



Carbohydrate modified ultrafine ceramic nanoparticles for allergen immunotherapy

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ARTICLE INFO

Article history:

Received 20 October 2010

Received in revised form 24 January 2011

Accepted 1 February 2011

Available online 18 February 2011

Keywords:

Allergy

Aquasomes

Ceramic

Specific immunotherapy

ABSTRACT

The uses of drug-delivery systems in allergen specific immunotherapy appear to be a promising approach due to their ability to act as adjuvants, transport the allergens to immune-competent cells and tissues and reduce the number of administrations. The aim of this work was to evaluate the carbohydrate modified ultrafine ceramic core based nanoparticles (aquasomes) as adjuvant/delivery vehicle in specific immunotherapy using ovalbumin (OVA) as an allergen model. Prepared nanoparticles were characterized for size, shape, zeta potential, antigen integrity, surface adsorption efficiency and in vitro release. The humoral and cellular-induced immune responses generated by OVA adsorbed aquasomes were studied by two intradermal immunizations in BALB/c mice. OVA sensitized mice were treated with OVA adsorbed aquasomes and OVA adsorbed aluminum hydroxide following established protocol. Fifteen days after therapy, animals were challenged with OVA and different signs of anaphylactic shock were evaluated. Developed aquasomes possessed a negative zeta potential (-11.3 mV) and an average size of 47 nm with OVA adsorption efficiency of $\sim 60.2 \mu\text{g mg}^{-1}$ of hydroxyapatite core. In vivo immune response after two intradermal injections with OVA adsorbed aquasomes resulted in a mixed Th1/Th2-type immune response. OVA-sensitized mice model, treatment with OVA adsorbed aquasomes elicited lower levels of IgE ($p < 0.05$), serum histamine and higher survival rate in comparison with alum adsorbed OVA. Symptoms of anaphylactic shock in OVA aquasome-treated mice were weaker than the one induced in the alum adsorbed OVA group. Results from this study demonstrate the valuable use of aquasomes in allergen immunotherapy.

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1. Introduction

Allergy, or hypersensitivity type I, applies to an abnormal reaction against innocuous environmental compounds (allergens) and involves complex interactions between exogenous and genetically determined factors. The prevalence of type I allergy has increased which have doubled in prevalence in industrialized countries over the last two decades [1]. The pathological features of allergic diseases include the production of allergen-specific IgE, which results in immediate phase responses with mast cell and basophil degranulation and the release of mediators including histamine, proteases, cytokines, and arachadonic acid products, including leukotrienes. In addition, immediate phase responses are usually followed by the development of late phase reactions and eosinophilic inflammatory processes driven by the production by Th2 lymphokines such as IL-4, IL-5, IL-9 and IL-13 [2,3].

So far, the only curative treatment for allergy is specific immunotherapy (SIT), where an immunologic hyporesponsiveness

is induced by intradermal injections of gradually increasing quantities of allergen extract. SIT is accompanied by suppression of Th2 cells and generation of allergen-specific regulatory T cells (Treg), with increased secretion of IL-10 and TGF- β and development of peripheral tolerance [4]. Although the precise mechanisms involved remain uncertain; there is a substantial body of clinical evidence and practice to support the use of SIT and is considered to be the only effective, specific treatment for IgE mediated allergic diseases [3,5,6].

Despite the efficacy of SIT, there are concerns with the treatment including difficulties in standardization of allergen extracts, patient compliance due to frequent injections, long treatment duration and safety issues comprising local side-effects and risk of anaphylaxis [1,7]. One of the strategies to improve vaccines for SIT is to use adjuvants that can modulate the allergen-specific T cell response. For this purpose, nanoparticle based antigen-delivery systems have shown promising results [1,8–11]. Besides the advantage of targeting to immune-competent cells, these systems act like reservoirs to release the molecules in either a continuous or a pulsatile manner, avoiding a multiple-injection schedule [8]. Apart from these advantages, these carriers protect the antigen from harsh pH conditions and enzymatic degradation and thus requiring lower doses [9]. As a consequence, the chance of suffering an anaphylactic shock and the

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cost of the therapy would decrease. All these factors lead to patient friendly treatment and would minimize side effects.

In the last decade, ceramic/carbohydrate based biocompatible and biodegradable nanoparticulate system called “aquasomes” has shown promise as an adjuvant for a variety of antigens (11–13). Aquasomes consist of a ceramic core, the surface of which is non-covalently modified with carbohydrates to obtain a sugar ball, which is then adsorbed with therapeutic proteins. Core provides structural stability to largely immutable solid. The surface modification creates a glassy molecular stabilization film that adsorbs antigen/protein with minimal structural denaturation and preserves the protein conformational state [11–15]. One of the features that distinguish aquasomes from other adjuvant(s) is that surface coated polyhydroxyloligomers maintain the nanometer scale architecture, which appears to be central to their ability to produce both humoral and cellular immune response [13,15,16].

In this perspective, the aim of the present work was to prepare and evaluate the immune-adjuvant capacity of aquasomes as carriers for allergens using ovalbumin (OVA) as a model. OVA adsorbed aquasomes (OVA aquasome) were prepared by simple adsorption method to maintain the antigenic conformational properties. The immuno-stimulatory response induced by administration of two doses of OVA aquasome by the intradermal route and protective capability of aquasomes in OVA sensitized BALB/c mice against an anaphylactic shock were evaluated.

