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Stability of calcium phosphate nanoparticle adjuvanted outer membrane protein in term of zetapotential

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Abstract
The nanoparticles are widely used in various areas such as drug delivery, vaccine adjuvants, as an antibacterial and so on. The property of these nanoparticles is retained only if they are stable in aqueous solution. The nanoparticles have high surface energy given to their large surface to volume ratio that generally makes them highly unstable and have the tendency to aggregate. There are various methods by which, the aggregation can be prevented such as altering ionic environment, pH or incorporation of protein. The stability can be assessed by measuring the zeta potential. In this study, we have determined the zeta-potential of the *Salmonella* Typhimurium outer membrane protein (OMP) entrapped in calcium phosphate nanoparticles (CAP-NP) at pH 6.0, 6.5, 7.0 and 7.5. It was observed that there was no significant difference among the zeta-potential of calcium phosphate nanoparticle-OMP complex suspended in PBS of pH 6.0, 7.0 and 7.5. However, the zeta-potential at pH 6.5 was significantly lower. The vaccine was found to be stable at pH 7.0 to 7.5 and highly unstable at pH 6.5 in term of zetapotential.

Keywords: Zeta potential, calcium phosphate nanoparticle, outer membrane protein vaccine, stability, pH

Introduction
The zeta-potential is a measure of the electrical charge of particles that are suspended in aqueous solution. It is often the only value that can be used to describe double-layer properties of a colloidal dispersion in colloids, typically the higher the zeta-potential (negative or positive); the more stable the colloid due to the particle repulsion (Hanoar *et al*., 2012) [5]. A value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from highly charged surfaces (Priya *et al*., 2014) [10]. The value of zeta-potential changes depending on some properties of solid/ liquid interface, ionic strength are being considered, and the most important factor that affects zeta-potential is pH. The degree of dissociation of the functional groups depends of the pH of the suspension; therefore the zeta-potential is pH dependent. As far as the biochemical systems are concerned, it is known that enzyme-ligand binding is favored under conditions of electrostatic attraction (Wade *et al*., 1998) [20]. Also, enzyme immobilization is known to depend not only on the chemical interaction specificity, but also on the difference in the surface potentials between the enzyme molecule and the matrix carrier (Schultz *et al*., 2008) [16]. Deviations of zeta-potential of cells from the normal range of values have been used as an indicator of membrane abnormalities (Tokumasu *et al*., 2009) [17]. Charge on the cell membrane, originating from phosphoryl and carboxyl groups of macromolecules that constitute it (Wilson *et al*., 2001) [22], can be manipulated to prevent cellular aggregation, which is an effect detrimental for cellular electrophoresis techniques (Klodzinska *et al*., 2010) [8]. Electrostatic effects have been regularly used for the electrophoretic separation of peptides, and the protein adsorption has been shown to be directly dependent on the magnitude of the difference between the zeta-potentials of the protein and the adsorbent (Cai *et al*., 2006) [3]. It was recently proposed that zeta-potential may play a role in viral-host interactions (Rowell *et al*., 2005) [14], whereas zeta-potential of polioviruses was used as a control parameter during their removal from contaminated waters (Kondo *et al*., 2004) [9]. Zeta-potential has also been used to explain the effect of ions on coagulation in blood, including the effect of thrombosis (Riddick *et al*., 1968) [11]. Recently, the same concept was applied to explain the aggregation of cholesterol particles, demonstrating how a control over zeta-potential may be used to prevent the formation of

pathological cholesteric deposits, including atherosclerotic plaque and gallstones (Uskokovic, 2008) [18]. The idea to manipulate surface charges of interacting species in order to generate complex soft matter morphologies has been, however, pursued to a lesser extent.

In the present study, calcium phosphate nanoparticle adjuvanted outer membrane protein of *Salmonella based vaccine* (CAP-OMP) vaccine was prepared and stability of calcium phosphate nanoparticle-OMP Evaluated at different pH (pH 6, 6.5, 7 and 7.5) of phosphate buffered saline in terms of zeta potential. Finally zeta-potential was estimated experimentally by a device called a Zeta sizer (Nano-zs90).

