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Aquasomes—A Nanoparticulate Approach for the Delivery of Antigen

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The development of compound that enhances immune responses to recombinant or synthetic epitopes is of considerable importance in vaccine research. Of the many different types of immunopotentiating compounds that have been researched, aquasomes are of considerable promise, because of their potency and adjuvanticity. Aquasomes were prepared by self-assembling of hydroxyapatite by co-precipitation method and thereafter preliminary coated with polyhydroxyl oligomers (cellobiose and trehalose) and subsequently adsorbed with bovine serum albumin (BSA) as a model antigen. The prepared systems were characterized for size, shape, antigen-loading efficiency, in vitro antigen stability, and in vivo performance. BSA-immobilized aquasomes were around 200 nm in diameter and spherical in shape and had approximately 20–30% BSA-loading efficiency. The immunological activity of the formulated aquasomes was compared with plain BSA and better results were observed. Studies also indicated that aquasome formulations could elicit combined T-helper 1 (Th1) and Th2 immune response.

Keywords aquasomes; nanoparticles; hydroxyapatite; vaccines; bovine serum albumin; trehalose; cellobiose

INTRODUCTION

Vaccines are amongst the most widely used pharmaceuticals, providing significant benefits to human health through the eradication of smallpox, conferring protection against dreadful diseases such as polio, measles, whooping cough, cancer, malaria, AIDS, diphtheria, and hepatitis. Most vaccines are designed as a prophylactic measure, that is, to stimulate the immune response so that on subsequent exposure to the particular infectious agent, the extent of infection in the vaccinated individual is so low that disease does not occur.

Recombinant production constitutes one of the dominating strategies for the generation of subunit vaccine candidates. Protein immunogens (subunit vaccine candidates), which are typically not immunogenic in themselves, are normally administered with an adjuvant to improve their immunogenicity. All currently licensed vaccines induce mainly a humoral immune response owing to the use of adjuvants such as alum. Alum has a good safety record, but comparative studies show that it is a weak adjuvant for antibody induction to protein subunits and a poor adjuvant for cell-mediated immunity (CMI). Moreover, alum adjuvants can induce IgE antibody responses and have been associated with allergic reactions in some subjects. Storage and distribution practices can often challenge the integrity of many alum-based vaccines (Brandau, Jones, Wiefthoff, Rexroad, & Middaugh 2003; Gupta & Siber, 1995; He et al., 2000; O'Hagan, 2001).

Several adjuvants at various stages of preclinical and clinical testing have proven effective as adjuvant for enhancing both humoral and cellular immune responses against immunogens. These novel carrier adjuvants have the potential to present immunogens through desired pathway (cytosolic or endocytic pathway) and enhance the Th1 or Th2 type of immune response. The use of novel carrier adjuvants (e.g., microparticles, nanoparticles, emulsions, liposomes, niosomes, virus-like particles, and vesosomes) has been evaluated for improving the immunological response against week immunogens (Brennan & Dougan, 2005; Gupta et al., 2005; Hillman, 2002; Jaganathan & Vyas, 2006; Mishra et al., 2006).

Aquasomes-based vaccines offer many advantages as a vaccine delivery system. Both cellular and humoral immune responses can be elicited to antigens adsorbed onto the surface of aquasomes. Aquasomes are ceramic-based nano-sized carriers that consist of hydroxyapatite (HA), oligosaccharide (trehalose, cellobiose), and the target protein antigen. Outer surface of aquasomes on which antigens are noncovalently linked consists of polyhydroxyl oligomers or sugar molecules such as cellobiose, trehalose, maltose, sorbitol, and lactose, in addition to allosteric effectors such as pyridoxal-5-phosphate and sodium citrate which create a quasi-aqueous film that

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prevents the denaturation or degradation of the protein. Carbohydrate film on ceramic particle retains the drugs spatial properties, for example, three-dimensional conformations, a freedom of internal molecular rearrangement induced by intermolecular interactions, and a freedom of bulk movement (Arakawa & Timsheff, 1992; Goyal et al., 2006; Hatley & Blair, 1999; Irma, Rodrigo, Jose, & Carlos, 2007; Kossovsky, Gelman, Hnatyszyn, Sponsler, & Chow 1993; Kossovsky, Sponsler, & Hnatyszyn 1995; Rexroad, Wiethoff, Jones, & Middaugh, 2002).

Aquasome-based antigen carriers may reduce the side effects and enhance the duration of the immune response. Furthermore, aquasome-based vaccines would be biocompatible, economical, and simple to manufacture. In addition, it would have the potential to selectively trigger a defined class of immune response such as the T-helper 1 (Th1) $CD4^+$ T-cell response and CMI and have equal applicability for any new-generation antigens.