2. Materials and methods

2.1. Materials

The $(\text{NH}_4)_2\cdot\text{HPO}_4$, $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, and trehalose were obtained from Himedia Pvt. Ltd. (Mumbai, India). Aluminum hydroxide gel (Alhydrogel®) was procured from Brannntag Biosector, Frederikssund, Denmark. The reagents used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and biconinonic acid (BCA) protein assay kit were obtained from Genei (Bangaluru, India). Bovine serum albumin, horseradish peroxidase (HRP) conjugated goat anti mouse IgG, IgG1, IgG2a and IgE antibodies were procured from Sigma (Sigma-Aldrich Pvt. Ltd., St. Louis, USA). Cytokine assay kits were from eBioscience, San Diego, CA. All other chemicals and reagents were of analytical grade, purchased from local suppliers and were used as received. Distilled deionized water (Milli-Q™ Water system, Millipore Corporation, Massachusetts, USA) was used throughout the study.

2.2. Preparation of hydroxyapatite core and its characterization

Hydroxyapatite (HA) core was prepared by self-precipitation method as previously reported [17]. 0.5 M $(\text{NH}_4)_2\text{HPO}_4$ was added quickly to 0.5 M $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$ ethanol solution with vigorous stirring. pHs of both solutions were kept above 10 by adding concentrated NH_4OH solution drop wise. Mixture was stirred vigorously for 1 h using mechanical stirrer (REMI C-24, Mumbai, India) followed by overnight drying at 40 °C in oven. The precipitated hydroxyapatite particles were washed first with ethanol and then with distilled deionized water. Finally, ceramic particle suspension was again dried overnight at 40 °C overnight. Fourier transformed infrared spectroscopy (FT-IR) was used for structure analysis of ceramic core. The KBr sample disk was prepared using 1% (w/w) of hydroxyapatite powder and was compressed and dried at 100 °C. Infrared spectra were recorded in the wave number range of 4000 to 400 cm^{-1} (resolution 4.0 cm^{-1}) using FTIR spectrophotometer (Perkin-Elmer-1600, Waltham, Massachusetts). Phase analysis of hydroxyapatite core was performed using X-ray diffraction (XRD). Hydroxyapatite ceramic cores were exposed to $\text{Cu K}\alpha$ radiation (45 kV $\times$ 40 mA) in a wide-angle X-ray diffractometer (XRD-6000, Shimadzu, Japan). The instrument was operated in the step-scan

mode in increments of 0.03° 2 θ . The angular range was 10–50° 2 θ , and counts were accumulated for 1 s at each step. The particle morphology was determined by transmission electron microscopy (Philips EM268D, The Netherlands). One drop of aqueous dispersion was placed over a 400-mesh carbon-coated copper grid followed by negative staining with phosphotungstic acid (3%, w/v, adjusted to pH 4.7 with KOH) and placed at the accelerating voltage of 80 kV. The mean hydrodynamic diameter, polydispersity and zeta potential of core was measured by photon correlation spectroscopy (PCS) using Zeta Nano ZS 90 (Malvern, UK) after appropriate dilution (1:200) with PBS pH 7.4 prior to analysis.

2.3. Preparation of OVA adsorbed aquasomes and its characterization

Aquasomes were prepared by a method reported previously with slight modifications [16]. Briefly; hydroxyapatite weighing approximately 15 mg was initially suspended in 30 ml of 29.2 mM trehalose solution in a 50 ml clean beaker by vigorous stirring. The resultant suspension was sonicated for 10 min at a frequency of approximately 20 kHz at 25 °C using a probe sonicator (Soniweld India Ltd., Mumbai, India). The dispersion was clarified by centrifugation (REMI C-24, Mumbai, India) at 10,000 rpm for 20 min at 4 °C. The remaining pellets were then discarded and the mixture was lyophilized overnight at a condenser temperature of –82 °C and pressure of less than 10^{–1} mbar. Unadsorbed trehalose was removed by centrifugation against 20 ml of 25 mM phosphate buffer (pH 7.4). Further, 10 mg of sugar coated particles was dispersed in OVA solution (8 mg/ml in PBS pH 7.4) and kept at 4 °C over night. OVA adsorbed aquasomes were washed three times with de-ionized water by using centrifugation at 10,000 rpm for 30 min (REMI C-24, Mumbai, India) and stored at 4 °C until used. The morphological examination, mean particle size and size distribution of prepared aquasomes were determined as described above in Section 2.2 for ceramic core. Sugar content on surface of hydroxyapatite was determined by anthrone method [18] using glucose as standard. The adsorption of sugar on the surface of hydroxyapatite was confirmed by change in zeta potential using Zetasizer Nano ZS 90 (Malvern, UK). SDS-PAGE experiment was carried out to analyze the integrity of OVA antigen under non-denaturing condition. Protein bands were detected by Coomassie blue staining. SDS-PAGE experiment was performed in the presence of strong anionic reducing agent, i.e. sodium dodecyl sulfates (SDS). In the presence of strong reducing agent and heat, proteins get dissociated before they were applied on the gel [19]. OVA was extracted by dissolving the aquasomes in 2 ml of 5% (w/v) SDS in 0.1 N HCl solution. The extracted allergen was concentrated and adsorbed onto a 3.5% stacking gel and subjected to electrophoresis on a 12% separating gel at 200 V (Bio-Rad, USA) until the Coomassie dye stained protein band reached the gel bottom.

