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Enhanced stability of horseradish peroxidase encapsulated in acetalated dextran microparticles stored outside cold chain conditions

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ABSTRACT

Micro- and nanoparticles have been shown to improve the efficacy of safer protein-based (subunit) vaccines. Here, we evaluate a method of improving the vaccine stability outside cold chain conditions by encapsulation of a model enzyme, horseradish peroxidase (HRP), in an acid-sensitive, tunable biodegradable polymer, acetalated dextran (Ac-DEX). Vaccines that are stable outside the cold chain would be desirable for use in developing nations. Ac-DEX particles encapsulating HRP were prepared using two different methods, probe sonication and homogenization. These particles were stored under different storage conditions (−20 °C, 4 °C, 25 °C or 45 °C) for a period of 3 months. On different days, the particles were characterized for various physical and chemical measurements. At all conditions, Ac-DEX particles remained spherical in nature, as compared to PLGA particles that fused together starting at day 3 at 45 °C. Furthermore, our results indicated that encapsulation of HRP in Ac-DEX reduces its storage temperature dependence and enhances its stability outside cold chain conditions. Homogenized particles performed better than probe sonicated particles and retained 70% of the enzyme's initial activity as compared to free HRP that retained only 40% of the initial activity after 3 months of storage at 25 °C or 45 °C. Additionally, HRP activity was more stable when encapsulated in Ac-DEX, and the variance in enzyme activity between the different storage temperatures was not observed for either particle preparation. This suggests that storage at a constant temperature is not required with vaccines encapsulated in Ac-DEX particles. Overall, our results suggest that an Ac-DEX based micro-/nanoparticles system has wide applications as vaccines and drug delivery carriers, including those in developing nations.

1. Introduction

Diseases caused by bacteria, viruses, fungi and other parasites (*e.g.* HIV/AID, malaria, tuberculosis) result in approximately 15 million deaths each year, out of which 50% occur in developing nations (Rogers et al., 2010). For many of these diseases, a vaccine may have already been developed. However, high health care costs, inadequate distribution systems, socio-cultural phobias, required sterility with injectable vaccines, and the loss of vaccine potency during storage and transport limit the success of prevention efforts where they are most needed in these developing nations.

The cold chain is a system designed to protect and maintain vaccine viability during storage and transport (Rogers et al., 2010). It begins with the cold storage unit at the vaccine manufacturing plant, extends through the transfer of vaccine to the distributor and then to the provider's office, and ends with vaccine administration. Most vaccines are stable only within a very narrow temperature range and the excursions outside this range severely limit their efficacy (Chen and Kristensen, 2009). The high cost of cold chain maintenance and difficulties in ensuring its quality control due to limited resources and remote point-of-care sites, especially in developing countries, necessitate the development of improved vaccine formulations that are stable under a broader range of temperature (25–50 °C) conditions long-term.

A few new approaches have been developed in the recent years that allow for the storage and distribution of vaccines while avoiding cold chain. Generally, a dried solid state formulation of proteins prepared using methods such as lyophilization have been found to improve their storage stability as compared to liquid

Abbreviations: PLGA, polylactic-*co*-glycolic acid; Ac-DEX, acetalated dextran; MW, molecular weight; 71k, 71,400 molecular weight; HRP, horseradish peroxidase; DCs, dendritic cells; DCM, dichloromethane; PBS, phosphate buffered saline; PVA, poly(vinyl alcohol); BCA, bicinchoninic acid assay; TMB, tetramethyl benzidine; EE, encapsulation efficiency.

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state formulations (Carpenter et al., 1997). Some recent stability studies of vaccines have also demonstrated that dried powder formulations are more stable as compared to liquid formulations outside cold chain (Amorij et al., 2008; Chen and Kristensen, 2009).

Besides improved vaccine stability outside the cold chain, newly developed vaccines are also needed to aid heath care in developing countries. Historically, vaccines prepared from attenuated or heat-inactivated viruses have been very successful in treating diseases such as smallpox, polio and diphtheria. However, due to safety concerns, attenuated viruses are not used for pathogens such as HIV and Hepatitis C (Lauring et al., 2010). Subunit (protein-based) vaccines are considered to be a safer alternative to live or attenuated virus vaccine formulations because while they lack the molecular machinery to cause an infection, they are still capable of inducing protective immunity (Perrie et al., 2008). Yet subunit vaccines are typically weakly immunogenic and require vaccine adjuvants or delivery systems that enhance immunogenicity. The use of polymeric micro- and nanoparticles as a vaccine delivery system is a novel approach that has gained considerable interest recently (Singh et al., 2007; Rice-Ficht et al., 2010). Sub-micron particulate carriers are considered ideal for "passively targeting" of antigen-presenting cells (APCs) *in vivo* due to their phagocytic properties. Since the particulate carrier is too large to be internalized by non-phagocytic cells, it selectively passively targets phagocytes (Manolova et al., 2008). Antigens can be encapsulated in a particulate carrier, injected *in vivo*, and reside at the injection site until being phagocytosed by resident APCs. Additionally, encapsulated antigens can be sterilized by γ-irradiation in a cost-effective manner as compared to the more laborious and expensive aseptic production (Mohanan et al., 2011).

For these passively targeting particle vaccine systems, biodegradable polyesters such as polycaprolactone (PCL) and poly(lactic-co-glycolic) (PLGA) are two FDA approved polymers that have been used (Lu et al., 2000; Solbrig et al., 2007). However, degradation rates of both PCL and PLGA are slow and not tunable on timescales relevant to antigen presentation (Broaders et al., 2009), thus limiting their potential for use in viable protein-based vaccines. Additionally, the utility of polyester-based materials has been somewhat restricted as their degradation produces acidic by-products (Lu et al., 2000), which cannot only harm the local cellular environment but also the protein payload. An ideal vaccine delivery system should not only be biodegradable but also sensitive to the increased acidic conditions present upon phagocytosis. Acetalated dextran (Ac-DEX) is a novel material with the flexibility and biocompatibility of polyester materials, along with the additional benefit of a change in rate of payload release sensitive to physiologically relevant acidic conditions present in the phagosomes of APCs (Bachelder et al., 2008). Ac-DEX is prepared by acetalation of the hydroxyl groups of dextran, a homopolysaccharide of glucose. Dextran is a FDA approved material, and has been extensively used for cryoprotection and various biomedical applications due to its wide availability, biocompatibility, biodegradability, and ease of modification (Hermanson, 1996; Naessens et al., 2005). Ac-DEX is a pH-sensitive polymer and degrades much faster in lysosomal conditions (pH ~5) as compared to physiological pH 7.4 (Bachelder et al., 2008). This pH-sensitivity allows for a significant increase to both CD8+ (major histocompatibility complex I; MHC I) and CD4+ (MHC II) T cells relative to other carrier materials (Broaders et al., 2009). Also, in contrast to above-mentioned polymers, Ac-DEX has tunable degradation rates due to the formation of both cyclic and acyclic acetal groups with significantly different rates of hydrolysis (Broaders et al., 2009). Furthermore, the higher glass transition temperature (160–190 °C) of Ac-DEX as compared to that of PLGA (45–50 °C) indicates a higher stability outside cold chain conditions.