In the present experiment, aquasomes containing HA coated with oligosaccharide and antigen were prepared. Aquasomes were prepared by self-assembling of HA by co-precipitation method and thereafter by preliminary coating of polyhydroxyl oligomers (cellobiose and trehalose) and subsequently by coating of bovine serum albumin (BSA) as a model antigen. Main formulation parameters that were tested included the type of oligosaccharide/antigen (cellobiose or trehalose) and their concentration. BSA was used as a model antigen to examine adjuvanticity and antigen preservation properties of these aquasomes. The prepared systems were characterized for size, shape, surface charge, entrapment efficiency, and in-process protein stability studies. Differential scanning calorimetry (DSC) was used to study the interaction of protein antigen with oligosaccharides and also for the determination of storage stability of aquasomes. The specific immunological responses elicited by the prepared systems were evaluated by subcutaneous administration.

MATERIAL AND METHODS

Materials

All materials used in this study were of analytical grade. The $(NH_4)_2 \cdot HPO_4$, Ca $(NO_3)_2 \cdot 4H_2O$, cellobiose, and trehalose were obtained from Himedia (Mumbai, India). BSA and Triton X-100 were purchased from Sigma Chemicals Co. (Bangalore, India). All the chemicals for ELISA and SDS electrophoresis were procured from Genei Pvt. Ltd., (Bangalore, India). Antibody estimation kit for IgG, IgG-1, and IgG-2a was purchased from Sigma Chemicals Co.

Preparation of Hydroxyapatite by Co-Precipitation Method

HA was prepared by co-precipitation method as reported by Tas (2001). In brief, 3 mL of aliquot of 0.1% (wt/vol) methylcellulose solution was mixed (to act as a dispersant) with 1,440 mL of deionized water containing 0.152 M of Ca $(NO_3)_2$. $4H_2O$ and 0.090 M NH_4 $(PO_4)_2$. Subsequently, 115 mL of 24% NH_4OH was added into the above described opaque solution, and then solution was heated and vigorously stirred using mechanical stirrer (Remi, Mumbai, India) at a temperature of 60–70°C for 3 h on a hot plate. The precipitate that formed was recovered from the supernatant by filtration and subsequently washed five times with deionized water. The filtrate was dried over night at 100°C. HA precursors were finally calcined in an air atmosphere at 1,000°C for 6 h followed by light grinding by hand with a mortar and pestle.

Optimization of Sugars and Antigen Concentration on Ceramic Core by Langmuir Adsorption Isotherm

The stock solution (1,000 mg/mL) of different polyhydroxyl oligomers, e.g., trehalose and cellobiose, was prepared in deionized water. Accurately weighed 50 mg of HA was placed into the each volumetric flask. Cited quantity of sample (trehalose and cellobiose) was taken in the range of 1–10 mL in different volumetric flask and volume was made up to 10 mL. Each flask was shaken vigorously for about 20 min and sonicated for 20 min at 250 W at 2–10°C using a probe sonicator (Soniweld, Mumbai, India). Resultant suspension was stored over night at 2–8°C in refrigerator. Finally, the suspensions were centrifuged at 8765 g for 15 min and the supernatants (1 mL) of each flask were taken, and the amount of adsorbed sugar was determined by the anthrone method.

After optimizing the sugar concentration on HA ceramic core, it was subjected to optimization of antigen (BSA) concentration on preformed aquasomes by following the same method for sugar coating by using different concentrations of BSA in the range of 10–100 μg/mL. The protein concentration in solution was determined by micro-BCA methods while setting a blank of unloaded aquasomes formulation. Finally, unadsorbed antigen was removed by microcentrifugation against 20 mL of phosphate buffer saline (PBS, pH 7.4). The residual pellets were lyophilized overnight (Heto-Holten, Gydevang, Denmark). Concentration of HA-coated sugars and BSA was calculated from the respective absorbance, and Langmuir adsorption isotherm was then prepared and different variables were calculated (b = binding constant; Y_m = optimum concentration for the formation of mono layer on the aquasomal surface).

Characterization of Hydroxyapatite Ceramic Core

Electron Microscopic (SEM) Studies of Hydroxyapatite

The morphological examination of aquasomes was performed using a transmission electron microscope (Philips EM-60, Eindhoven, Netherlands) following the negative staining of phosphotungstic acid solution (2% wt/vol).

Fourier-Transformed Infrared of Hydroxyapatite

Fourier-transformed infrared (FTIR) spectrophotometer was used for structural analysis. The KBr sample disk was prepared using 1% (wt/wt) of HA powder compressed and dried at 100°C. Infrared spectra were recorded in the wave number range of 4,000–400 cm^{-1} (resolution 4.0 cm^{-1}) using FTIR spectrophotometer (Shimadzu, Singapore Science Park, Singapore).

X-ray Diffraction Analysis of Hydroxyapatite

HA ceramic cores were exposed to CuKα radiation (45 kV × 40 mA) in a wide-angle X-ray diffractometer (Philips XRD-6000). The instrument was operated in the step-scan mode in increments of 0.03° 2θ. The angular range was 10–70° 2θ, and counts were accumulated for 1 s at each step.