2.4. Adsorption efficiency and OVA release from aquasomes

The percentage OVA adsorption efficiency of aquasomes was determined as reported previously [16]. Briefly, accurately weighed OVA adsorbed aquasome formulations (200 mg) were suspended in Triton-X 100 (0.01%, w/v) and incubated in a wrist action shaker for 1 h. The samples were centrifuged at 14,000 rpm for 15 min and antigen concentration in the supernatant was determined using micro BCA method while setting a blank of plain aquasome formulation treated in the same manner. For in vitro release studies, 200 mg of OVA adsorbed aquasomes was suspended in phosphate buffer (pH 7.4, 10 ml) with slow stirring in magnetic stirrer (Remi, Mumbai, India). Samples were withdrawn (1 ml) after 15 min each followed by centrifugation (1700 rpm for 10 min at 4 °C) and the released protein in the supernatant was measured by BCA protein assay at various times. Equal quantity of fresh buffer was added after each sampling to maintain the sink conditions.

2.5. *In vivo* immunological response

Female BALB/c mice (age 7–9 weeks; weight 15–20 g) were used in all experiments. Animals were housed in climate ($23 \pm 2^\circ\text{C}$; RH: 60%) and photoperiod (12 h light–dark cycles) controlled animal quarters at the animal house and resource facility in group of six ($n=6$). They were fed standard rodent pellet and had free access to drinking water. After one week of acclimatization with the environment, immunization protocols were followed. They were withdrawn of any food intake 3 h before immunization. The study protocol including handling, care and immunization were approved by Institutional Animal Ethics Committee and studies were carried out as per the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Animals were immunized by two intradermal injections with two weeks interval, i.e. at day 0 (priming) and at day 14 (boosting) with one of the following: (i) free OVA (10 μg per mouse) in 50 μl of PBS; (ii) blank aquasomes 100 μg in 50 μl of PBS; (iii) OVA aquasomes (equivalent of 10 μg of OVA per mouse); and (iv) OVA in alum (OVA alum) and (v) sterile PBS 50 μl only. OVA alum was prepared by dissolving the protein (50 $\mu\text{g}/\text{ml}$) in PBS (pH 7.4, 0.01 M) and subsequently mixing by sonication with aluminum hydroxide gel (1 mg/ml).

2.5.1. Sample collection

Blood was collected from the saphenous vein of mice (under mild ether anesthesia) at day 0 (pre-immune sera, 3 h before immunization), 14 days and 42 days (terminal samples) of priming. The terminal blood samples were obtained by cardiac puncture post euthanasia after 4 weeks of booster immunization. Serum was separated by centrifugation ($1500 \times g$ for 5 min) from collected blood samples after allowing coagulation for 30 min at room temperature. Serum samples were stored at -22°C until analyzed for antibody. Spleen of three mice from each group was surgically removed at the end of the experiment for cytokine assay.

2.5.2. Measurement of humoral response

An indirect ELISA was used to determine the level of OVA-specific IgG antibodies and isotypes in the serum. Each well of microtiter plate (Nunc-Immune Plate® Fb96 Maxisorb, Nunc, India) was coated with antigen (100 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 25 $\mu\text{g}/\text{ml}$ OVA in bicarbonate buffer, pH 9.6). Each well was dispensed with 100 μl volume and incubated at 4°C overnight. The plate was then washed three times with PBS-Tween 20 (PBS-T, 0.05%, v/v) followed by blocking with 150 μl of 3% gelatin in PBS for 90 min. at 37°C and washed thrice with PBS-T. 100 μl of serum (1:800) in gelatin PBS-T20 was added in each well and incubated for 90 min at room temperature. The plate was then washed three times as described above. 100 μl of peroxidase labeled goat anti-mouse IgG, IgG2a or IgG1 (1:1000) was added to each well. The plates were covered and after incubation for 1 h at room temperature washing was repeated. 100 μl of substrate O-phenylenediamine dihydrochloride (OPD) (prepared in citrate buffer + H_2O_2) was added to each well followed by addition of 50 μl of H_2SO_4 after 90 min. After development of color, the reaction was stopped by adding 25 μl of 2.5 M H_2SO_4 and absorbance was measured at 492 nm (ELISA microplate Reader, Bio-Rad, USA). Values are expressed as mean $\pm$ S.D.

2.5.3. Estimation of cytokine levels

Endogenous levels of IL-4 and IFN- γ in mouse spleen homogenates were measured by using separate ELISA kits for these cytokines as previously described [20]. Individual spleens from euthanized mice at the end of experiment were separated, weighed and homogenized in ice-cold RPMI containing 1% 3-(cholamidopropyl-*o*-dimethylammonio)-1-propanesulphonate (CHAPS; Sigma) in a micro tissue homogenizer (MAC, New Delhi, India). 10% (w/v) homogenates were stored

for 1 h at $2-4^\circ\text{C}$ and insoluble debris were removed by centrifugation at $2000 \times g$ for 20 min (REMI C-24, Mumbai India). Capture ELISA based mouse cytokine assay kits from eBioscience (San Diego, CA) were used to determine the presence of cytokines in spleen homogenates according to the manufacturer's instructions. Data are expressed as picogram per milliliter in the spleen homogenates as computed by comparison with the standard curve provided by the manufacturer for IL-4 and IFN- γ .

