Preparation of calcium phosphate nanoparticles and evaluation of their effects on muscle cells of rat

Shantanu Tamuly and Mumtesh Kumar Saxena*

Animal Biotechnology Center,
Department of Veterinary Physiology and Biochemistry,
College of Veterinary and Animal Sciences,
G.B. Pant University of Agriculture and Technology,
Pantnagar 263 145, India

Calcium phosphate has been used for many years as a DNA delivery system. It has not been approved for use in the vaccine adjuvant system since it has been known for site-specific reaction in the muscle cells for irritant effects. Calcium phosphate nanoparticles have been shown to be a lesser irritant than the macroparticles. In the present study calcium phosphate nanoparticles were prepared with bovine serum albumin using different stirring times and each analysed for entrapment efficiency. The void calcium phosphate nanoparticles were injected intramuscularly into rats for testing the site-specific inflammation. The muscle samples were collected on the 14th day of injection. The smallest particle size of about 40 μm of calcium phosphate nanoparticle–BSA complex was obtained by stirring for 1 h. The void calcium phosphate nanoparticles did not elicit any site-specific reaction in rats, as revealed by in histopathological examination. Hence calcium phosphate nanoparticles can be efficiently used as an adjuvant for non-live vaccines. However, further analysis of effects of calcium phosphate nanoparticles on the vital organs such as brain, liver, kidney is warranted.

Keywords: Adjuvant, calcium phosphate, muscle cells, nanoparticles, rat.

THE area of nanoparticles of inorganic compounds has assumed great significance in entrapping biomolecules in veterinary and medical sciences. These inorganic nanoparticles have many advantages over organic ones, such as better keeping quality and also being inexpensive. These nanoparticles have found their way in a number of biomedical applications such as gene therapy, adjuvants and drug-delivery systems[1–8]. Calcium phosphate has been used for more than 30 years to deliver genetic material to mammalian cells. Apart from using calcium phosphate nanoparticles (CAP) in gene delivery systems, it has also been utilized as an adjuvant for protein-based vaccines[4,5]. The exact mechanism of action of calcium phosphate nanoparticles as an adjuvant is not clear; however it is believed that it acts as a depot and slowly releases the antigen entrapped in it for a longer duration of time. The calcium phosphate nanoparticles do not stimulate site-specific reaction upon intramuscular injection. These nanoparticles have also been found to be safe in terms of generation of IgE response[5]. Among the various methods of manufacture of calcium phosphate nanoparticles, the co-precipitation method is easier and can be utilized in the industrial scale. In the present study, the calcium phosphate nanoparticles were prepared with bovine serum albumin (BSA) using different stirring times and their inflammatory effect on rats was studied. The basic protocol was slightly modified from that mentioned by He *et al.*[5] and Joyappa *et al.*[1]. One millilitre of BSA solution (1 mg/ml) was taken in a flask with a magnetic bar in stirring mode, followed by drop-by-drop addition of 7.5 ml of calcium chloride (12.5 mM). To this was added drop by drop 1.5 ml of sodium citrate and 7.5 ml of dibasic sodium phosphate (12.5 mM). The solution was stirred for different time intervals of 1, 2, 3 and 4 h. The suspension thus prepared was then centrifuged at 800 *g* for 20 min. The pellet was washed thrice with 0.1 M PBS (pH 7.2) and resuspended in 1 ml of 0.1 M PBS (pH 7.2). The entrapment efficiency of calcium phosphate nanoparticles was determined using the method by Joyappa *et al.*[1] Next, 1 ml of the CAP–BSA complex was centrifuged at 800 *g* for 20 min and the pellet was weighed. The pellet was resuspended in 100 mM EDTA followed by incubation for 1 h at room temperature. The protein released from the calcium phosphate nanoparticles was retrieved by centrifugation of the suspension at 800 *g* for 10 min. The supernatant was collected and the protein content was estimated by measuring the optical density of the suspension at 280 nm wavelength.

The entrapment efficiency was calculated using the formulae[1]

$$E\% = \frac{r - \text{Protein}}{i - \text{Protein}} \times 100,$$

where *E*% indicates percentage of protein bound to calcium phosphate nanoparticles, *r*-Protein indicates the amount of protein entrapped in calcium phosphate nanoparticles and *i*-Protein indicates the initial amount of protein added to the calcium phosphate nanoparticles solution.