Characterization of Aquasomes

Confirmation of Coating of Polyhydroxyl Oligomer on Aquasomes

Coating of the polyhydroxyl oligomers, i.e., cellobiose and trehalose, over the surface of aquasomes system was confirmed by concanavalin-A-induced aggregation assay as reported previously (Merz, Horsch, Ruffner, & Rast, 1999; So & Goldstein, 1967). Concanavalin-A solution (100 μg/mL) was added to different sugar-coated HA core suspensions (10 μg/mL) in quartz cuvettes and absorbance was determined at 450 nm as a function of time of 5 min interval using UV-visible spectrophotometer (Shimadzu). Data were subtracted from blank experiment conducted in the absence of concanavalin-A. The adsorption of sugar and antigen on the surface of HA was further confirmed by IR spectroscopy using FTIR spectrophotometer (Shimadzu).

Size and Zeta Potential

The mean particle size and zeta potential of prepared aquasome systems were determined by a photon correlation spectroscopy using Malvern Zetasizer nano ZS 90 (Malvern Instruments Co., Worcestershire, UK). Zeta potential of various aquasomal formulations was obtained from the electrophoretic mobility measurements taken with a Zeta-sizer. Fifty milligrams of different aquasomal formulations was shaken with 1 mL of PBS (pH 7.4) buffer at 37°C for 1 h. The HA was then washed with PBS buffer and resuspended in 10 mL of deionized water. The average of 22 measurements was used. All measurements were made at a constant scattering angle of 90° at 25°C.

Antigen-Loading Efficiency

The antigen-loading efficiency for the aquasomes formulations was determined as reported previously (Lewis, 1996). Briefly, accurately weight antigen-loaded aquasome formulations were suspended in Triton X-100 (0.01%) and incubated in a wrist shaker for 1 h. Then, samples were centrifuged at 14,000 rpm for 15 min and absorbance was determined using micro-BCA methods with set a blank of unloaded aquasomes formulation. Antigen loading was expressed as per unit weight of aquasomes particles (μg of antigen/mg of sample).

In Vitro Antigen Release Studies

In vitro antigen release kinetics indicates the release pattern and availability of protein immunogen (BSA) under simulated condition. In vitro release studies were performed by incubating 100 mg of aquasome formulations in PBS (pH 7.4) at 37°C with continuous stirring. After predetermined time, samples were collected and centrifuged at 14,000 rpm for 15 min and supernatant was collected. The equal volume of medium was replaced immediately after the withdrawal and samples were analyzed for protein contents by micro BCA method.

In-Process Stability Studies

SDS–PAGE was performed to investigate the protein stability and integrity of BSA during formulation of aquasome systems (Laemmli, 1970). BSA was extracted by dissolving the aquasome systems in 2 mL of 5% (wt/vol) SDS in 0.1 N HCl solution. The extracted antigen was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 10% separating gel at 200 V (SCIP, Bio Rad, Hercules, CA, USA) until the Coomassie dye-stained protein band reached the gel bottom.

Differential Scanning Calorimetric Analysis

In this study, differential scanning calorimetric (DSC) was used to analyze the effect of cellobiose and trehalose on the antigen (BSA). DSC analysis of aquasomes formulations were performed by DSC analyzer (M DSC V1.1A TA INST, 2000) having a sample cell (containing formulation) and a reference cell (filled with buffer only).

In Vivo Studies

Female Balb/c mice aged 6–8 weeks, weighing 15–20 g, were used for in vivo studies. Animals were housed in groups of five (six mice in each experimental group) with free access to food and water. Each mouse was immunized subcutaneously with an equivalent dose of 20 μg BSA followed by booster dose after 2 weeks with the same formulations. The Institutional Animals Ethical Committee of Dr. Harisingh Gour University approved the study. The studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. The immune responses of different aquasomal formulations were compared with same dose of alum-adsorbed antigen (20 μg equivalent dose) following the subcutaneous administration. Serum samples were collected from retro-orbital plexus of the mice at the regular interval and assayed for quantification of specific antibodies (IgG, IgG-1, and IgG-2a) by using ELISA-based Sigma Isotyping kit (ISO type-2, Sigma).

Anti-BSA Antibody Detection

Antibody responses in immunized animals were monitored using a microplate ELISA procedure. Micro-titer plates (Nunc-Immuno Plate® Fb 96 Mexisorp, NUNC, Wiesbaden, Germany) were coated with 100 µL/well of serially diluted serum samples in PBS (pH 7.4) and incubated for 1 h at 4 °C. The plates were thoroughly washed with PBS–Tween 20 (0.05%, vol/vol) (PBST). One hundred microliters of each isotype antibody (IgG, IgG-1, and IgG-2a) was added to each well of coated ELISA plates. The plates were incubated for 30 min at room temperature and washed three times with PBST. One hundred microliters of peroxidase labeled goat anti-mouse IgG (Fab Specific) was added to each well. The plates were covered, and after incubation for 30 min at room temperature, washing was repeated. One hundred microliters of tetramethyl benzidine (TMB) solution was added to each well followed by the addition of 50 µL of H_2SO_4 after 15 min. After 15 min developed color was measured at 450 nm using ELISA plate reader (Bio Rad). Endpoint titers were expressed as the log 2 of the reciprocal of last dilution, which gave an optical density (OD) at 450 nm above the OD of negative controls. IgG-2a/IgG-1 fraction was calculated from respective log 2 values of endpoint titers.