2.5.4. Sensitization, vaccination and challenge studies

For sensitization studies, previously published protocol was followed [21]. BALB/c mice were sensitized by intraperitoneal injection of 50 μg of OVA emulsified in 1 mg alum adjuvant (Branntag Biosector, Denmark) in a total volume of 150 μl on days 1 and 8. On days 14, 17 and 20, the animals (5 mice per group) received intradermal injections with 3 μg of OVA each incorporated in either OVA-aquasomes, OVA-alum and OVA in PBS. Finally, on day 42 the animals were challenged by an injection of 1 mg of OVA by intraperitoneal route. To monitor serum IgE antibody responses, blood samples from saphenous vein were obtained along the sensitization and immunotherapy periods and stored at $-80 \pm 1^\circ\text{C}$ until their analysis.

2.6. Evaluation of anaphylaxis

IgE antibody level was determined by following the same protocol as described in Section 2.5 except the serum dilution ratio was 1:400 and peroxidase labeled goat anti-mouse IgE antibody was used. Histamine release test was performed on heparinized whole blood from the saphenous vein obtained before and 30 min after the challenge. Histamine release was assayed by a fluorometric method as previously described [22]. The body temperature changes associated with anaphylactic shock were monitored by measuring the rectal temperature without general anesthesia before and 10 min after the challenge. Anaphylactic symptoms (activity/lethargy, piloerection or ruffled or erect fur and cyanosis) were evaluated 30 min after the challenge and reaction severity was classified according to the categories depending on their gravity: (i) (–) absent; (ii) (+) weak; (iii) (3) moderate; and (iv) (+++) strong. Finally, the survival rate was recorded 24 h after intraperitoneal challenge as described in literature [21,22].

2.7. Statistical analysis

Data from *in vivo* studies were compared using ANOVA followed by the Tukey's post-test to assess statistical significance. Results were considered to be statistically significant if $p < 0.05$. All calculations were performed using GraphPad® Prism® version 5.03 for Windows, GraphPad Software, San Diego, California, USA.

3. Results

3.1. Preparation of hydroxyapatite core and its characterization

Tin oxide, diamond and brushite (calcium phosphate dihydrate) are generally used as core for preparation of aquasomes [12,13,15,16,23]. Owing to biodegradability, cost, stability and safety, hydroxyapatite (HA) was selected as a core for the preparation of aquasomes. Moreover, it is widely used for the preparation of implants, drug and antigen delivery [24,25]. They are particularly suitable for protein delivery because of their high adsorption capability [26].

HA particles prepared by self-precipitation method under controlled process parameters were smaller in size and crystalline in nature. The prepared HA core was analyzed by FTIR spectroscopy to evaluate their crystallinity. The bands for PO_4^{3-} of the calcined powder observed at

507, 604, 945, 964, 1024, and 1184 cm^{-1} , whereas the medium sharp peak at 633 , 2910 and 3570 cm^{-1} was due to the OH^{-1} bending deformation (Fig. 1). The FTIR spectrum of prepared HA core was in accordance with previous findings [17,24].

Hydroxyapatite ceramic core was characterized by X-ray diffraction patterns (Fig. 2) to assess their crystalline nature. Characteristic intense absorption peaks at $31\text{--}32$, $49\text{--}50$; $25\text{--}27$ (2θ angle) indicates the crystalline behavior of HA. The XRD pattern of prepared sintered hydroxyapatite ceramic core complied with the standard HA core [16,27]. The morphology of the HA core is shown in TEM image in Fig. 3A. The microstructure of HA core reveals that the ultrafine nanosized spherical particles are dense with rough surfaces. Some aggregation was also observed which may be due to drying. DLS results support the finding of TEM and particle size of HA was found to be 39 nm with polydispersity index 0.28 .

3.2. Preparation and characterization of aquasomes

Aquasome formulations were also characterized for the size, shape, and antigen adsorption efficiency. TEM image of aquasome systems indicated that the systems were smaller in size, spherical and elongated in shape (Fig. 3B). Photon correlation spectroscopy also confirmed the nanometric architecture of prepared aquasomes. Both transmission electron microscopy and photon correlation spectroscopy results revealed that the aquasomes ranged from 47 nm (Table 1).

3.3. Adsorption efficiency and release studies

Coating of trehalose using freeze drying showed that hydroxyapatite core adsorbed 2.887% of the trehalose initially added. The zeta potential of the hydroxyapatite ceramic core was slightly positive in pH 7.4 that was reduced to slightly negative value after coating of trehalose, which confirm the coating of sugar layer on ceramic core. Further adsorption of OVA results in negative value of $-11.34 \pm 1.4\text{ mV}$. Percent antigen adsorption on aquasome was found to be $60.22 \pm 2.24\text{ }\mu\text{g/mg}$ of core (Table 1). It was previously demonstrated that hemoglobin and insulin intercalated between the trehalose loose epitaxial adsorption around the ceramic core [28], similar trend is also postulated in this study, and thus it was assumed that OVA was tightly packed between the trehalose molecules. Fig. 4 indicates the amount of OVA released into the medium during each 10 min period when the medium was exchanged every