Materials and Methods

The whole outer membrane protein of *Salmonella* Typhimurium was isolated by Cho-Kim *et al*., (1991) [4]. Briefly, the *Salmonella* Typhimurium was grown in BHI broth up to mid-log phase. The cells were harvested by centrifugation at 10,000×g for 10 minutes. The cells were resuspended in HEPES buffer. The cell membrane was broken by ultrasonication. The cell debris was separated by centrifugation at 10,000×g for 10 minutes. The membrane was precipitated by ultracentrifugation at 100,000×g for 1 hour followed by incubation of membrane fraction in 2% sodium lauryl sarcisonate for 1 hour at room temperature (25 °C). The suspension was ultracentrifuged again at 100,000×g for 1 hour at 4 °C. The pellet of OMP was resuspended in sterile triple glass water.

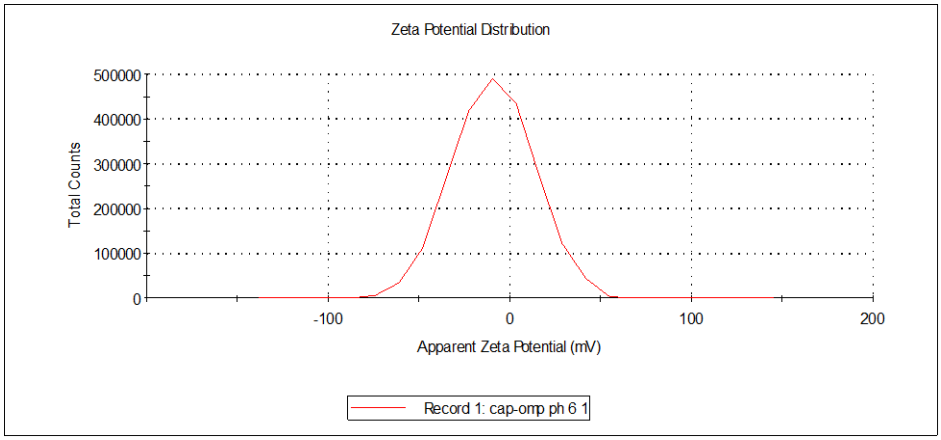
Preparation of calcium phosphate nanoparticle adsorbed outer membrane protein (CAP-OMP) vaccine.

The CAP-OMP was prepared as per the method described by He *et al*. (2002) [6]. Briefly, 1 mg of the OMP was added to a stirring conical flask followed by addition of 7.5 ml of 12.5 mM of calcium chloride and 12.5 mM sodium phosphate (dibasic) in the presence of 1.5 ml of 15.6 mM sodium citrate. The solution was stirred for one hour. The suspension was coated with cellobiose followed by addition of 4 mg of OMP. The resultant CAP-OMP was lyophilized. The total protein (entrapped protein plus the protein coated outside the nanoparticle by cellobiose) of calcium phosphate nanoparticle was estimated by modified Lowry's method. Finally pellets redissolved with PBS (pH 6, 6.5, 7 and 7.5) just before determining the zeta potential. Finally the zeta-potential of CAP-OMP was determined by zeta sizer (Nano-zs90) in IASST institute, Guwahati. The zeta-potential determination was carried out in triplicates.