Statistical Analysis

Analysis of antibody titers was performed on logarithmically transformed data, and the data were presented as *SD*. ANOVA test was used to compare mean values of different groups. Statistical significance was considered at $p < .05$.

RESULTS AND DISCUSSION

Aquasomes consist of a ceramic core with noncovalently modified carbohydrates surface to obtain nanometric immutable solids, which are then adsorbed with antigen. The main requirement of the core particles is that they should be in nano size. In this study, HA was selected as a core for the preparation of aquasomes. Electron microscopy and particle size analysis by zeta sizer (Malvern, Worcestershire, UK) revealed that prepared HA ceramic cores were smaller and nanometric in size (Figure 1 and Table 1). Crystalline behavior of HA was further confirmed by X-ray diffraction (XRD) analysis (Figure 2). The XRD pattern containing strong extraneous peaks at 31–32, 49–50, and 25–27 (2θ angle) indicates crystalline behavior of HA, which was very identical with the standard diffractogram of HA of International Center for Diffraction Data (ICDD). The prepared HA core was also analyzed by FTIR spectroscopy. The characteristic bands for PO_4^{3-} of HA appear at 473, 564, 602, 962, 1,033, and 1,093 cm^{-1}. The medium sharp peak at 633 cm^{-1} can be assigned to the OH-bending deformation mode and indicates the presence of HA phase. The bands for PO_4^{3-} of the calcined powder at 1,000°C appear at 430, 474, 567, 604, 946, 979, 1,043, 1,093, and 1,126 cm^{-1}, which can be attributed to HA phases (Figure 3) (Tas, 2001).

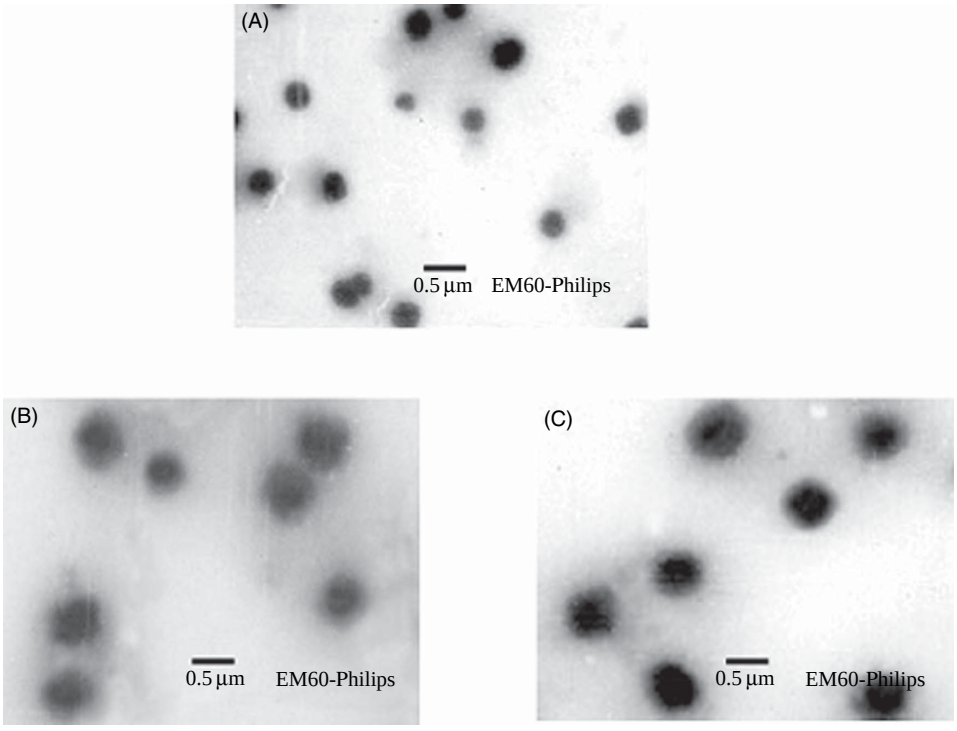

FIGURE 1. Electron microphotograph of different formulations: (A) transmission electron microscope (TEM) image plain hydroxyapatite; (B) TEM image of cellobiose-coated aquasomes; (C) TEM image of trehalose-coated aquasomes.

TABLE 1
General Characterization of Aquasome-Based Formulations

Formulations	Zeta Potential (mV)	% Entrapment Efficiency[a]	*b*	Y_m	PDI	Particle Size Average (nm)
Hydroxyapatite core	+2.73	–	–	–	0.084	150.32
Hydroxyapatite core coated with cellobiose	–10.2	–	6. 425	4.065	0.18	247.26
Hydroxyapatite core coated with trehalose	11.34	–	4.258	3.875	0.14	264.14
BSA-loaded aquasomes (trehalose)	–18.3	25.32 ± 1.26	2.113	0.979	0.24	286.56
BSA-loaded aquasomes (cellobiose)	–20.8	28.52 ± 1.48	2.968	0.995	0.21	291.24
Hydroxyapatite core coated with BSA	–8.57	20.57 ± 2.14	3.427	3.56	0.16	262.18

[a]Mean ± *SD* (n = 4); *b*, binding constant; Y_m, amount adsorbed in mg/g.