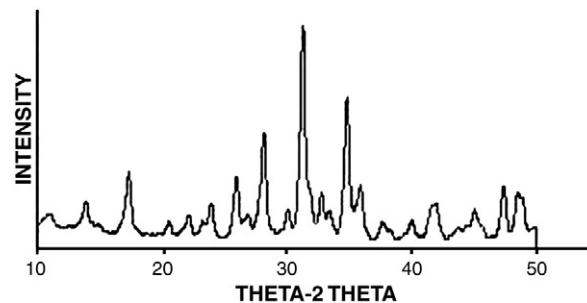


Fig. 2. X-ray diffraction pattern of hydroxyapatite core particles obtained from the mixture of calcium nitrate and di-ammonium hydrogen phosphate.

10 min . During the first period, about 40% of the absorbed OVA was released into the medium. Subsequent release of OVA was very small and decreased over time. The cumulative amounts of OVA released at 50 min reached at least 90% .

3.4. In process stability studies

The ultimate stability of a protein containing preparations can often be a function of the physical and chemical conditions to which it was exposed during processing. Therefore, the primary aim of the in vitro stability studies was to determine the structural integrity of antigen/protein during the preparation of aquasomes by SDS-PAGE. Fig. 5 clearly indicates a visible band of pure as well as extracted antigen at the same loci. This in turns reveals that the formulation procedures used do not irreversibly aggregate or cleave the antigen. The data suggest that there was not any deleterious effect on the OVA integrity during the

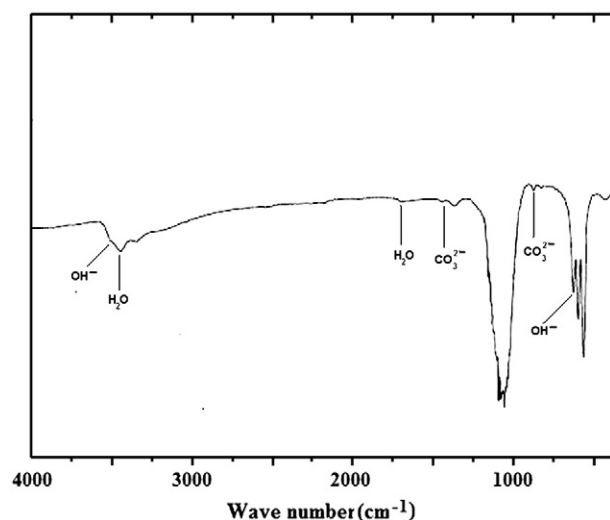


Fig. 1. FTIR spectra of hydroxyapatite core particles obtained from the mixture of calcium nitrate and di-ammonium hydrogen phosphate.

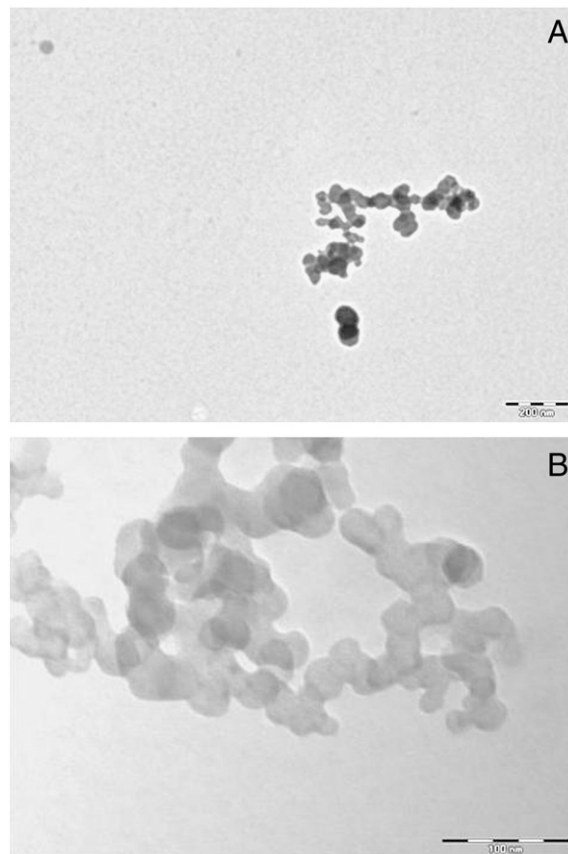


Fig. 3. TEM image of antigen-loaded formulation of: (A) plain hydroxyapatite ceramic core and (B) OVA adsorbed aquasomes.

Table 1
Characterization of formulations.

	Sugar adsorbed (%)	Zeta potential (mV)	Size (nm)	Polydispersity	OVA loading ($\mu\text{g mg}^{-1}$)
Hydroxyapatite core	NA ^a	$+3.20 \pm 1.72$	39 ± 11	0.28	ND ^b
Blank Aquasomes	2.887 ± 0.46	-6.12 ± 1.1	43 ± 14	0.31	NA
OVA adsorbed aquasomes	ND	-11.34 ± 1.40	47 ± 19	0.32	60.22 ± 2.24

Values expressed as mean $\pm$ SEM.^a NA: Not applicable.^b ND: Not determined.

preparation of aquasome formulations. It has been reported that trehalose increases the transition temperature (ΔT_m) of proteins to the maximum and polyhydroxyoligomeric coating provides water like environment in extreme desiccated conditions thus it proved to be one of the best candidates as a universal stabilizer because of its inertness toward protein surfaces [14].