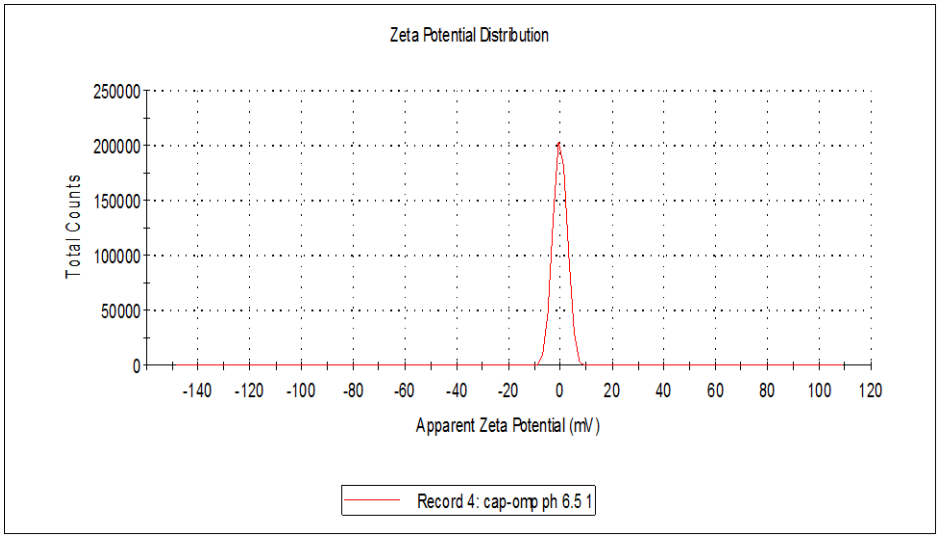
Statistical Analysis

The data were expressed in terms of mean ± standard error of mean. The difference between the different pH was analyzed by one factor ANOVA. The post-hoc multiple comparison of the mean at 95% family-wise confidence level was carried using Tukey's HSD test. The p-value less than 0.05 were considered statistically significant. All of the analysis was carried out in statistical software R (R Core Team, 2018) [12].

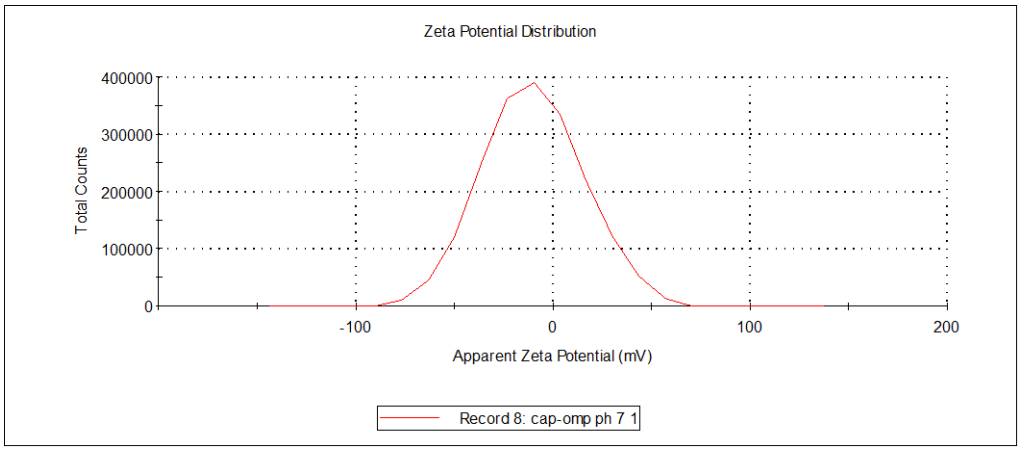
Results and Discussion



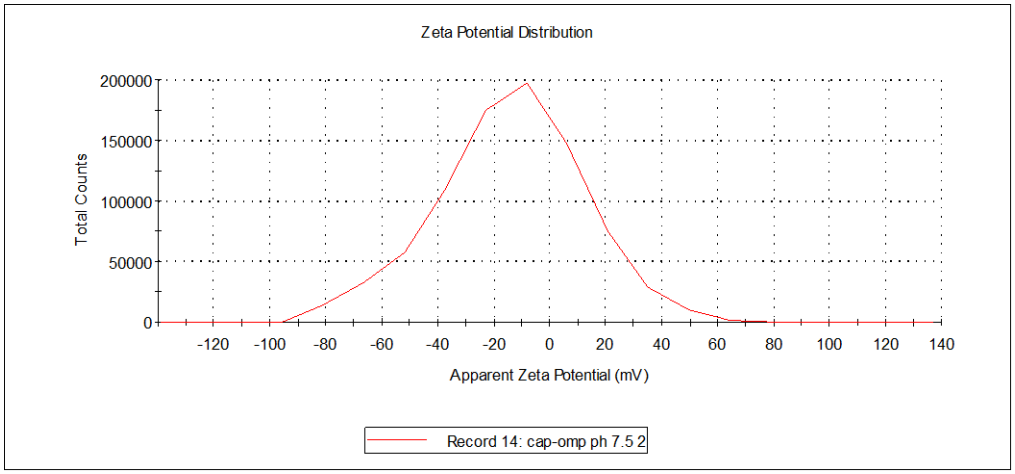
Graph 1: Shows The Highest Zeta-Potential Record of Cap-Omp Vaccine at pH6.0