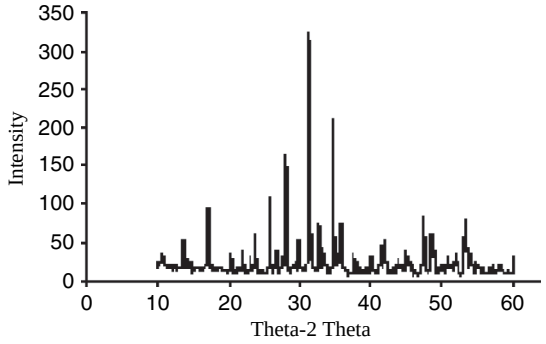

FIGURE 2. X-ray diffraction (XRD) of hydroxyapatite.

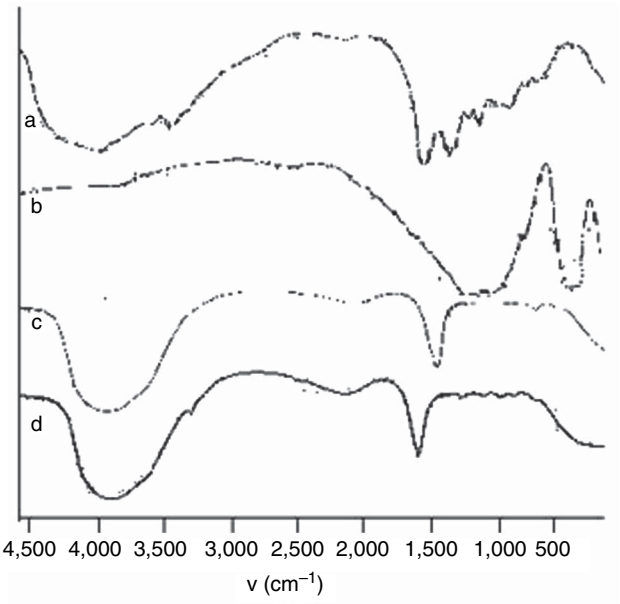

FIGURE 3. Fourier-transformed infrared (FTIR) spectra of different formulation at 24°C. (a) Plain BSA; (b) plain hydroxyapatite; (c) aquasomes formulation coated with cellobiose; (d) aquasomes formulation coated with trehalose.

Adsorption of polyhydroxyl oligomers and antigen onto the surface of HA were optimized by Langmuir adsorption isotherm and shown in Table 1. Both isotherms follow the Langmuir adsorption isotherm pattern satisfactorily. The correlation coefficient of Langmuir isotherm (log C vs. log x/m) was found to be in the range of .980–.999. These adsorption isotherms are very much similar to type IV Langmuir isotherm, which indicated the porous nature of HA and multiple-layer formation and condensation within the pores of solid. The Langmuir adsorption parameters, i.e., binding constant *b* and sugar adsorbed per milligram of HA Y_m, are shown in Table 1. From the observations, it was found that binding constants were in the order of uncoated plain HA > cellobiose-coated aquasomes > trehalose-coated aquasomes, whereas the sugar adsorbed per milligram of HA Y_m was found in the order of uncoated plain HA > trehalose-coated aquasomes > cellobiose-coated aquasomes. So it can be concluded that the adsorption efficiency of trehalose was less than cellobiose, but formed stronger bond with well-packed structure. This can be explained on the basis of epitaxial arrangement of sugars adsorbed or the packing of adjacent sugar molecules adsorbed on HA. Packing of cellobiose is comparatively greater than trehalose, whereas the trehalose particles try to arrange them in a manner in which lowest energy of adsorption is achieved. The sugar bonds to the HA surface because the high interfacial energy of HA is reduced by the adsorbed film, thus lowering the free energy of the system. Hydrogen bonding within the glassy sugar matrix confers three-dimensional stability and retards dissolution. The driving forces for protein adsorption are the formation of protein–trehalose hydrogen bonds and the enthalpically favorable release of water from the surfaces of the trehalose and protein to the bulk aqueous phase. Because the protein is adsorbed, rather than embedded in the matrix (as with conventional adjuvants), most of the protein surface can probably retain its mobility, remaining hydrated and accessible to antibodies.