3.5. In vivo immunological response

The serum levels of OVA-specific IgG isotypes elicited in BALB/c mice after two doses of 10 μg of OVA (equivalent dose with alum or adsorbed on aquasomes) are depicted in Fig. 6. Comparable titer of IgG1 antibodies was detected in sera of the animals, immunized with OVA adsorbed aquasomes or inoculated with OVA-alum. On the other hand, immunization with naïve OVA in PBS or blank aquasomes was not able to induce any OVA-specific antibody response. Furthermore, an enhanced and significant immune response was also observed after the booster injection in OVA aquasomes immunized mice as compared to OVA in PBS ($p < 0.001$). After the boost, OVA adsorbed aquasomes induced higher antibody response compared with the primary response, indicating induction of memory cells after priming ($p < 0.05$). Negligible IgG2a primary and secondary response was elicited by the OVA alone, while a weak IgG2a antibody titer was elicited by OVA alum in the secondary response only. In contrast, mice injected with OVA adsorbed aquasomes induced high levels of IgG2a. The consistently higher IgG2a anti-OVA titers obtained, in both primary ($p < 0.001$) and secondary ($p < 0.02$) responses, in mice treated with OVA adsorbed aquasomes than in mice treated with OVA alum were observed in all mice. Because IgG1 is driven by IL-4 (Th2), and IgG2a is driven by IFN- γ (Th1) an increase of IgG1/IgG2a ratio after vaccination indicates a Th2 response, and a decrease in this ratio after immunization indicates a Th1 response. Alum induces almost only a Th2 response, while OVA adsorbed aquasomes elicit both Th1 and Th2 responses as demonstrated by the high IgG2a and IgG1 anti-OVA antibody responses. These data were confirmed by studying the cellular immune response. Significantly elevated levels of antigen specific IFN- γ and IL-4 release were detected in spleens of

mice immunized with OVA aquasomes as compared to mice treated with OVA alum ($p < 0.01$) and OVA in PBS ($p < 0.001$) (Fig. 7).

3.6. Mice sensitization and induction of active systemic anaphylaxis

BALB/c mice were sensitized with 50 mg OVA-Alum by intraperitoneal route at days 0 and 7. Once the allergic status to OVA was confirmed, mice were immunized intradermally at days 13, 16 and 19 with either OVA-aquasome or OVA-alum (as positive control; 3 mg OVA per immunization). Finally, at day 34, mice were challenged with 1 mg of OVA (intraperitoneal) to provoke a systemic anaphylaxis. At day 42, IgE antibody serum titer was significantly lower in group immunized with OVA-aquasomes as compared to the group immunized with OVA-alum ($p < 0.001$). Present observation is consistent with previous report which demonstrated that calcium phosphate nanoparticles (CAP) induced lower IgE titer as compared to alum [29].

In order to estimate the IgE-mediated mast cell degranulation, the serum histamine level from the immunized animals was quantified 30 min after the intraperitoneal challenge. Fig. 8 summarizes these results. The histamine secretion in mice treated with OVA aquasomes was significantly reduced as compared to the OVA-alum ($p < 0.05$). Likewise, the decrease of the body temperature was significantly lower in animals treated with OVA aquasomes than with OVA Alum ($p < 0.01$ and $p < 0.001$) compared with basal body temperature (Table 2). The severity of the anaphylactic shock induced in the animals by both

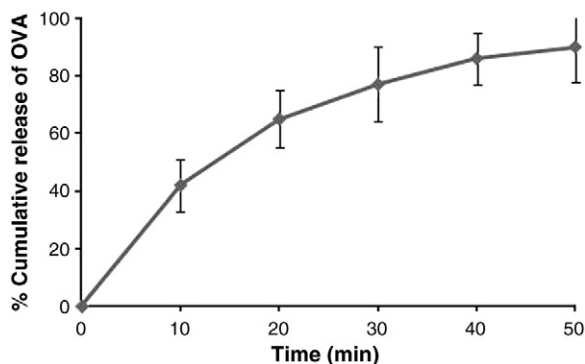


Fig. 4. In vitro release of OVA from aquasomes in PBS. Data express the mean of the cumulative amount of OVA released vs. time (mean $\pm$ S.D, $n = 3$).