In this study, we have presented a method to improve vaccine stability outside cold chain by encapsulation in Ac-DEX. Horseradish peroxidase (HRP) enzyme, a heme-containing oxidoreductase, with a molecular weight of 44-kDa was chosen as a model antigen to demonstrate the applicability of our approach. To evaluate degradation of the enzyme, we monitored HRP's capability of reducing hydrogen peroxide using tetramethyl benzidine (TMB) reagent. Ac-DEX particles were prepared using probe sonication or homogenization techniques as described previously (Kauffman et al., in press). These particles were stored at −20, 4, 25 or 45 °C for up to 90 days. On days 0, 15, 30, 60 and 90, the particles were removed from storage and various physical and chemical characterization studies were carried out. Scanning electron microscopy was used (SEM) to characterize the morphology of the Ac-DEX particles. The activity of the encapsulated enzyme was monitored over time by adding TMB and measuring the resultant color change spectroscopically. Overall, Ac-DEX particle physical and chemical characterizations were used to evaluate the effects of temperature on encapsulated protein to determine the potential application of this system as a vaccine carrier for application in developing nations.

2. Materials and methods

2.1. Materials

The following materials were obtained from Sigma Aldrich (St. Louis, MO): Horseradish peroxidase type VI (44-kDa, EC 1.11.1.7 at 250–330 units/mg as defined by the manufacturer as 1 unit oxidizes 1 μmol of ABTS per minute at 25 °C at pH 5.0), dextran from *Leuconostoc mesenteroides* (71,400 MW), pyridinium p-toluenesulfonate (98%), 2-methoxypropene (97%, stabilized), 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system, triethylamine (≥99%), fluorescamine, poly(vinyl alcohol) (PVA, 87–89% hydrolyzed, 13–23k MW), poly(DL-lactide-co-glycolide) (PLGA, 85:15 lactide:glycolide, 50,000–75,000 MW), sodium acetate, dimethyl sulfoxide (DMSO; anhydrous, 99.9%), deuterium oxide (D_2O, 99.9 at.% D, 0.75 wt% TSP), and deuterium chloride (35 wt% solution in D_2O, 99 at.% D). Glacial acetic acid (ACS grade), anhydrous ethanol, acetone, and phosphate buffered saline (10× PBS, pH 7.4) were obtained from Thermo Fisher Scientific (USA). Dichloromethane (DCM) was purchased from Honeywell Burdick & Jackson (Muskegon, MI) and Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA), respectively. All the biomaterials, reagents, solvents purchased from commercial sources and used without further purification.

Basic water was prepared by adding 0.01% of triethylamine to nanopure water (Millipore Milli-Q Quantum® BioPak™ water filter) to attain a pH of 9.0.

2.2. Methods

2.2.1. Synthesis of Ac-DEX

Ac-DEX was synthesized from lyophilized dextran as described elsewhere (Bachelder et al., 2008) with some modifications. Briefly, 1.0 g of lyophilized dextran (71k) and pyridinium p-toluenesulfonate (0.0617 mmol) were dissolved in anhydrous DMSO. The resultant solution was reacted with 2-methoxypropene (37 mmol) under nitrogen gas for 5 min before it was quenched with triethylamine. The resultant reaction mixture was then precipitated in basic water (pH 9), vacuum filtered, and lyophilized (−70 °C, 25 mTorr) for two days to yield a fluffy white powder. The white powder was further purified by dissolving in ethanol, re-precipitated, filtered, and lyophilized again to yield a fine white powder.

2.2.2. NMR analysis of Ac-DEX

The relative cyclic to acyclic ratio of acetal substitution of the Ac-DEX systems was determined *via* ^{1}H NMR using a 300 MHz Bruker 300 Ultrashield NMR, as described previously (Broaders et al., 2009). Briefly, Ac-DEX was placed in an NMR tube with D_2O and DCl. Upon complete degradation of Ac-DEX, the degradation products were analyzed by NMR. Acyclic acetals hydrolyze to produce methanol and acetone whereas cycle acetals produce only acetone. The cyclic acetal coverage of Ac-DEX was then determined *via* the relative ratio of acetone to methanol by comparing the integration of the acetone peak (2.08 ppm) to methanol peak (3.34 ppm). All integrations were normalized to the number of protons on each molecule.

2.2.3. Preparation of PLGA or Ac-DEX particles encapsulating HRP by probe sonication method

HRP loaded Ac-DEX or PLGA particles were prepared using a double-emulsion water/oil/water (w/o/w) method similar to that described by (Bachelder et al., 2008). Briefly, HRP solution in phosphate buffer saline (PBS) was added to an organic phase (dichloromethane) containing Ac-DEX (1% weight loading). The resulting mixture was probe sonicated on ice bath for 5 s at 30 W with a 0.5-in. flat tip using Branson Sonifier 450. Then a 3% polyvinyl alcohol (PVA) solution was added to the primary emulsion and probe sonicated again for 30 s at 30 W to form a w/o/w emulsion. The double emulsion was then added to a second PVA solution (0.3%, w/w in PBS) and stirred for 3 h to evaporate the organic solvent. To recover the nanoparticles, each formulation was subjected to centrifugation (Beckman RA-21, Los Angeles, CA, USA) for 10 min at 16,500 rpm and 4 °C. The supernatant was discarded, and the resulting sediment was washed twice with basic water. Finally, the particles were freeze-dried at −70 °C and 25 mTorr for 24 h (Labconco, Kansas City, MO, USA) and stored under specified conditions until further use. Blank particles were prepared using the similar method as described above but without the addition of the enzyme.

2.2.4. Preparation of Ac-DEX particles encapsulating HRP by homogenization method

HRP dissolved in PBS was added to an organic phase (dichloromethane) containing Ac-DEX. The resulting mixture was homogenized for 30 s at 20k rpm using Polytron PT 10-35 Homogenizer (Westbury, NY). To this primary emulsion a 3% polyvinyl alcohol (PVA) solution was added and homogenized again for 30 s to form a w/o/w emulsion. The formed primary emulsion was then immediately added to a second PVA solution (0.3%, w/w in PBS) and stirred for 3 h to evaporate the organic solvent. To recover the particles, each formulation was centrifuged at 4 °C for 10 min at 9000 RCF (relative centrifugal force). The supernatant was discarded and the particles were washed twice with basic water, freeze-dried and stored under specified conditions until further use. Blank particles were prepared using similar method but without the addition of HRP.