Calcium phosphate void nanoparticles (with no incorporation of protein) were synthesized using the protocol of He *et al.*[5]. The particle size was measured by transmission electron microscopy (Department of Veterinary Anatomy, G.B. Pant University of Agriculture and Technology, Pantnagar) and X-ray diffraction (Advanced Research Instrumentation Facility, Jawaharlal Nehru University (AIRF, JNU), New Delhi) using the formulae

$$Dn = \frac{0.9\lambda}{\beta \cos\theta}.$$

*For correspondence. (e-mail: mumtesh@rediffmail.com)

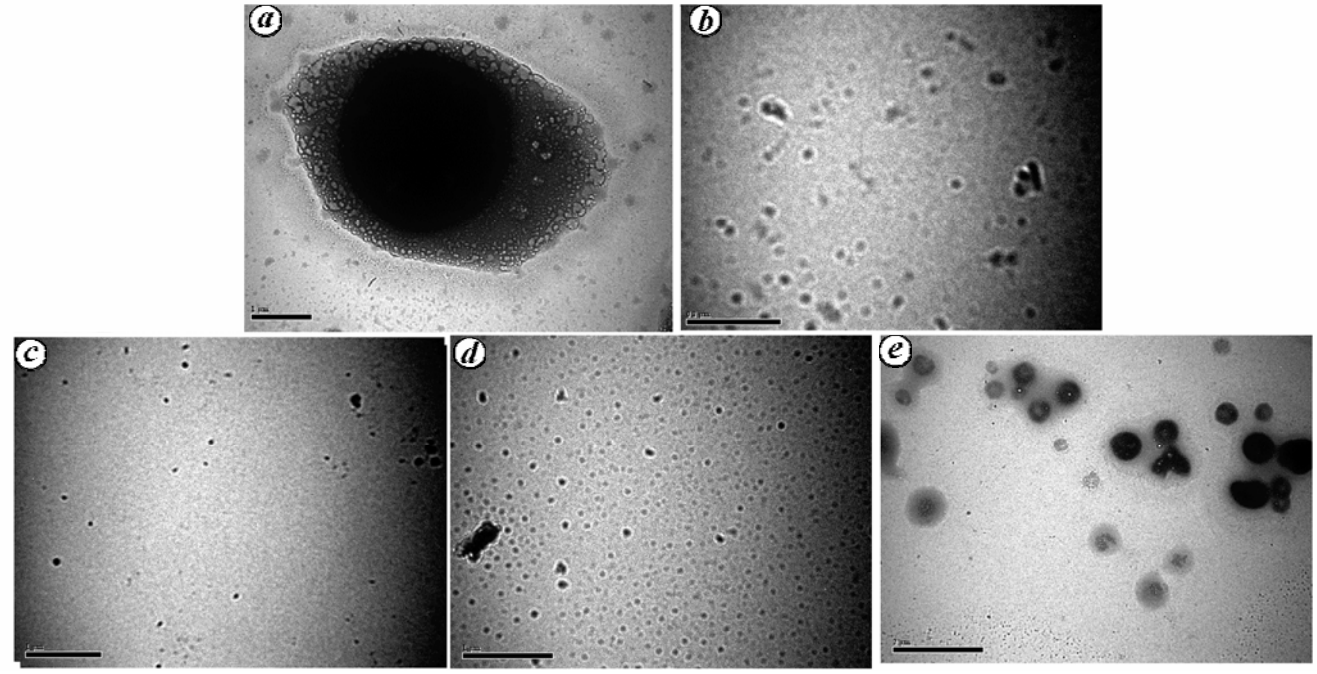

Figure 1. Electron micrograph of CAP–BSA complex at (***a***) 0 h; (***b***) 1 h; (***c***) 2 h; (***d***) 3 h and (***e***) 4 h of stirring.

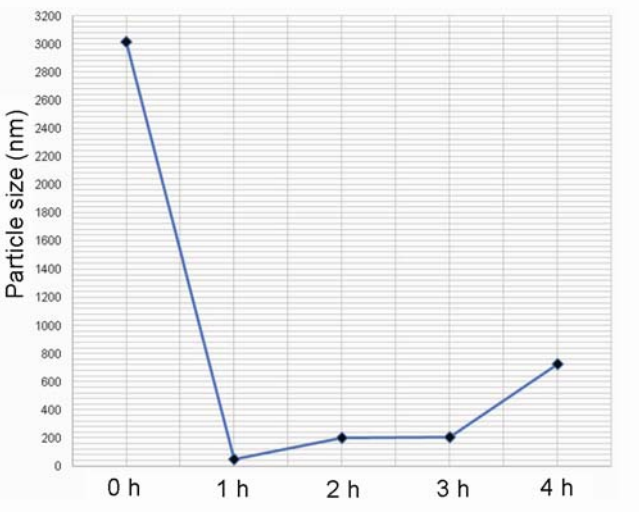

Figure 2. Alteration of particle size in different time intervals of stirring.

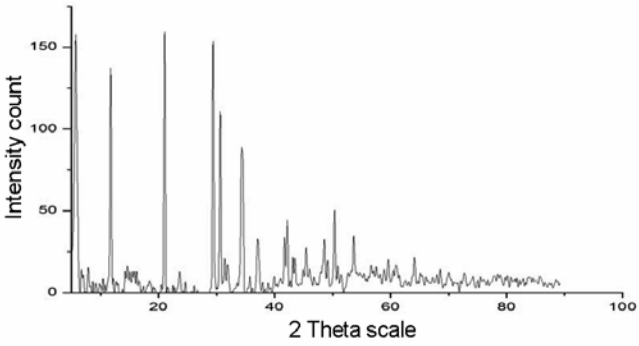

Figure 3. XRD analysis of calcium phosphate nanoparticles.