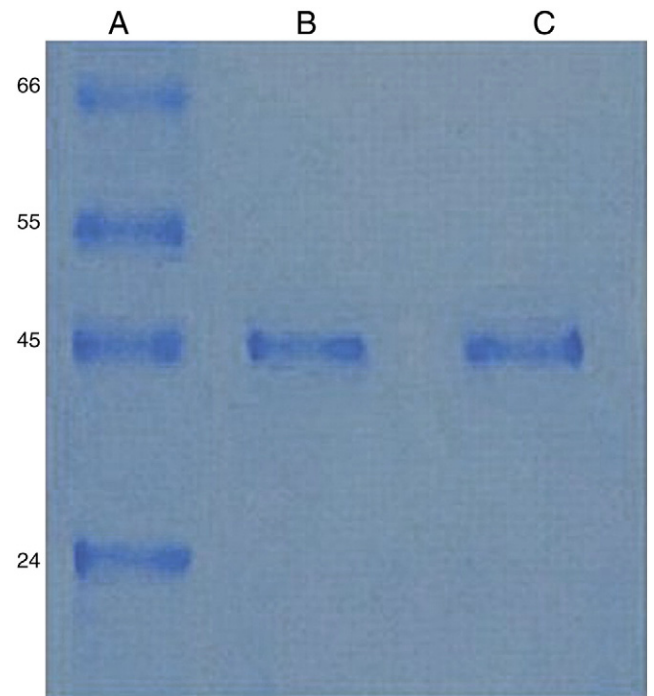


Fig. 5. SDS-PAGE analysis of allergen formulations. Left to right—lane 1: protein marker; lane 2: plain antigen; lane 3: OVA adsorbed aquasomes.

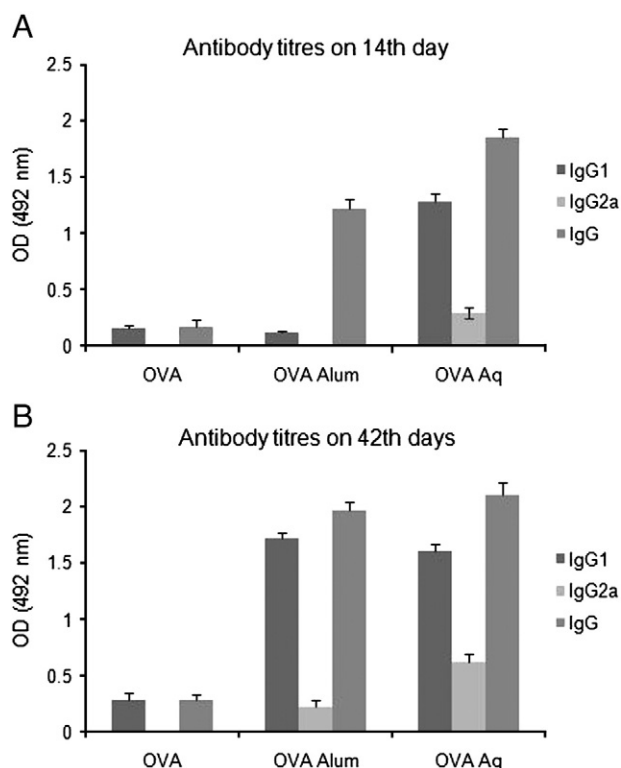


Fig. 6. Serum OVA-specific IgG isotype titers at weeks 2 and 6 (mean $\pm$ S.D., $n=6$). Female BALB/c mice were intradermally immunized twice at weeks 0 and 2 with OVA (10 μ g) alone as a control, or in combination with alum or aquasomes. Blood samples were collected at weeks 2 and 6. OVA-specific antibody titers were measured by an indirect ELISA as described in the text.

formulations was evaluated by measuring cyanosis, activity and piloerection (ruffled or erect fur). OVA aquasome immunized mice displayed a normal activity and motion with no cyanosis, while mice treated with OVA-alum exhibited a total lack of mobility and a high degree of cyanosis. So, animals treated with OVA aquasome displayed less intense symptoms than the control group. Finally, the death rate was also lower for the aquasome immunized animals than those treated with OVA-alum (50% vs. 80%, respectively).

4. Discussion

Aluminum salts are widely used adjuvants for human vaccines. Over the past 25 years, multiple attempts have been made to find

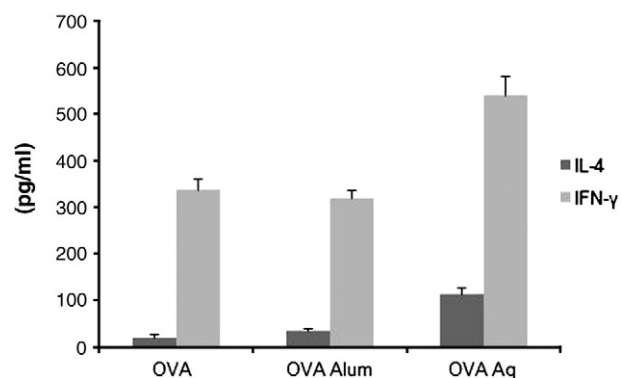


Fig. 7. Cytokine level of IFN- γ and IL-4 in spleen homogenates (in pg/ml) obtained from BALB/c mice 42 days after intradermal immunization 10 μ g ovalbumin (OVA) with one of the following: OVA in solution (OVA), alum adsorbed OVA and OVA adsorbed aquasomes (mean $\pm$ S.D., $n=6$).

