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COMMUNICATION

# Nonliposomal Approach—A Study of Preparation of Egg Albumin Nanospheres Containing Amphotericin-B

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**ABSTRACT**

*The stability of liposomes after introduction into the body is presently being discussed and needs thorough understanding. Hence, as a nonliposomal approach, egg albumin nanospheres were prepared by the pH-coacervation method, and a preliminary study was carried out of the influence of process variables on the size and shape of nanospheres by changing the pH of the albumin solution, concentration of albumin solution, and volume of cross-linking agent. The batch prepared with an albumin medium of pH 9, 2% concentration, and 100 μl of 4% glutaraldehyde-ethanol solution was found to have a spherical uniform shape with an average size of 497.6 nm. The ideal batch was loaded with the systemic antifungal drug amphotericin-B. Drug-loaded nanospheres were evaluated to study their in vitro release. They were found to exhibit a biphasic pattern with a cumulative percentage release of 97.7%.*

## INTRODUCTION

In recent years, considerable energy has been devoted to the formulation of colloidal drug delivery systems acceptable for general systemic use and capable of carrying a drug to its target at the cellular (or tissue) level (1). Among the various particulate carriers (e.g., microspheres, liposomes, nanocapsules, and nanoparticles), the nanoparticles are used as potential colloidal vectors in a most promising way to reduce various toxicities efficiently and to modify the distribution of the drug. However, microspheres, due to their relatively large size, can produce microembolization, and some problems associated with liposome stability remain unsolved (2). Colloi-

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dal carriers in the form of nanospheres (or particles) have advantages over soluble carriers in terms of drug-loading capacity (3), stability (4), and use of relatively cheaper carrier material. Thus, as a nonliposomal approach, a study of the preparation of nanospheres was carried out.

The most widely utilized drug carrier is albumin as it meets many of the requirements of the ideal carrier (5). Albumin nanospheres are biodegradable, nonimmunogenic, and capable of holding a wide variety of drug molecules due to their increased protein-binding capacity; therefore, it was decided to use egg albumin as a carrier for the present study.

Albumin nanospheres (or microspheres) are most often prepared using emulsion methods. The main disadvantage of using emulsion methods (6) for the preparation of small nanospheres is that large amounts of organic solvents are required to remove oil residues from the particles (7). The complete removal of dispersion agent that remains on the particle surface is difficult. Thus, preparation of nanospheres using pH-coacervation enables a fine degree of control over the size. No surfactant is employed.

The object of the present work was to study the influence of process variables such as pH of the albumin medium, concentration of the albumin medium, and volume of cross-linking agent on the size and shape of nanospheres prepared by the pH-coacervation method and also to study the release profile of nanospheres loaded with the systemic antifungal agent Amphotericin-B, a drug that is already marketed in a costly liposomal carrier.

## MATERIALS AND METHODS

### Materials

Amphotericin-B was generously donated by Sarabhai Chemicals, Baroda, India. Other materials and equipment used included egg albumin flakes LR (Lobachem), acetone (analytic reagent grade), glutaraldehyde 25% (analytic reagent grade), ethanol, 0.5 M sodium hydroxide solution, and a Millipore filter unit (CPSMM 75541) was used for filtration throughout the study.

### Preparation of Nanospheres by the pH-Coacervation Method

Egg albumin (0.2 g) was dissolved in 10 ml of distilled water. The egg albumin solution was filtered using a Millipore filter with a cutoff range of 0.8 to 1 μm. The filtrate was put in a beaker and adjusted to a particular pH using 0.5 M sodium hydroxide solution. The solution was stirred on a magnetic stirrer, and the required amount of acetone was added dropwise from a syringe until the solution became turbid. The egg albumin nanospheres so formed were cross-linked by adding 50 μl, 100 μl, or 150 μl of a 4% gluteraldehyde-ethanol solution, and the solution was mixed continuously for 3 hr at room temperature. After the cross-linking stage, the egg albumin nanospheres were again filtered through the Millipore membrane filter. The filtrate was centrifuged at 20,000 rpm at 20°C for 30 min. After centrifugation, the supernatant was removed, and the suspension was washed four times with acetone. Finally, the nanospheres obtained were suspended in an acetone-water mixture.

### Influence of Process Variables on the Size and Shape of Nanospheres

To investigate the influence of process variables on the size and shape of nanospheres, nine batches of nanospheres were prepared as follows.

1. Preparation of nanospheres by changing the pH of albumin solution: three (A, B, C) batches of nanospheres were prepared by changing the pH to 8, 9, and 10, respectively. For the preparation of such batches each time, only the pH of the albumin solution and volume of cross-linking agent were kept at 2% and 100 μl, respectively.
2. Preparation of nanospheres by changing concentration of albumin: three batches (D, E, F) were prepared by changing the albumin concentration to 2%, 3%, and 4%, respectively. For the preparation of these batches each time, only the concentration of albumin solution was varied. The variables pH of the albumin solution and volume of cross-linking agent were kept at 9 and 100 μl, respectively.
3. Preparation of nanospheres by changing the volume of cross-linking agent: three batches of nanospheres (G, H, I) were prepared by changing the amount of cross-linking agent to 50 μl, 100 μl, and 150 μl, respectively. For the preparation of such batches each time, only the amount of cross-linking agent was varied. The variables pH of the albumin solution and concentration of albumin were kept at 9 and 2%, respectively.