2.2.5. Encapsulation efficiency

The encapsulation efficiency of HRP was determined using a fluorescamine (4-phenyl-spiro [furan-2(3H), 1′-phthalan]-3,3′-dione) assay (Le Goff et al., 2011). Briefly, HRP encapsulated Ac-DEX particles were degraded in PBS and 50-vol.% formic acid nanopure water mixture (pH 3.0) at 37 °C for 24 h. After this, the pH of solution was adjusted to 7.4 with a NaOH solution and the samples were aliquoted in 96-well plate. To this, fluorescamine solution (3 mg/ml in acetone) was added and the fluorescence of the resulting solution was read at 400 nm excitation and 460 nm emission *via* FlexStation 3 Benchtop Multi-Mode Microplate Reader (Sunnyvale, CA). The amount of encapsulated protein was determined using HRP as standard. The encapsulation efficiency (EE) of HRP was determined using Eq. (1):

$$\mathrm{EE} = \frac{\mathrm{Protein}_{\mathrm{encapsulated}}}{\mathrm{Protein}_{\mathrm{theoretical}}} \times 100\% \quad (1)$$

2.2.6. Enzyme activity

For enzyme activity measurement, Ac-DEX particles containing HRP were suspended in triplicate in 0.3 M sodium acetate buffer (pH 5.0) at 37 °C for 24 h to facilitate full degradation of the polymer. After this, aliquots were withdrawn and placed in a 96-well plate. Then, TMB was added to the samples and reacted to form a blue-colored product. TMB contains hydrogen peroxide, and this method is similar to the one reported by Temocin and Yigitoglu (2009). The reaction was then quenched with a 0.1 N sulphuric acid solution to form a yellow product. The activity of HRP was determined by measuring the absorbance at 450 nm *via* a plate reader. Blank Ac-DEX particles were analyzed in a similar fashion to determine background absorbance. A standard curve of HRP activity in sodium acetate buffer was prepared to relate absorbance to protein concentration and determine the percent of enzyme activity.

2.2.7. Degradation studies

Degradation studies were carried out as described elsewhere (Bachelder et al., 2008; Broaders et al., 2009) with some modifications. Blank Ac-DEX particles were suspended in triplicate in sodium acetate buffer (pH 5) or in PBS (pH 7.4). The samples were kept at 37 °C on a shaker plate at 150 rpm. At various time points (0–96 h), aliquots were withdrawn and centrifuged (15,000 × *g*, 4 °C, 5 min), and the supernatants were stored at −20 °C in a 96-well polystyrene plate until further use. The supernatants were analyzed using a microplate reductometric bicinchoninic acid (BCA) based assay according to the manufacturer's protocol (Protein Assay Kit; Pierce, Rockford, IN).

2.2.8. Enzyme activity of released protein

Activity characterization of the released HRP from Ac-DEX particles was performed by collecting aliquots in the same manner as described for the degradation analysis. The aliquots were centrifuged (21,000 × *g*, 4 °C, 10 min) and the supernatants were analyzed using TMB reagent as described for the enzyme activity.

2.2.9. Physical and chemical characterization studies of particles

2.2.9.1. Scanning electron microscopy. The surface morphology of the prepared formulation was imaged using FEI NOVA NanoSEM 400. Particles were suspended at 10 mg/ml concentrations in basic water and a small amount (20 μl) was placed on SEM (aluminum) pin stub (Ted Pella; Redding, CA). The samples were allowed to air dry, and then sputter coated with a layer of gold alloy for 120 s before analyzing with SEM.

2.2.9.2. Determination of HRP activity of samples stored under various temperature conditions at different time points. At each time point, particles and lyophilized free HRP from the different temperature conditions were suspended at 1 mg/ml in sodium acetate buffer (pH 5.0) and incubated at 37 °C on a shaker plate (150 rpm). After 24 h, aliquots were withdrawn and analyzed for HRP content using TMB reagent as described previously. The normalized enzyme activity (NEA) of HRP was defined as the ratio of the enzyme activity at a particular time point (EA_t) to the enzyme activity at day 0 (EA_0) and calculated using Eq. (2).

$$\mathrm{NEA} = \frac{\mathrm{EA}_t}{\mathrm{EA}_0} \times 100\% \quad (2)$$

Table 1
Drug loading, yield, encapsulation efficiency (EE), baseline enzyme activity (BEA), and degradation half-lives at pH 5.0 and 7.4 for HRP encapsulated Ac-DEX particles prepared by probe sonication and homogenization techniques. EE and BEA are presented as mean ± standard error ($n = 3$).

Particle type	Protein loading (w/w) (%)	Yield (%)	Encapsulation efficiency (%)	Baseline enzyme activity (%)	Degradation half life (h)	
					pH 5.0	pH 7.4
Sonicated HRP loaded particles	0.736 ± 0.016	55.0	73.6 ± 1.6	54.8 ± 2.9	1.2	72.0
Homogenized HRP loaded particles	0.995 ± 0.013	89.0	99.5 ± 1.3	86.5 ± 1.6	1.2	74.4

2.2.9.3. Differential scanning calorimetry. To investigate the structural changes, if any, in Ac-DEX polymer stored at various temperatures, glass transition temperature was determined using differential scanning calorimetry (DSC) (Mettler Toledo, UK) as described by (Rouse et al., 2007). Briefly, particles were weighed into an aluminum pan, which was then hermetically sealed with a pinhole in the lid. An empty aluminum pan with a pinhole in the id acted as a reference. The sample cell containing the aluminum pans was flushed with low-pressure argon to stabilize the atmosphere before heating to 450 °C at a scan rate of 10 °C/min under the argon atmosphere. The temperature calibration of the instrument was previously performed with indium and zinc to have accurate measurements of both low and moderate temperatures. DSC scans of blank particles prepared by probe sonication and homogenization techniques immediately after lyophilization were used as controls.

2.2.9.4. Statistics. Differences in enzyme activities for the samples prepared using probe sonication or homogenization compared to that of free HRP stored at various temperatures at different time points were evaluated by ANOVA. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of HRP encapsulated acetalated dextran microparticles

The Ac-DEX polymer used in this study was prepared using 71 kDa dextran and had relative cyclic acetal coverage of 43%. Relative cyclic acetal coverage relates to the degradation properties of the polymer (Broaders et al., 2009). Lower cyclic acetal coverage relates to fast-degrading acyclic acetals dominating the acetal population, forming a faster-degrading polymer compared to one with greater cyclic acetal coverage. Using this polymer, HRP was encapsulated in Ac-DEX particles using a double emulsion process (water/oil/water) followed by solvent evaporation. These emulsions were prepared by either probe sonication or high-speed homogenization. Particles prepared using homogenization technique showed a higher yield (89%) and better encapsulation efficiency (99.5%) of HRP as compared to those prepared by probe sonication method, 55% and 73.6%, respectively (Table 1).