Graph 2: Shows The Lowest Zeta-Potential Record of Cap-Omp Vaccine At pH6.5



Graph 3: Shows The Highest Zeta-Potential Record of Cap-Omp Vaccine At pH7.0

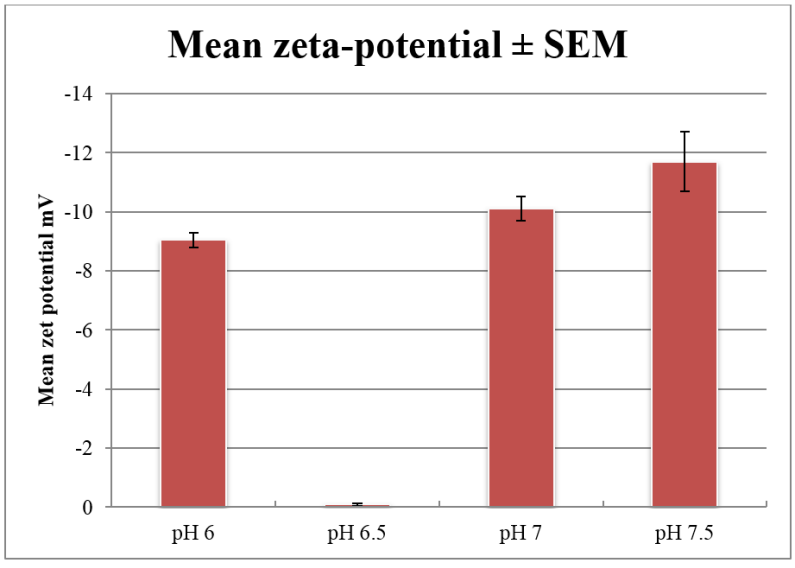


Graph 1: Shows The Highest Zeta-Potential Record of Cap-Omp Vaccine At pH7.5

Zeta-potential determination was carried out in triplicates. There was no significant difference among the zeta-potential of calcium phosphate nanoparticle-OMP complex suspended in PBS at pH 6.0, 7.0 and 7.5. However, the zeta-potential at pH 6.5 was significantly lower. ANOVA showing the mean zeta-potential in different pH is depicted in the Table 3.1.

Table 1: Difference in Mean of Zeta-Potential with Standard Error of Mean. (Means Bearing The Same Superscript Do Not Differ Significantly.

pH	Mean zeta-potential ± SEM
6.0	-9.04a± 0.24
6.5	-0.09b± 0.04
7.0	-10.1a± 0.4
7.5	-11.7a± 1.02



Graph 3: Graphical representation of the mean Zeta-Potential at pH (6.0, 6.5, 7.0 AND 7.5)

The area of nanoparticles of inorganic compounds has assumed great significance in entrapping biomolecules in veterinary and medical sciences. These inorganic nano-particles have many advantages over organic ones, such as better keeping quality and also being inexpensive. These nanoparticles have found their way in a number of biomedical applications such as gene therapy, drug-delivery systems and adjuvants. Calcium phosphate has been used for more than three decades to deliver genetic material to mammalian cells. Apart from using calcium phosphate nanoparticles (CAP) in gene delivery systems, it has also been utilized as an adjuvant for protein-based vaccines (He *et al***.,** 2002) [6].