Coating of polyhydroxyl oligomers (cellobiose and trehalose) onto the surface of HA was further confirmed by concanavalin-A-induced assay and zeta potential measurement. Studies revealed that the trehalose-coated formulation

aggregates much faster than the cellobiose-coated preparation of ceramic core (Figure 4). The adsorption of sugars on HA ceramic core was also confirmed by zeta potential measurement (Table 1). It was noted that on increasing the core (HA) : sugar (cellobiose and trehalose) ratio, the zeta potential of the aquasome decreased from +2.73 to −20.8 mV. The zeta potential of the coated particles decreased up to 1:4 core-to-coat ratios, and then no increase in size was observed. This would be due to the saturation of the free surfaces of the core with the coating material. But, on increasing the concentration of sugars, the size and shape of the aquasomes changed from small uniform particles to irregular large particles; the aggregates of small particles were observed. Data of zeta potentials for different formulations are shown in Table 1. The zeta potential of the plain HA was +2.73, which was reversed to negative value such as −10.2 and −11.34 for trehalose- and cellobiose-coated preparation, respectively. Moreover, the zeta potential was further reduced to negative value after coating of BSA on plain HA, trehalose, and cellobiose-coated HA core − 8. 57, −18.3, and −20.8, respectively.

Simultaneous identification of sugar and antigen on ceramic core was confirmed by FTIR spectroscopy. Figure 3 shows the IR spectra of different formulations and revealed that amide I and amide II bands were observed at 1,657–1,650 cm^{-1} and 1,550–1,540 cm^{-1}, respectively, and OH and NH stretch bands at 3,200–3,400 cm^{-1} and 950–1,050 cm^{-1}, respectively, in infrared spectrum confirm the presence of sugar and protein; broad peaks at 3,200–3,400 cm^{-1} also show the presence of hydrogen bonds between them. Peak of PO_4^- at 536, 950, and 1,015 cm^{-1} also confirm the presence of HA in aquasomes preparation. Antigen loading of the aquasomes was found almost 2.5 times lower in magnitude than the plain HA core without cellobiose coating. Antigen loading of the different aquasomes formulation was shown in Table 1.

In vitro release rate studies were conducted on different aquasomal formulation in PBS (pH 7.4). Samples were withdrawn at regular intervals and antigen content was measured. Cumulative amount of antigen released was plotted against time in order to construct release profile. Figure 5 describes the BSA release pattern from various aquasomal formulations. It was noted that antigen desorbed from nano-carriers follow a typical biphasic pattern. An initial faster release rate was observed within hours followed by slow release rate in second phase. The possible region might be surface desorption of antigen followed by sustained release of antigen from aquasomes matrix. It was noted that cellobiose coated aquasomes released antigen faster than trehalose coated aquasomes and plain HA ceramic core, this may be attributed to the zeta potential differences and adsorption patterns of BSA antigen onto the surface of core material, which restricts its movement from matrix to external aqueous phase.

In SDS–PAGE experiments, protein bands of BSA appeared as a single band in all the formulations in the molecular weight region of ~67 kDa (Figure 6) that corresponds to marker protein BSA at 67 kD, which confirms the in-process stability of antigen in formulation. DSC has been extensively used to study glass transition temperature (T_g) of carbohydrates and

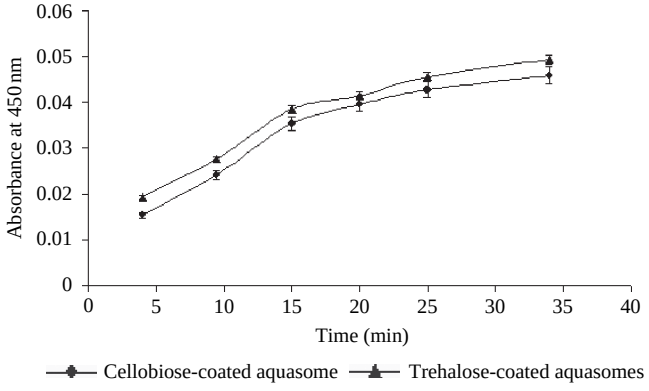

FIGURE 4. Concanavalin-A-induced aggregation of various aquasomal formulations.

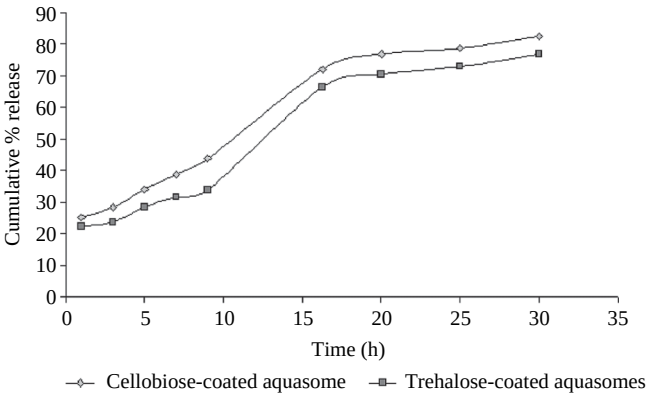

FIGURE 5. Cumulative percentage release rate of different aquasomal formulations.