In order to determine the effect of encapsulation procedure on the HRP enzyme activity, the activity of HRP enzyme after encapsulation was also measured and compared to the free HRP. Table 1 shows the percent baseline enzyme activities obtained for probe sonicated and homogenized particles. Particles prepared using homogenization method were able to encapsulate 99.5% of the loaded HRP while 86.5% of the enzyme activity was retained. On the other hand, particles prepared by probe sonication method were able to encapsulate 73.6% of the loaded HRP while 54.8% of the enzyme activity was retained. This data suggests that homogenization process is relatively more benign for encapsulating biologically active components as compared to probe sonication method. We anticipate that the high energy associated with probe sonication as compared to mechanical homogenization causes more
denaturation of HRP (Mundargi et al., 2008). It is well realized that all types of encapsulation techniques pose mechanical, thermal and chemical stresses on the system under investigation. However, the information available in literature about the influence of these encapsulation techniques on the integrity and stability of the encapsulated products is rather limited (Mundargi et al., 2008). It is known that proteins encapsulated by emulsion techniques into particulates are susceptible to denaturation, aggregation,

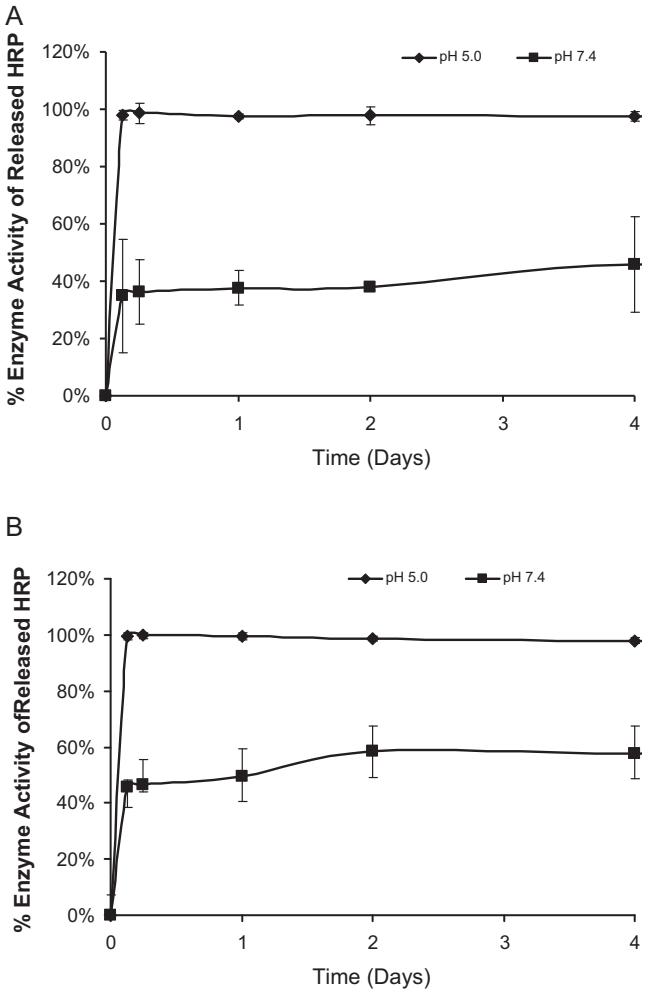

Fig. 1. Enzyme activity of the released HRP from Ac-DEX particles prepared by (A) probe sonication or (B) homogenization at phagosomal (pH 5.0) and physiological conditions (pH 7.4). Each data point is presented as the mean ± standard error ($n = 3$).

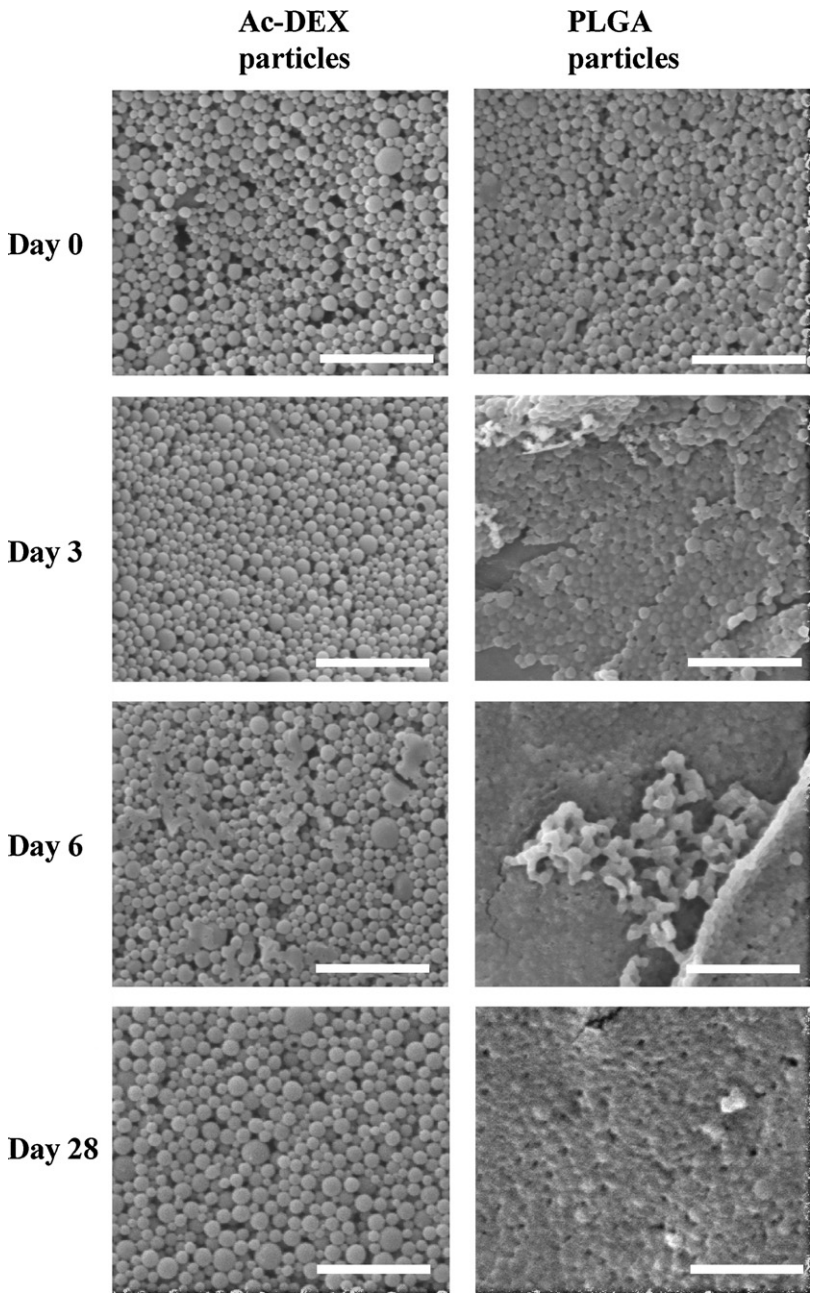

Fig. 2. Scanning electron micrographs of blank Ac-DEX and PLGA particles prepared by probe sonication method. The particles were stored at 45 °C temperatures and imaged on days 0, 3, 6 and 28 (scale bar = 2 μm).