### Determination of Particle Size

The prepared nanospheres were subjected to scanning electron microscopic analysis (Figs. 1 and 2), and the av-

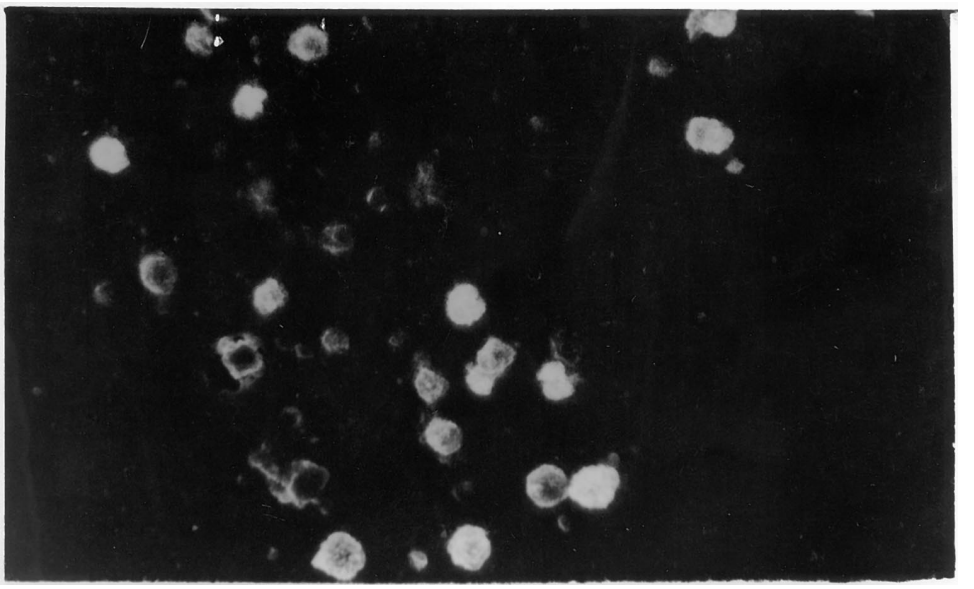

**Figure 1**. Scanning electron micrograph of nanospheres of the ideal batch (magnification 6000 × 3).

erage particle size of nanospheres in each batch was determined by measuring the diameter of all the spheres. The size and shape of nanospheres were found to vary with change of variables. The ideal batch was selected based on the shape and size of the nanosphere (Fig. 1).

**Preparation of Drug-Loaded Nanospheres**

Through the preliminary investigations, the ideal batch was selected based on its size and shape. The drug amphotericin-B was loaded into the ideal batch as fol-

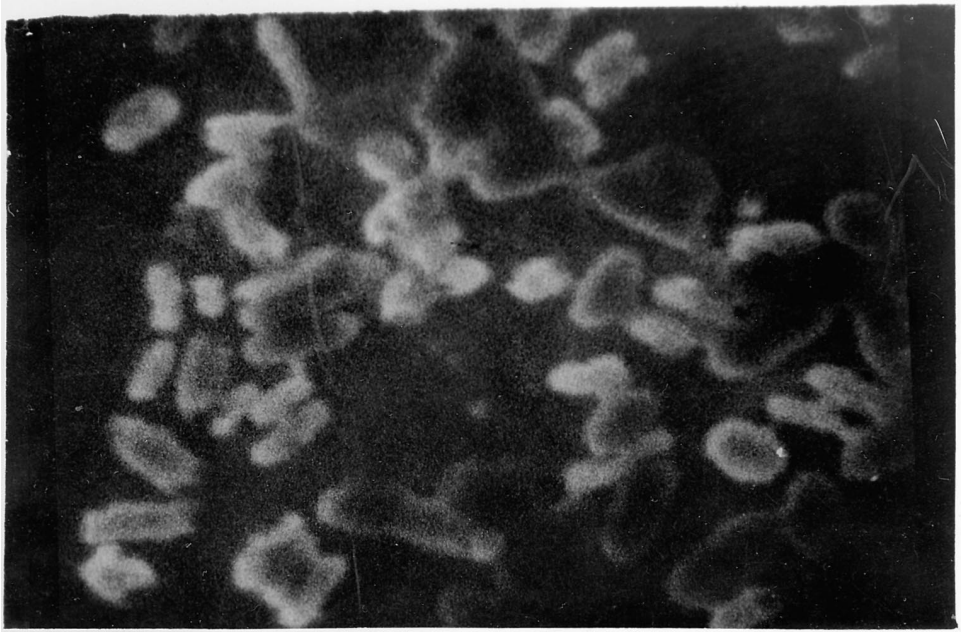

**Figure 2**. Scanning electron micrograph of nanospheres preserved in phosphate buffer saline (magnification 6000 × 1.7).

lows. Egg albumin (1.2 g) was dissolved in 60 ml of distilled water, and the solution was filtered using a Millipore membrane with a filter size range of 0.8 to 1 μm. The filtrate was put into a beaker, and the pH was adjusted to 9 using 0.5 M sodium hydroxide solution. The drug (amphotericin-B) was mixed with albumin solution. The solution was stirred on a magnetic stirrer. Subsequent steps were followed in manner similar to the general method of preparation.

