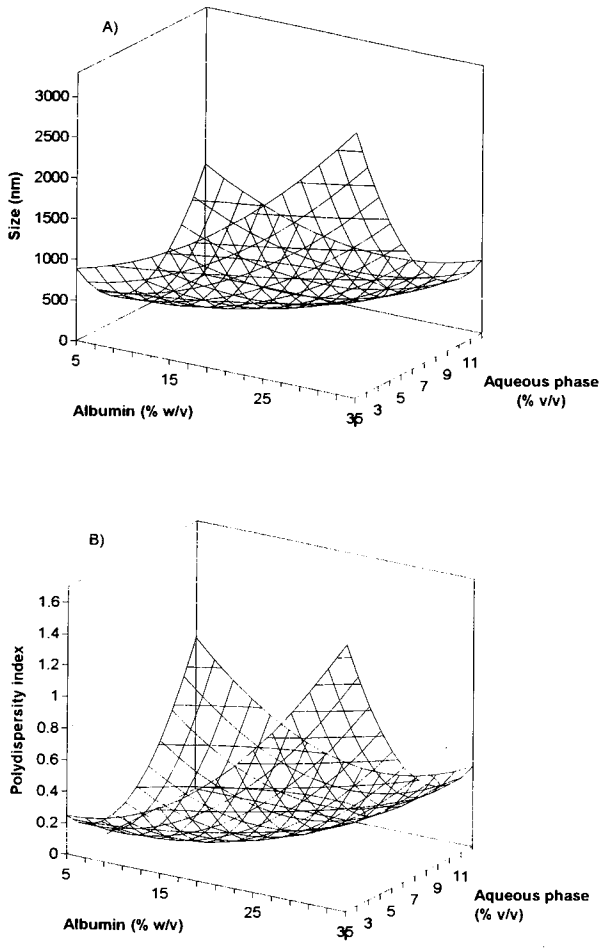


Fig. 3.

narrow distribution should be obtained. Using ultrasound, liquids can be dispersed with cavitation energy levels up to 100 W/cm$^2$. Together with a specially designed in-line continuous flow-through cell which forced the emulsion to pass across the surface of the sonication horn, an uniform and thermostatically controlled treatment of the emulsion was possible. By integrating a static mixer into the flow-through system an additional homogenizing effect could be observed.

Apart from design parameters of the equipment, the average size of the droplets is also determined by factors such as viscosity and phase volume ratios of the emulsion system. As shown in Fig. 3 particle size and polydispersity are mainly influenced by the albumin concentration and aqueous volume ratio. An increase in albumin concentration increased both the mean particle size and distribution. We attribute this effect to a higher relative viscosity of the aqueous phase which leads to higher resistance of the emulsion droplets against deformation and disintegration. Increased internal phase volume resulted in larger sphere size and increased size range. With large internal phase volume the mean distance between the droplets is smaller leading to coalescence.

The yield of albumin nanoparticles is also an important factor in the preparation of colloidal systems. As shown in Fig. 4A) the yield is significantly influenced by the albumin concentration. An average yield of 80% (m/m) could be obtained with albumin concentrations up to 15% (m/v). Higher amount of albumin resulted in aggregation and deposition of protein on the glasswalls.

A hydrophilic macromolecule, FITC-labelled Dextran with a molecular mass of 40 kDa, was selected to investigate the influence of drug loading on micromeretic properties. Addition of drugs to the aqueous phase has shown to influence the size of the particles (8). At levels beyond the solubility limit of the drug, the size of the particles begins to increase, due to the formation of microsuspensions. However, in this study no significant influence of drug concentration on albumin particle size could be observed with the FITC-dextran up to concentrations of 30 mg/ml.

The energy input and duration of ultrasonication may affect size and size distribution of albumin particles (8). These results could not be confirmed in our study, since an optimum emulsification was already reached after 5 min. Cross-linking was found to have no significant influence neither on mean diameter nor on size of the particles nor on yield. Other studies in which the crosslinking of albumin microspheres has been investigated report similar results (13). An optimum region for all investigated response factors could clearly be characterized using an overall desirability function.

In conclusion, a new equipment for preparing albumin nanospheres was developed, which allows a highly reproducible preparation of albumin particles with spherical

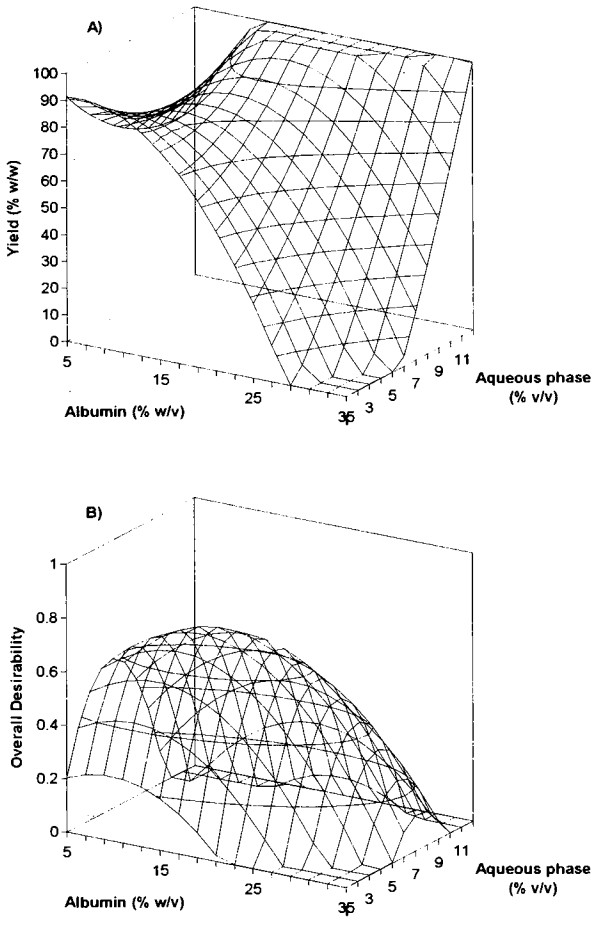


Fig. 4.

shape, smooth surfaces and sizes below 200 nm. Since a very narrow size distribution was obtained, further fractionation steps, such as size exclusion chromatography or centrifugation are not necessary. The reaction chamber and tubes are autoclavable and using ultrasonic generators with higher power output would allow scale up for larger batch sizes. Among the five process parameters investigated, only the albumin concentration and the aqueous volume ratio showed a significant influence on particle size, polydispersity and yield.

Albumin nanospheres may offer especially for macromolecular hydrophilic drug molecules such as proteins or peptides interesting applications for site-specific drug delivery. The effects of particle size and surface modifications on phagocytic uptake in-vitro and distribution in-vivo are currently under investigation.

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