oxidation and cleavage, particularly at the aqueous phase-solvent interface. In addition to these factors, protein denaturation results in a further loss of biological activity of the encapsulated protein. Addition of stabilizers such as surfactants during the primary emulsion phase (*e.g.* tween, polyvinyl alcohol) or molecules such as trehalose and mannitol to the protein phase can improve the protein integrity. Protein stability may also be enhanced if it is encapsulated as a solid rather than in solution (Mundargi et al., 2008).

Particle degradation was investigated at two pH conditions: pH 5.0, which closely mimics APC phagosomal conditions, and pH 7.4, which is that of physiological conditions. Degradation half-life, $t_{1/2}$, was defined as the time required for 50% of the particles to degrade. Due to the acid-sensitivity of Ac-DEX polymer, particles prepared by both techniques showed faster degradation at pH 5.0 ($t_{1/2} \leq 3$ h) as compared to pH 7.4 ($t_{1/2}$ = 72–75 h) (Table 1). The $t_{1/2}$ values obtained here suggest that Ac-DEX particles take over 10 times longer to fully degrade at pH 7.4 as compared to that at 5.0.

Fig. 1 displays the profiles for the enzyme activity of the released HRP encapsulated Ac-DEX particles at pH 5.0 and 7.4. Due to the acid sensitivity of Ac-DEX, HRP was released at a faster rate at low pH when compared to physiological conditions. This observation is consistent with the degradation profiles of probe-sonicated and homogenized particles in phagosomal (pH 5.0) or physiological conditions (pH 7.4) (Supplementary Fig. S1). Accelerated degradation of particles at lower pH indicates the triggered release of encapsulated agents in the phagosomal compartments of phagocytes upon uptake, thus limiting payload release systemically. Rapid vehicle degradation and triggered release of encapsulated protein are essential for achieving efficient antigen

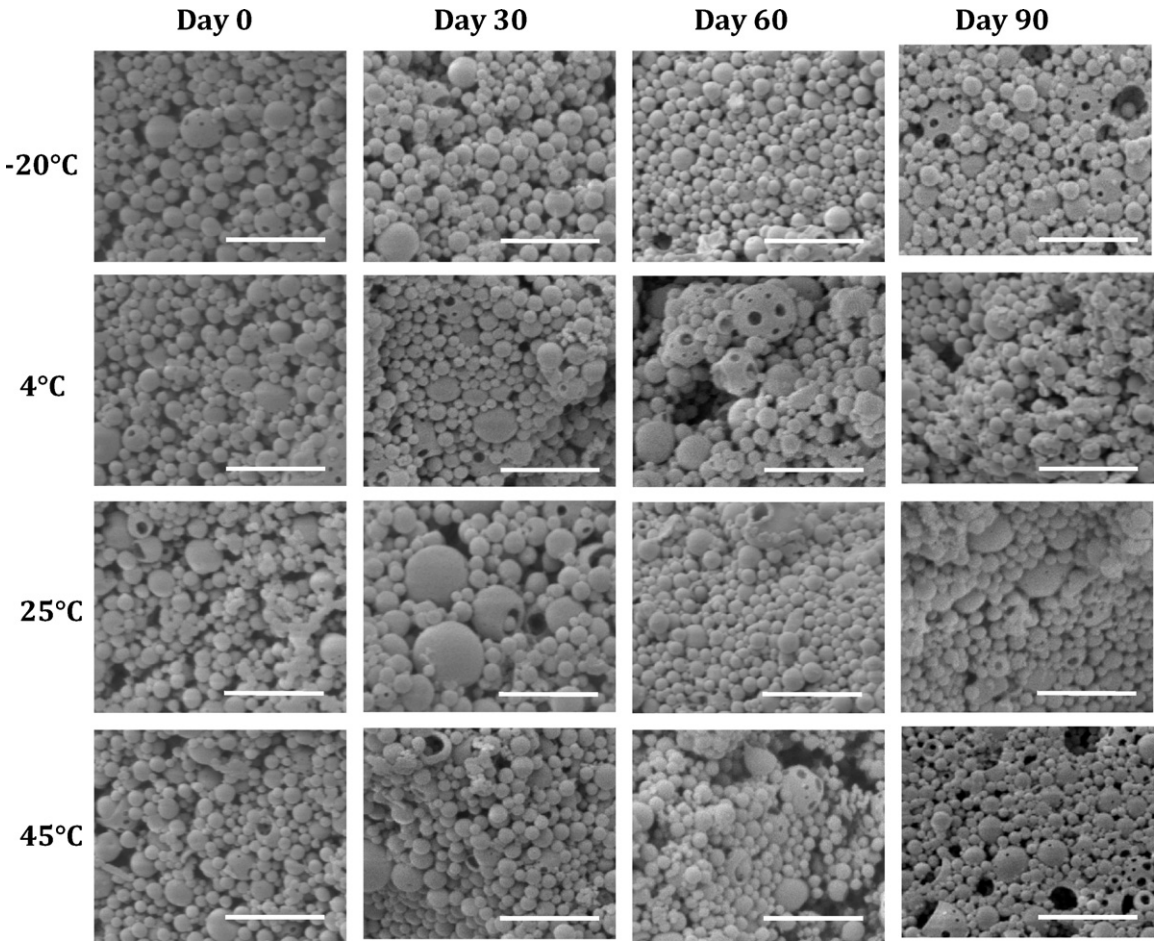

Fig. 3. Scanning electron micrographs of HRP encapsulated Ac-DEX particles prepared by probe sonication method. The particles were stored at different temperatures (−20 °C, 4 °C, 25 °C and 45 °C) and imaged on days 0, 30, 60 and 90 (scale bar = 1 μm).

cross-presentation from APCs (Broaders et al., 2009; Howland and Wittrup, 2008).