One drop of the CAP–BSA complex was loaded onto a copper grid and kept for 1 h for drying. The dried sample was viewed under transmission electron microscope. For XRD analysis, 10 ml of suspension containing the CAP–BSA complex was lyophilized and sent to AIRF, JNU for XRD analysis. The following parameters were selected: scan rate = 2°/min; scan range = 5° to 90° and X-ray source being Cu-Kα.

The rats were divided in two groups containing seven rats each. The first group (treatment) was administered void calcium phosphate nanoparticles at the rate of 1 mg per kg body weight and the second group (control) was administered PBS (pH 7.2) intramuscularly. After 14 days, the muscle samples were collected for histopathological studies. The animal experiments were carried out according to the ethical standards laid down by our university guidelines.

The initial size of the CAP–BSA complex was found to be more than 1 μm (Figure 1 *a*), which was subjected to stirring at different time interval. On stirring, the minimum size (30 nm) was recorded at 1 h (Figure 1 *b*). The size of the complex kept increasing and reached 600 nm at 4 h (Figures 1 *c–e* and 2). The findings were confirmed by XRD analysis (Figure 3). In case of DNA delivery system, as observed by Welzel *et al.*[9], higher concentration of DNA caused blocking of the surface of nuclei formation and resulted into lower transfection efficiency. Similar findings were observed in the case of the protein in the present study. Particle size less than 1 μm is considered as a nanoparticle[4]. The particle size obtained in the present study was smaller than that reported earlier.

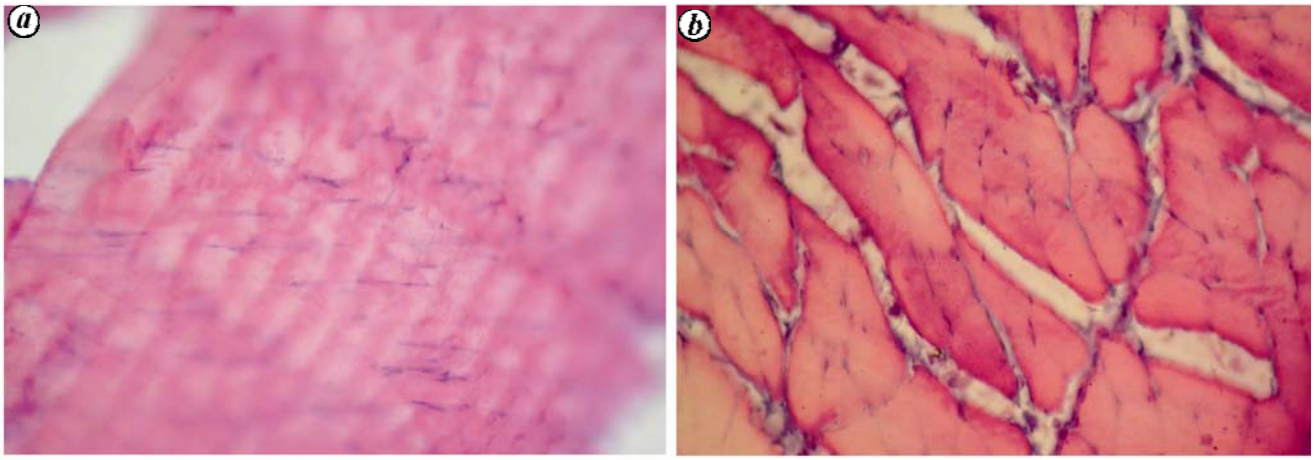

Figure 4. Histopathological slide of muscle tissue of calcium phosphate nanoparticle treated group (***a***) and control group (***b***).

Further, the protein entrapment efficiency of calcium phosphate nanoparticles was found to be 30%. The maximum amount of protein that could be loaded in the nanoparticles was estimated to be 50 μg/mg of nanoparticle, which was estimated spectrophotometrically at a wavelength of 280 nm. The nanoparticle pellet could be easily re-dispersed in aqueous buffer (PBS, pH 7.2). After 14 days of study the muscles were collected at the site of injection of calcium phosphate nanoparticles for histopathological examination. The muscles did not reveal any inflamatory reaction. The muscle samples of both treated and control group did not reveal any lesions (Figure 4). This is probably due to the fact that the nanoparticles are not recognized by the inflammatory cells due to their smaller size. Our observations are in agreement with those of He *et al.*[5]. The exact mechanism of formation of nanoparticles is not clearly understood. However, it is postulated that mixing protein/$CaCl_2$ with phosphate-containing buffer leads to the precipitation of the sparingly soluble calcium phosphate which incorporates the protein. In the present study, it was noted that calcium phosphate nanoparticles are safe for administration in animal systems and do not cause site-specific reactions. However, further toxicity analysis is warrented in terms of its biodegradibility and its effect on the vital organs such as liver, kidney and brain, since Liu *et al.*[10] have repoted that calcium phosphate nanoparticles increased the apoptosis rate in human granulosa cells by arresting them in the S phase of cell division.

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