In the present study, calcium phosphate nanoparticles were prepared with outer membrane proteins of *Salmonella* Typhimurium (MTCC-98). Calcium phosphate nanoparticles-OMP complex can be synthesized by top down method which utilizes the high energy wave such as ultra-sonication that breaks the microparticles into nanoparticles (Roy *et al.*, 2003) [15]. The top down method is generally not suitable for entrapment of protein or DNA in calcium phosphate nanoparticles as it may lead to degradation of entrapped biomolecule due to high energy wave (Bisht *et al.*, 2005) [2]. In the present study CAP-OMP was synthesized by the top up method. The mechanism behind calcium phosphate nanoparticle formulation is not clearly understood. However, it is assumed that mixing protein and CaCl2 with phosphate buffer leads to a precipitation of sparingly soluble calcium phosphate which incorporate proteins. Depending upon the time of stirring, precipitation is effectively prevented due to surface blocking; otherwise growth of calcium phosphate crystal continues leading to larger particles as observed by Welzel *et al*. (2004) [21]. Calcium phosphate nanoparticles interact with the OMP by electrostatic interaction.

In the present study, the CAP-OMP showed a mean zeta-potential ranging from -10.1 to – 11.7 in PBS at pH 7.0 to 7.5. Similar finding was also reported by Bisht *et al.* (2005) [2] for CAP-DNA complex. Priya *et al*. (2014) [10] reported that the average zeta-potential of phytosynthesized calcium phosphate nanoparticles by Zetasizer (Malvern) ZS90 was -20.25 mV. Banik and Basu (2014) [1] reported zeta-potential of -23 mV for the calcium phosphate nanoparticles synthesized by chemical method. Such variations might be due to the type of antigens being loaded in calcium phosphate nanoparticles. Vuk *et al*. (2011) [19] studied the Effects of calcium and phosphate on zeta-potential of amelogenin (rH174), the main protein of the developing enamel matrix. In that study, calcium and phosphate salts were introduced to rH174; the zeta-potential of the protein particles was affected more by the negatively charged ions (HxPO4x-3, Cl-), suggesting their tendency to locate within the double layer; same trend of affection has seen in this research, the ζ-potential of the outer membrane protein particles was affected more by the negatively charged ions of calcium phosphate nanoparticles.

Comparing the zeta-potential of hydroxyapatite (HAP) (the main mineral component of hard tissues), rH174 and CAP-OMP at pH 7.40, respectively, following the addition of CaCl2 and KH2PO4 up to 15 mM. In case of HAP, a shift towards positive side of zeta-potential values is observed following the addition of Ca2+ and the opposite shift is detected following the addition of KH2PO4, suggesting that both calcium and phosphate species tend to preferentially localize within the surface of HAP particles. In contrast, a trend in zeta-potential change observed for rH174 at pH 7.4 that was reported by Vuk *et al*. (2011) [19] is in agreement with the CAP-OMP reported in the present study. It demonstrates a preferential adsorption of negatively charged ions (HxPO4x-3, Cl-) onto rH174 particles compared to those of Ca2+ and K+.

Conclusion

Zeta-potential is an important factor for studying the stability of protein in aqueous solution. In the present study, the zeta-potential of CAP-OMP vaccine was evaluated. There was no significant difference among the zeta-potential of calcium phosphate nanoparticle-OMP complex suspended in PBS at pH 6.0, 7.0 and 7.5. However, the zeta-potential at pH 6.5 was significantly lower. From the present study, it could be concluded that: CAP-OMP *Salmonella* vaccine is stable at pH 7.0 to 7.5.