3.2. Comparison of morphological stability of blank Ac-DEX and PLGA particles

As PLGA is FDA approved and the most common polymer for vaccine delivery research, we compared the morphological stability of blank PLGA and Ac-DEX particles. These particles were prepared by probe sonication technique and stored at 45 °C for a period of four weeks (Fig. 2). On day 0, both PLGA and Ac-DEX particles were spherically shaped. From day three onwards PLGA particles started losing their structural integrity, and by day 28, PLGA particles completely lost their spherical shape and fused into a sheet-like structure. Since the glass transition temperature for PLGA is around 45–50 °C (Singh et al., 2004), the polymer most likely melted or agglomerated. This kind of phenomenon is detrimental to the potency of PLGA encapsulated vaccines that are accidentally exposed to high temperatures or stored at the higher ambient temperatures that exist in many developing nations. In contrast to PLGA particles, Ac-DEX particles maintained their spherical nature and morphological integrity for four weeks even at such a high temperature. This may be attributed to higher glass transition temperature, T_g, (160–190 °C) of Ac-DEX (Supplementary Fig. S2A). The DSC scans (Supplementary Fig. S2) show no significant activity at temperatures lower than 100 °C, indicating good thermal stability of the particles up to 90 days at various storage temperatures. There is one discrepancy with the probe sonicated sample stored at 45 °C, in which a transition appeared at 60 °C in the scan after 90 days. The presence of this dip is indicative of some change in the sample that is not influencing the higher temperature behavior since the higher temperature transition is still present. We do not have an explanation for this unusual behavior at this point and further studies will need to be performed to identify this discrepancy.

3.3. Morphological characterization and stability of HRP encapsulated Ac-DEX particles

Figs. 3 and 4 show the SEM images of HRP encapsulated Ac-DEX particles prepared by probe sonication and homogenization methods. All the particles were stored at different temperatures (−20 °C, 4 °C, 25 °C, 45 °C) and imaged on days 0, 30, 60, and 90. The particles prepared using probe sonication technique were spherical in shape, 100–200 nm in size, and maintained their morphological integrity over the entire duration of study even at the temperatures outside cold chain conditions (*i.e.* 25 °C and 45 °C) (Fig. 3). In addition, the blank and HRP encapsulating probe sonicated Ac-DEX particles had similar morphology (Figs. 2 and 3) indicating that HRP encapsulation did not affect the particle morphology and long-term stability. The particles prepared using homogenization technique were 1–2 μm in size and porous in nature (Fig. 4). Particle porosity may arise due to the fusion of the water droplets trapped inside the particles during stirring phase of emulsion preparation that are then sublimated during lyophilization (Yang et al., 2000, 2001).

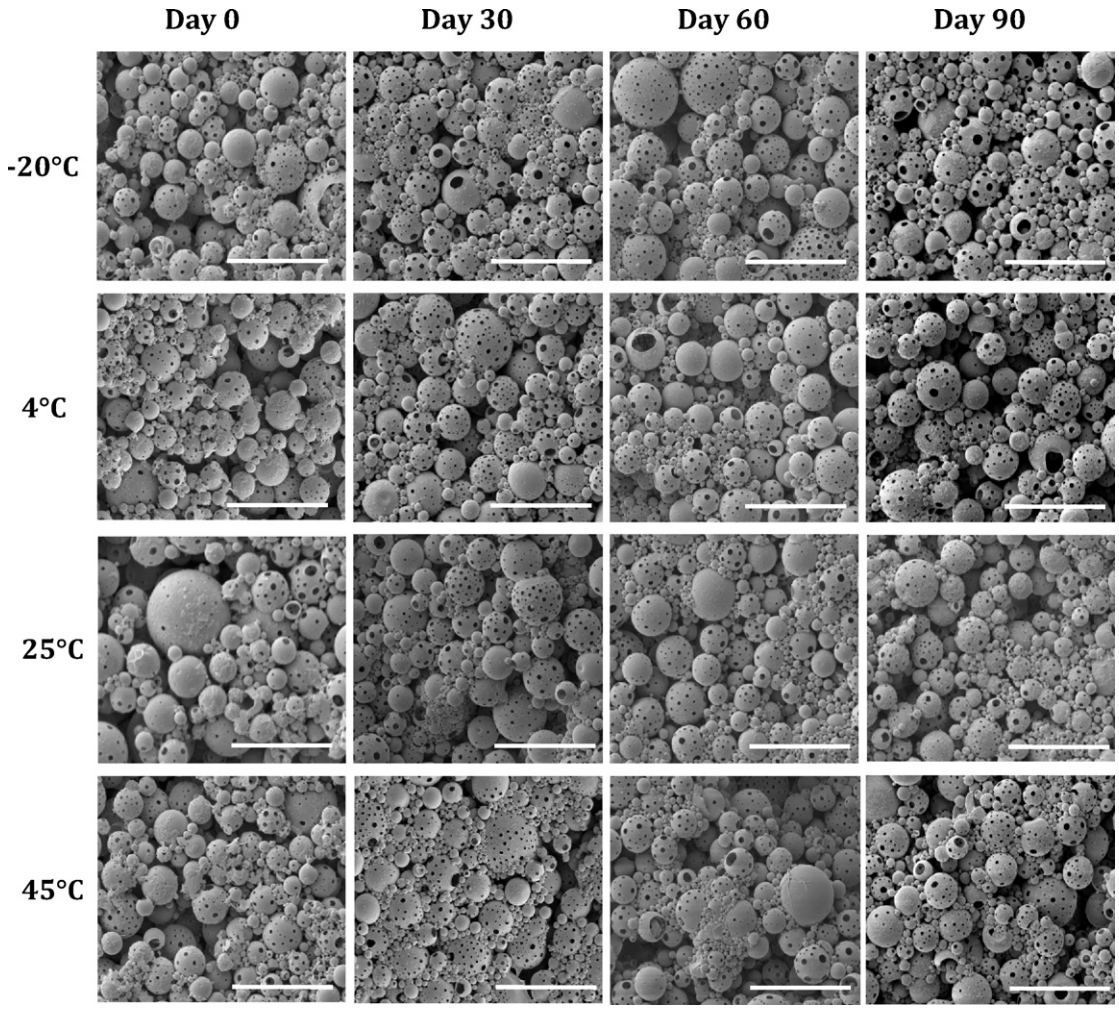

Fig. 4. Scanning electron micrographs of HRP encapsulated Ac-DEX particles prepared by homogenization method. The particles were stored at different temperatures (−20 °C, 4 °C, 25 °C and 45 °C) and imaged on days 0, 30, 60 and 90 (scale bar = 10 μm).

In general, all the homogenized particles maintained spherical morphology and porosity over the entire duration of study under all storage conditions, including high temperatures of 25 °C and 45 °C, without any signs of agglomeration or degradation.