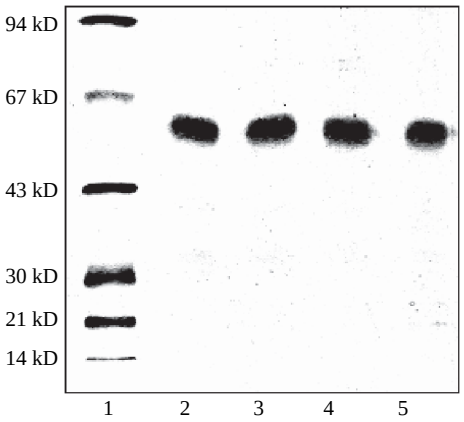

FIGURE 6. SDS–PAGE of aquasome formulations. Left to right—lane 1: protein marker; lane 2: plain antigen; lane 3: hydroxyapatite-adsorbed antigen; lane 4: cellobiose-coated aquasomes; lane 5: trehalose-coated aquasomes.

protein. The glass transition from the glass to rubbery state can be measured using DSC as a change in temperature upon melting of glass. Figure 7 shows DSC thermograms of different aquasome formulations containing BSA as a model protein antigen; the difference in the T_g between different aquasome formulations was relatively small in comparison with plain BSA. As found by others, the T_g of BSA was ~45°C; when adsorbed onto the aquasomes systems, T_g increases to ± 20°C after lyophilization of aquasome formulations. DSC isotherm revealed that all prepared aquasome formulations might be stable at room temperature. Presence of sugars and calcium HA may impart synergistic affect on the stability of prepared aquasome formulations. Many workers have reported the modification of some physical properties of sugars by salts and synergistic effects of sugars and divalent cations on protein stabilization (Carpenter, Crowe, & Arakawa, 1986; Mazzobre & Buera, 1999; Miller, dePablo, & Corti, 1997). The data from the current study's IR, zeta potential, and DSC add strength to the evidence that there is a biochemical interaction between HA, sugars, and BSA (protein), which may extend the protein stability in formulation.

In vivo studies of prepared aquasomes were evaluated following subcutaneous immunization. The antibody titers in serum obtained after subcutaneous administration of aquasomes, alum, and plain BSA is shown in Figure 8. After 28 days, antibody titer from aquasome formulations produced better response as compared with alum, plain HA, and plain BSA groups. This may be accounted for the biochemical nature of aquasomes' nano-ceramic and prolonged release of antigen, and leads to production of better humoral response as compared with pure antigen. However, trehalose-coated aquasomes show better adjuvanticity as compared with other formulation, which could be due to the high degree of molecular preservation by virtue of a significant degree of retained biological/antigenic activity. The polyhydroxyl oligomers act like water because they are enriched with hydroxyl groups and give water-like atmosphere. It was also observed from the results that HA-based BSA formulation exhibited satisfactory antibody titer, but the response was lower when compared with alum-based formulation (Khopade, Khopade, & Jain, 2002; Patil, Khopade, Nagaich, Agrawal, Jain, 2004). However, adsorption of BSA on HA with controlled release of BSA resulted in better immunological response in comparison with plain BSA. Results showed that higher levels of antibodies were detected in the serum of mice immunized with aquasomes, alum, and HA after 2 weeks, and the titer value was comparable with that elicited by pure BSA after 4 weeks (no statistical significant difference $p > .05$).

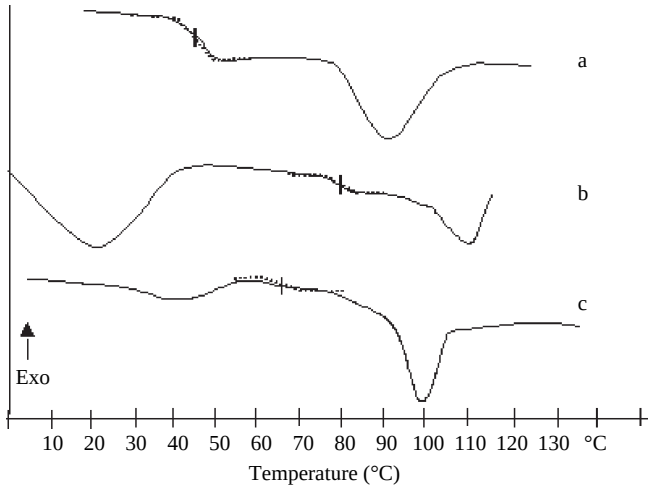

FIGURE 7. DSC thermograms of various aquasomal formulations: (a) plain BSA; (b) aquasome formulations coated with cellobiose; (c) aquasomes formulations coated with trehalose.