Reference

1. Banik M, Basu T. Calcium phosphate nanoparticles: a study of their synthesis, characterization and mode of interaction with salmon testis DNA. Dalton Trans. Doi 2014; 10:1039.
2. Bisht S, Bhakta G, Mitra S, Maitra A. pDNA loaded calcium phosphate nanoparticles: high efficient non-viral vector for gene delivery. Int. J pharm. 2005; 288:157-168.
3. Cai K, Frant M, Bossert J, Hilderbrand G, Liefeith K, Jandt KD. Surface functionalized titanium thin films: zeta-potential, protein adsorption and cell proliferation. Colloids and Surfaces B: Biointerfaces. 2006; 50:1-8.
4. Choi- Kim H, Maheswaran SK, Feelice LJ, Molitor TW. Relationship between the iron regulated membrane proteins and the outer membrane protein of *in vivo* grown Pasteurella multocida. Vet. Microbial 1991; 28:75-92.
5. Hanoar DAH, Michelazzi M, Leonelli C, Sorrel CC. The effect of carboxylic acids on the aqueous dispersion and electrophoretic deposition of ZrO2. Journal of the European ceramic society. 2012; 32(1):235-244.
6. He Q, Mitchell AR, Johnson ST, Wagner-Bartar C, Morcol T, Bell SDJ. Calcium Phosphate Nanoparticle induces mucosal immunity and protection against herpus simplex virus type 2. Clin. Diagn. Lab. Immunol. 2002; 5:1021-1024.
7. Joyappa DH, Kumar CA, Banumathi N, Reddy GR and Suryanarayana VV. Calcium phosphate nanoparticle prepared with foot and mouth disease virus P1-3CD gene construct protects mice and guinea pigs against the challenge virus. Vet. Microbiol 2009; 139(1-2):58–66.
8. Klodzinska E, Szumski M, Dziuabkiewicz E, Hrynkiewicz K, Skwarek E, Janusz W, Buszewski B. Effect of zeta-potential Value on Bacterial Behavior during Electrophoretic Separation. Electrophoresis. 2010; 31:1590-1596. [PubMed: 20422634]
9. Kondo Y, Morita Y, Yamada A, Kimura H. A highly effective method for removing suspended poliovirus from water using a positively charged carbon felt electrode. Microbiology and Immunology. 2004; 488:599-605. [PubMed: 15322340]
10. Priya P, Sudhir S, Aniket G, Mahendra R. Biofabrication of calcium phosphate nanoparticles using the plant Mimusops elengi. Environ Chem Lett, 2014, 2-8.
11. Riddick TM. Control of Colloid Stability through zeta-potential and its Relationship to Cardiovascular Disease. Livingston Publishing; Wynnewood, PA, 1968.
12. Core Team R. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2018.

13. URL https://www.R-project.org/.
14. Rowell RL, Fairhurst D, Key S, Morfesis A, Monahan IM, Mitchnick M *et al*. Microbicides for HIV/AIDS. 1. Electrophoretic Fingerprinting the H9 Cell Model System. Langmuir. 2005; 21:10165-10171. [PubMed: 16229541]
15. Roy I, Mitra S, Maitra AN, Mozumdar S. Calcium phosphate nanoparticles as non-viral vectors for targeted gene gene delivery. Int. J pharm. 2003; 250:25-33.
16. Schultz N, Metreveli G, Franzreb M, Frimmel FH, Syldatk C. Zeta-potential measurement as a diagnostic tool in enzyme immobilisation. Colloids and Surfaces B: Biointerfaces. 2008; 66:39-44.
17. Tokumasu F, Nardone GA, Ostera GR, Fairhurst RM, BEaudry SD, Hayakawa E *et al*. Altered Membrane Structure and Surface Potential in Homozygous Hemoglobin C Erythrocytes. PLoS ONE. 2009; 4:e5828. [PubMed: 19503809]
18. Uskokovic V. Surface Charge Effects Involved in the Control of Stability of Sols Comprising Uniform Cholesterol Particles. Mat & Manufacturing Processes. 2008; 23:620-623.
19. Vuk U, Roselyn O, Sonia D, Stefan H. Dynamic Light Scattering and zeta-potential of Colloidal Mixtures of Amelogenin and Hydroxyapatite in Calcium and Phosphate Rich Ionic Milieus. Arch. Oral Biol. 2011; 56(6):2-23.
20. Wade RC, Gabdoulline RR, Ludemann SK, Lounnas V. Electrostatic steering and ionic tethering in enzyme-ligand binding: Insights from simulations. Proceedings of the National Academy of Sciences. 1998; 95:5942-5949.
21. Welzel T, Radtke I, Meyer-Zaika W, Heumann R, Epple M. Transfection of cells with custom – made calcium phosphate nanoparticles coated with DNA. J Mater. Chem. 2004; 14:2213-2217.
22. Wilson W, Wade MM, Holman SC, Champlin FR. Status of methods for assessing bacterial cell surface charge properties based on zeta-potential measurements. Journal of Microbiological Methods. 2001; 43:153-164. [PubMed: 11118650].