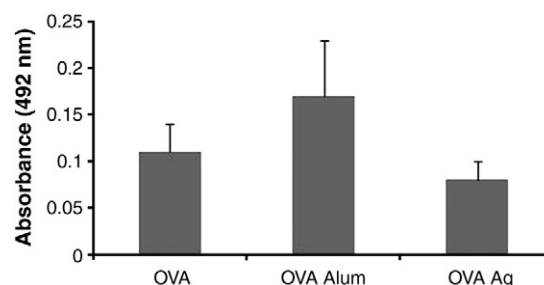


Fig. 8. Evaluation of total IgE in sera from BALB/c mice during the sensitization and challenge study. Mice were sensitized to OVA (50 mg OVA by an intraperitoneal route, days 0 and 7) and immunized with 10 μ g OVA, divided into three doses (mean $\pm$ S.D., $n=6$).

novel adjuvants or even replace them by vehicles which have the ability to induce cellular and humoral immune responses. In recent years, the potential of aquasomes as a vaccine delivery system has been investigated with a variety of antigens [12,13,18,30]; but none of the study reported the suitability of these fascinating protein delivery systems in the field of allergy. Aside from the ability of aquasomes to elicit antibody responses, there is accumulating evidence of their ability to elicit cell-mediated immune responses [18,30,31]. Moreover, when working with biological molecules such as proteins, an essential aspect is the maintenance of their conformational properties during the fabrication process and storage. Thus, in this work, aquasomes have been used as delivery vehicle/adjuvant and for the surface adsorption of OVA (model allergen) without altering the antigenic and immunogenic properties of the protein/allergen [12,14]. Size of the particles plays an important role if surface adsorption is the method of choice for antigen/allergen loading on the delivery systems. Therefore, we prepared ultrafine aquasomes with high allergen loading although the nature of protein/antigen also plays an important role in adsorption mechanism.

When mice are immunized with conventional adjuvant alum intradermally, the humoral type immune response, i.e. antibody response was more strong and effective than cellular type immune response, since the exogenous antigen is mainly presented by B cells through the MHC class II pathway to Th2 cells. In case of aquasomes, antigens are presented partly by the B cell through the MHC II pathway leading to a good antibody response, while part of the antigen is cleaved within the antigen presenting cells and presented through MHC class I pathway, leading to a Th1/CTL response [13,14,16,18]. As it is well known, immunotherapy in humans should be based on the decrease of a Th2 response by enhancing the Th1 response, and this balance is achieved by increasing the activity of the Treg lymphocytes [7]. Treg cytokines (IL-10, TNF- α) play a key role in preventing IgE and Th2 expansion [22,23].

Moreover, in this study, cytokine production (i.e. IFN- γ and IL-4) for the OVA-alum group was negligible; further confirming the poor immunogenicity of OVA in the presence of alum. On the contrary, the aquasome groups produced significant levels of IFN- γ and IL-4; a cytokine profile consistent with a Th1 cellular immune response [21,22].

In this study, we also compared the level of protection against an OVA challenge after immunization with different OVA vaccine formulations. The adsorption of OVA appears to be a more effective and safe strategy for protecting against an anaphylactic shock in sensitized mice.

When OVA-aquasome formulations were intradermally administered to mice, OVA-specific IgG2a levels (Th1 marker) increased. The high specific IgG levels induced by OVA aquasome and OVA-alum would be able to counteract the production of IgE. Early studies indicated that calcium phosphate particles produce strong adjuvant effects, induced less immunoglobulin E (IgE) than aluminum

Table 2

Systemic anaphylactic reactions and body temperature measurement 30 and 10 min after intraperitoneal challenge, respectively, and death rate 24 h later.

	Body temperature (°C)	Activity	Cyanosis	Piloerection	Death rate (%)	Plasma histamine increase ^a
OVA-alum	34.0 ± 1.5	—	+++	++	80	770 ± 29
OVA-aquasomes	35.7 ± 0.9	++	+	++	50	246 ± 17

^a Plasma histamine increase (ng/ml) 30 min after the intraperitoneal challenge (day 34) with 1 mg ovalbumin (OVA) in mice sensitized with OVA (50 mg OVA at days 0 and 7) and immunized with 3 mg OVA (adsorbed in alum or adsorbed in aquasomes at days 13, 16 and 19). Basal histamine level: 9.845 ng/ml. Data are reported as mean ± SD. $P < 0.05$ comparing both treatments with ANOVA, followed by Tukey's test.

adjuvants, and elicited only minimal local irritation in animal experiments and human clinical trials [29].

Nevertheless, the balance between both antibodies would be in favor of OVA-aquasome because of the lower IgE level. Thus, IgG antibodies would block the allergen, avoiding its interaction with IgE antibodies [22]. In this way, IgG competes with IgE for allergen binding and inhibit IgE-mediated granular release of mast cells and basophils. This partial deviation of the Th2 environment induced by OVA-aquasome would be responsible for the improvement in the symptoms associated with anaphylactic shock, such as a less increase of histamine level in serum, a lower decrease of the body temperature and a lower mortality rate than OVA-alum-treated mice.

As far as toxicity to ceramic nanoparticles is concerned, phase I study in healthy volunteers showed that calcium phosphate nanoparticles are safe and non-toxic when administered intradermally [31] although well designed acute and long term safety studies are warranted before these particles are accepted for clinical trials.

5. Conclusion

In summary, this report demonstrates that OVA adsorbed aquasomes are able to induce a strong T cell specific proliferative response with a cytokine profile suggestive of a Th1 response, prevention of anaphylactic reactions and maintenance of low titers of IgE, without abrogation of Th2-mediated responses. This suggests that aquasomes could have possible implications in the future of peptide-based vaccines against allergic disorders.

Acknowledgements

Authors are thankful to physics department of Guru Ghasidas Vishwavidyalaya, Bilaspur and All India Institute of Medical Sciences, New Delhi for conducting XRD and electron microscopy experiments respectively.

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