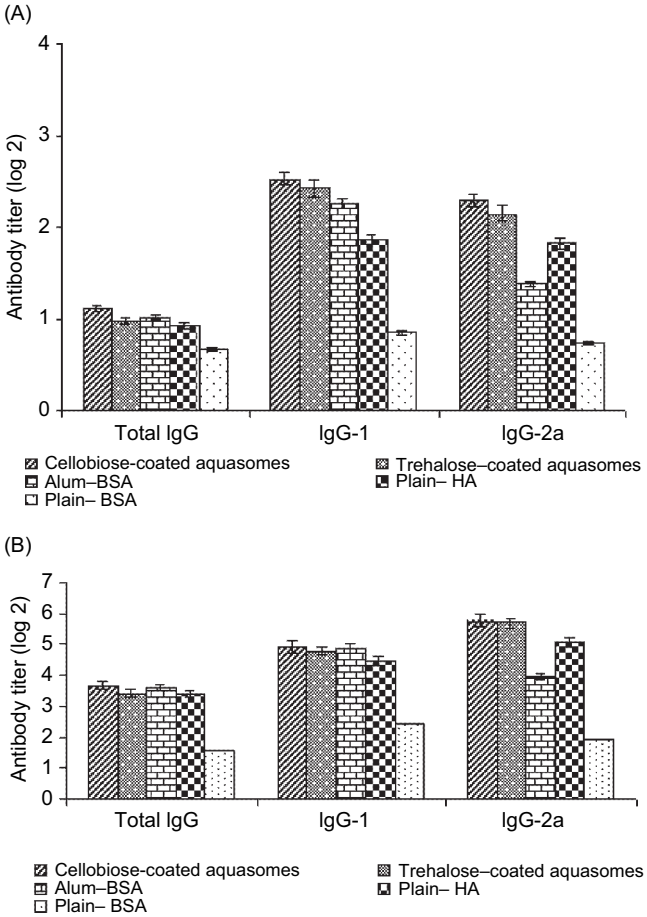

FIGURE 8. Anti-BSA titer of different formulations of aquasomes: (A) after 14th day without boosting; (B) after 28th day with boosting. Induction of serum IgG, IgG-1, and IgG-2a response elicited by the subcutaneous administration of BSA solution (group 5), BSA with HA (group 4), BSA with alum (group 3), BSA with trehalose-coated aquasomes (group 2), and BSA with cellobiose-coated aquasomes (group 1) at a constant dose of 20 µg BSA. Secondary immunization was done after 2 weeks. Values represent log 2 of endpoint dilutions ± *SD*.

Antibodies' isotype levels (IgG-1 and IgG-2a) were estimated in serum 4 weeks after secondary immunization of mice with different BSA vaccine formulations. Figure 8 shows that all the formulations are capable of eliciting a combined serum IgG-2a/IgG-1 response, with predominance of the IgG-2a response in the groups treated with aquasomes; HA plus BSA revealed high levels of IgG-2a/IgG-1. Results are consistent as reported previously by others and suggested that particulate antigens can be processed and presented either by MHC class I or MHC class II by dendritic cells and macrophages, which stimulates Th1 and Th2 lymphocyte subpopulations, whereas soluble antigens are exclusively presented by class II MHC, stimulating the Th2 response (Banchereau et al., 2004). This reflects that aquasomes formulation can elicit both Th1 and Th2 immune response.

Several mechanisms have been proposed for ceramic-based aquasomal antigen delivery system. The epitaxial adsorption of polyhydroxyl oligomer on the surface of HA and the presence of mannose-like binding lectins (MLBLs) (confirmed by concanavalin-A-induced assay) on the aquasome systems signify the immunopotentiation response. Polyhydroxyl oligomers on the surface of aquasome formulations serve as stabilizers and ligands for antigen-presenting cells (APCs) such as MLBLs. APCs such as macrophages and dendritic cells have been shown to express sugar-like mannose receptors on the cell surface. Enhanced immunological response of polyhydroxyl oligomer-coated aquasomes may be due to the better presentation and uptake of the oligosaccharide-cosseted antigen. Moreover, aquasomal nano-carriers could also act as rate-limiting barrier and serve as a local depot for the sustained release of adsorbed antigen. These might be the possible reasons for a well-sustained titer value using the aquasomes carriers. The results are in accordance with the previous findings that suggested transport of immunogen to APCs to be a promising target for gene therapy (Davis & Robinson, 2002; Hegde & Venkatesh, 2007; Saifuddin, Hart, Gewurz, Zhang, & Spear, 2000). Thus, the enhanced transport of conformationally stable antigen leads to better presentation to APCs. APCs contain both MHC-I and MHC-II molecules leading to processing and presentation of antigen via both endocytic and cytosolic pathways leading to elicit both humoral and cellular responses. The uptake of plain BSA by immunologically relevant cells is reported to be minimal and involves only a minor fraction of these cells. Aquasomes were found to be slightly better carrier system compared with other ceramic-based antigen nano-carriers. Thus, aquasomes are a stable, freeze-dried, ready-to-use formulation of chemically defined compounds that combines the efficacy of strong adjuvants with a safety profile of mild adjuvant.

CONCLUSION

Considerable evidence now exists that supports the contention that aquasomes formulations have shown better adjuvanticity. Nano-size aquasome formulations have superior surface inimitability by preserving structural integrity of protein and presenting in a better way to immunological cells; this is one of the major attributes responsible for better immunological response. Although this study suggests a better antibody titer, it needs an extensive work to prove the efficacy and safety of the aquasome formulations before they can be accepted clinically as alternative vaccine(s) versions.

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