### In Vitro Drug Release

This study was carried out according to the procedure described by Kim, Lee, and Lab (8) for microspheres. Drug-loaded nanospheres (10 mg) were put in a 250-ml conical flask, and to it 50 ml of 7.4 pH phosphate buffer saline was added. Then, the flask was kept in a shaker/incubator, and the shaker was adjusted to 80 horizontal strokes per min at 37°C. At various time intervals, 5 ml of drug-release media was withdrawn and replaced by the same volume of phosphate buffer saline solution. Each sample was filtered through a membrane filter with a pore size of 0.2 to 0.8 μm under vacuum. The drug was estimated by high-performance liquid chromatography (HPLC). The samples were withdrawn after 15 min and 30 min and 1, 2, 8, 16, and 24 hr (Table 1).

### Estimation of Drug

The drug estimation followed the HPLC assay procedure (9). An HPLC 484 (Waters) model was used for this purpose; it consisted of a $C_{18}$ reversed-phase Bondapack column. The column had a 3.9-mm internal diameter and was 300 mm in length. The mobile phase consisted of acetonitrile-10 M acetate buffer at pH 4.0 (37:63 v/v). The flow rate was adjusted so that a retention time of 14 min for amphotericin-B was obtained. The recovery efficiency was 70–97%.

***Table 1***

*In Vitro Release Study of Cumulative Percentage of Drug Release for Sample Batches*

| Time Interval | Cumulative Percentage of Drug Release | | | |
|---|---|---|---|---|
| | I | II | III | Average |
| 15 min | 34.90 | 35.10 | 33.90 | 34.60 |
| 30 min | 45.94 | 46.00 | 45.82 | 45.92 |
| 1 hr | 62.04 | 63.12 | 61.90 | 62.35 |
| 2 hr | 72.80 | 73.92 | 72.60 | 73.10 |
| 8 hr | 80.72 | 81.80 | 80.24 | 80.92 |
| 16 hr | 90.60 | 91.10 | 90.80 | 90.83 |
| 24 hr | 97.72 | 96.98 | 97.12 | 97.27 |

## RESULTS AND DISCUSSION

Preliminary study of the effect of process variables on the size and shape of nanospheres has revealed that nanospheres prepared at pH 9 were found to have uniform size and shape without agglomeration. The size of the nanospheres was found to be in the range between 380 and 600 nm. The average size of the particle was 497.6 nm (Fig. 1). Nanospheres prepared at pH 8 were found to possess an irregular size and shape. The size was in the range of 486 to 645 nm. The average size was 532 nm. Nanospheres prepared at pH 10 were found to have slight agglomeration, their size range was between 208 and 437 nm, with an average size of 287 nm. Investigations made by altering the pH of the albumin solution showed that the increase in the pH of the albumin solution increased the net charge on egg albumin, which counteracts the tendency of nanospheres to aggregate.

The nanospheres prepared using a 2% concentration of egg albumin showed particles with regular and uniform shape without agglomeration. The size range was between 372 and 620 nm. Nanospheres prepared using a 3% concentration of egg albumin had an irregular and larger shape. The size range was relatively increased from the previous batches, between 528 and 746 nm, with an average size of 546 nm. Preparation of nanospheres with a 4% concentration of egg albumin resulted in irregular shape. The size range was between 638 and 826 nm. As expected, changing the concentration of the albumin revealed that the increase in the concentration of albumin increased the size of the sphere and irregularity of the shape.

Investigations made by changing the volume of cross-linking agent lead to the conclusion that a decrease in the amount of cross-linking agent leads to the formation of partially cross-linked spheres due to inadequate stabilization. Through the overall investigations on process variables, it was evident that uniform-shape nanospheres can be produced by adjusting the pH of the albumin solution to 9, the concentration to 2%, and the volume of cross-linking agent to 100 μl.

The in vitro release of the drug from the nanospheres showed an interesting biphasic release. In the first half

hour, there was a burst release of 46% of the drug. Afterward, the drug release followed a steady pattern. Total percentage of drug released after 24 hr was found to be 97.7%, which may be considered satisfactory. When the nanospheres were preserved in phosphate buffer solution at pH 7.4 for more than 60 hr, they were found to have elongated, disrupted, and encapsulated structures (Fig. 2).

## CONCLUSION

When compared to various other particulate carriers like microspheres and liposomes, the nanospheres are also found to have satisfactory sphere morphology, size distribution, and in vitro release characteristics, which make them a suitable carrier for drug targeting instead of liposomes. However, a thorough stability analysis and in vivo targeting efficiency of these nanospheres are yet to be established.

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