3.4. HRP enzyme activity at various storage temperatures and time points

Fig. 5 relates the storage stability (as measured by normalized enzyme activity) of HRP encapsulated Ac-DEX particles prepared by probe sonication or homogenization method to free HRP at various temperatures (−20 °C, 4 °C, 25 °C, or 45 °C) over time. The maximum denaturation of HRP loaded in the Ac-DEX particles was observed within the first 30 days and later stabilized with time. This is in contrast to the free HRP where the activity decreased gradually with time at all the temperatures. This initial loss of encapsulated HRP activity could be due to the denaturation of the enzyme loosely bound to the particles' surface.

Fig. 6 compares the stability of encapsulated HRP, prepared by probe sonication or homogenization, with free HRP at a given storage temperature. At −20 °C or 4 °C after 15 days of storage, homogenized HRP particles retained enzyme activity of 97–100% of their initial activity, sonicated HRP particles: 72–73% of their initial activity, and free HRP: 89% of its initial activity. Prior research has shown that sonication, or an increase in the energy used to create the emulsion, results in denaturation of the encapsulated enzyme and is probably the reason why sonicated particles have lower enzymatic activity (Carrasquillo et al., 1999; Coleman and Lowman, 2011). On day 30, enzyme activity of homogenized HRP particles was reduced to 70% their initial activity while that for sonicated HRP particles and free HRP dropped down to 52% and 57–62% of their initial activity, respectively. Outside cold chain temperatures of 25 °C or 45 °C after 15 days of storage, homogenized HRP particles retained enzyme activity of 97–98% their initial activity, sonicated HRP particles: 71–72%, and free HRP: 70–83%. On day 30, enzyme activity of homogenized HRP particles was reduced to 68–69% while the activity for the sonicated particles and free HRP dropped down to 40–50% of its initial activity. Further significant losses in enzyme activity were not observed after day 30 under any storage condition for all the Ac-DEX encapsulated formulations. Overall, homogenized HRP particles provided enhanced stability to HRP enzyme under all storage conditions, more importantly outside cold chain conditions of 25 °C or 45 °C, as compared to free or sonicated HRP particles.

No significant differences in enzyme activity were observed at various time points and temperatures for probe-sonicated particles except at 45 °C-day 30 (Fig. 5A). By excluding this time point, the data presented storage temperature-independent stability ($p > 0.05$). The stability of HRP loaded Ac-DEX particles prepared by homogenization method was independent of the storage

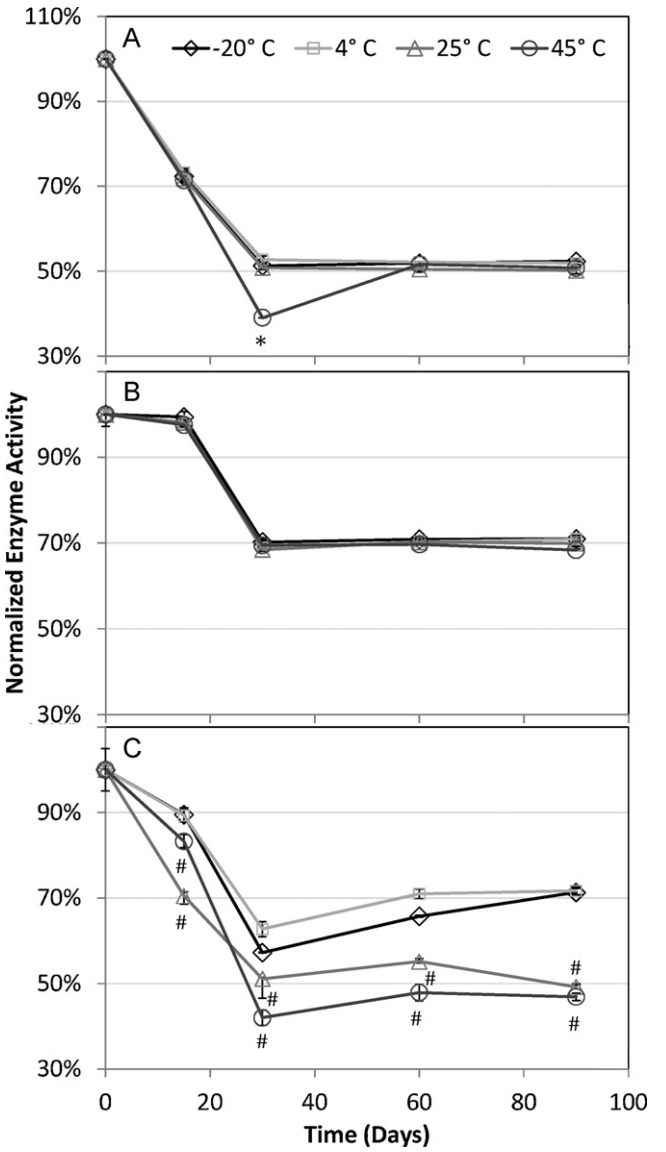

Fig. 5. Storage stability of HRP encapsulated in Ac-DEX particles prepared by (A) probe sonication method, and (B) homogenization method, as compared to (C) free HRP at various temperatures (−20 °C, 4 °C, 25 °C and 45 °C) over time. Measured enzyme activity at a particular time point was normalized to the enzyme activity at day 0. Each data point is presented as mean ± standard error ($n = 3$). An * indicates significance with respect to normalized enzyme activity at −20 °C, 4 °C, and 25 °C ($p \geq 0.05$). A # significance with respect to normalized enzyme activity at 4 °C ($p < 0.01$).

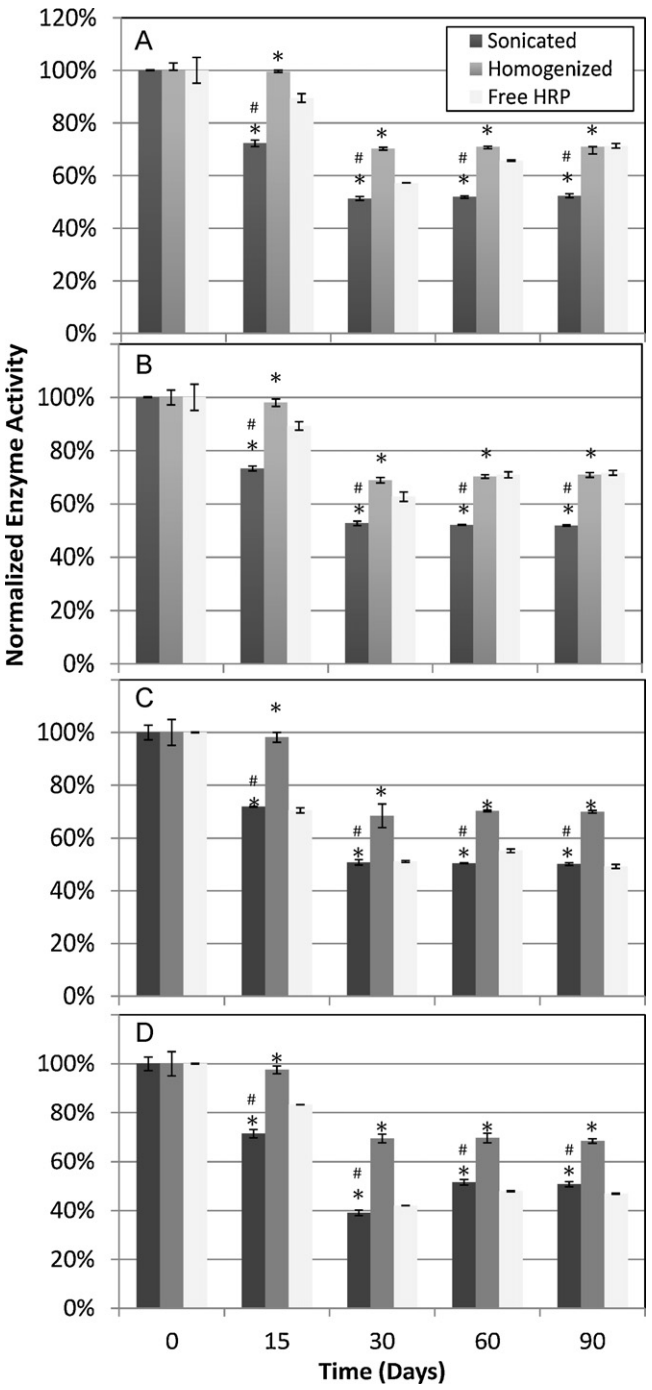

Fig. 6. Comparison of the storage stability of HRP encapsulated Ac-DEX particles prepared by probe sonication or homogenization methods, and free HRP at various time points at different temperatures. (A) −20 °C, (B) 4 °C, (C) 25 °C, and (D) 45 °C. Measured enzyme activity at a particular time point was normalized to the enzyme activity at day 0. Each data point is presented as mean ± standard error ($n = 3$). A * indicates significance ($p < 0.005$) with respect to free protein. A # significance ($p < 0.005$) with respect to homogenized particles.

temperature ($p > 0.05$) at all the time points as demonstrated by the overlapping time profiles at different temperatures (Fig. 5B). There were no significant differences ($p > 0.05$) in the normalized enzyme activity of free HRP samples stored at −20 °C when compared to 4 °C at all time points (Fig. 5C). However, the normalized enzyme activity values at all the time points were significantly less ($p < 0.01$) for 25 °C and 45 °C storage conditions, when compared to 4 °C. The storage stability of free HRP over time follows the order: 4 °C ≥ −20 °C > 25 °C > 45 °C. The maximum enzyme stability at 4 °C is consistent with the storage condition, 2–8 °C, recommended by the manufacturer (Sigma) although stability data provided by the vendor is very limited (only for 12 days). This temperature-independent stability for Ac-DEX encapsulated HRP may be due to the increased conformational stability provided by the Ac-DEX polymer surrounding the enzyme molecule (Temocin and Yigitoglu, 2009). Previous studies have also shown that enzyme denaturation can be limited by its encapsulation or immobilization to a support (Temocin and Yigitoglu, 2009). Immobilization of enzymes can be performed using techniques such as adsorption, covalent attachment or entrapment (Silva et al., 2007). Support material used to entrap the enzyme should be non-toxic, easily accessible and should have large surface area for the enzyme reaction and allow transport of both substrate and product with minimal diffusional restriction (Li et al., 2004).

Several vaccine stability studies have shown that the heat exposure, low temperature-freezing, and repeated freeze–thaw cycles are detrimental to vaccine potency (Matthias et al., 2007; Wirkas et al., 2007). *In vitro* and *in vivo* laboratory studies have demonstrated that freezing of most commonly used subunit aluminum salt (alum) adjuvanted vaccines damages the vaccine-adjuvant matrix, in that the formation of ice crystals overcomes repulsion forces between alum particles, resulting in coagulation and agglomeration of particles and an overall increase in particle size. The net result is a reduction in the potency of the vaccine (Chen and Kristensen, 2009; Matthias et al., 2007). Even though alum adjuvants are considered to be stable at high temperature, excessive heat can damage the protein based antigen portion of the vaccine by altering the tertiary structure of proteins. Similar denaturation is seen with live-attenuated vaccines as well (Chen and Kristensen, 2009). Other vaccine formulations also have stability issues at high temperatures. Those that are stabilized with saccharide additives like trehalose and mannitol, can have dissociation of the saccharides from the protein carrier (Chen and Kristensen, 2009). Further, the heat damage to a vaccine could be the direct result of inadvertent exposure to elevated temperatures or, in the case of lyophilized vaccines, heat shock from the addition of diluent that is too warm. Storage temperature-independent time profiles of encapsulated HRP obtained in this study suggest that Ac-DEX encapsulation improves the thermostability of our model system HRP and has great potential for the development of thermostable vaccines for various diseases.

Other methods have been used to improve vaccine and protein stability at non-ideal storage conditions. A recent study reported the stabilization of lecithin/glyceryl monostearate nanoparticles with protein antigens conjugated onto their surface by lyophilization in the presence of proper excipients (Sloat et al., 2010). An addition of 5% mannitol plus 1% polyvinylpyrrolidone during lyophilization maintained the immunogenicity of their model antigen conjugated onto the nanoparticles after 2.5 months of storage at room temperature or under accelerated conditions (37 °C). It should be noted that in this investigation Ac-DEX polymer by itself was able to improve the thermostability of HRP without the addition of any excipients. Nevertheless, excipients can be added, if desired, to Ac-DEX formulations to further enhance their stability.

4. Conclusions

Loss of vaccine potency due to excessive fluctuations in temperatures during storage and distribution is one of the major concerns for immunization programs. The maintenance of cold chain storage is difficult especially in developing countries with scarce resources. This necessitates the development of thermostable vaccine formulations that are effective. We have previously shown the applications of micro-/nano encapsulated Ac-DEX subunit vaccines in generating improved immune responses (Bachelder et al., 2010; Broaders et al., 2009). By taking HRP as our model protein in this study, we have shown that micro-/nano encapsulation in Ac-DEX polymer reduces the storage temperature dependence and improves the stability of the enzyme. More specifically, homogenized particles provided better insulation to HRP activity at elevated temperatures of 25 °C or 45 °C than probe-sonicated particles. Homogenized particles retained 70% of their initial activity as compared to free HRP that retained only 40% after 3 months of storage at 25 °C or 45 °C. This suggests that Ac-DEX based micro-/nano particulate system by itself can be used for the preparation of thermostable vaccines. By employing high-throughput and mass-production techniques such as spray drying, more affordable vaccine formulations can be manufactured, suitable for distribution in developing countries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.043.

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