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# Evaluation of Physicochemical Properties of Aluminium Phosphate Gel to Improve Adjuvanticity

**Anil Kumar Chawla*, Chandrani Das, Paramdeep Sngh, Mansha Tiwari, Seema Chaudhary**

*Department of Research & Development, Chimera Gentec Pvt. Ltd., Greater Noida, Uttar Pradesh (India)*
**Corresponding author email: anilkumarchawla@yahoo.com*

K E Y W O R D S: Adjuvants, Aluminium phosphate gel, complexometric titration, particulate antigens, settlement rate

**ABSTRACT:** Aluminium containing compounds are most popular class of adjuvants. They play active role as potency enhancer in a vaccine formulation, so it is important to have pre-information about adjutant's physical properties. The present study was aimed to prepare aluminium phosphate gel using different methods and to determine their physiochemical characteristics like Aluminium concentration, particle count and particle distribution along with their settling time. Aluminium phosphate gel was prepared by four different methods using chemical co-precipitation. Concentration of Aluminium was determined in gels using complexometric titration and Particle count & distribution, using liquid particle counter. Settling rate of aluminium phosphate gels were determined by measuring length of settling area with respect to time using Vernier calipers. Based on comparative results of all the four methods of aluminium phosphate gel preparation in terms of their physiochemical properties, we can select most relevant method for preparation of aluminium phosphate adjuvant.

## Introduction

Adjuvants (enhancers of vaccine potency) have been used for a long period to enhance the immune response to vaccine antigens as they have capability to turn soluble antigens in their particulate form (Muthurania et al., 2015). Particulate antigens are comparatively more immunogenic than soluble antigens as they can readily phagocytose, processed and presented by antigen presenting cells (Gamvrellis et al., 2004). Aluminum salts are the most widely used adjuvants in approved human and veterinary vaccines because of proven excellent safety profile and enhanced immune response(Gupta et al., 1995).These adjuvants have been used in regular vaccination practice and are generally regarded as safe (GRAS) and as stimulators of T-cell mediated immunity (Lindblad, 2004). In human vaccinations schedule, aluminium adjuvants have predominantly been used in Diphtheria, Pertussis, Tetanus and other viral vaccines in many countries for approximately 50 years (Lindblad, 2004). Use of aluminium adjuvants has also been initiated in Hepatitis A and Hepatitis B virus vaccines. Aluminium based adjuvants have been employed in formulation of vaccines for special risk groups; e.g Anthrax. Aluminium adjuvants have also been used in a large number of veterinary vaccine formulations against bacterial and viral diseases, and in attempts to make anti-parasite vaccines.

Some aluminium containing adjuvants are aluminium phosphate, aluminium hydroxide aluminium hydrochloride and aluminium silicate etc. Most of the vaccine manufacturers throughout the world prepare these adjuvants in-house. Usually, required antigens are adsorbed onto aluminium adjuvants for formulation of vaccine and act as carrier of vaccine (Petrovsky and Aguilar, 2004). Examples of commercially available aluminium containing adjuvants are Alhydrogel® (aluminium hydroxide adjuvant) and Adju-Phos® (aluminium phosphate adjuvant) (White and Hem, 2000). Physiochemical properties and quantity of aluminum gel can affect the final formulation of the vaccine and its immune-enhancing properties.

Aluminium phosphate gel is a colloidal mixture of aluminium phosphate which is negatively charged at physiological pH (5-7) which is suitable for adsorption of positively charged or neutral alkaline protein antigen. The pharmacopeias and WHO have set a maximum limit for the aluminium content in vaccine. Currently it is set as 1.25 mg/ single human dose (WHO TRS 595, 1976).

Physicochemical properties of aluminium phosphate gel can be confirmed by using several techniques. Concentration of aluminium present in aluminium phosphate gel can be determined by using complexometric titration. Aluminium phosphate gel sample is first dissolved by heating with acid. The released $Al^{+3}$ ions form a complex with disodium EDTA, the excess of disodium EDTA is then titrated with cupric sulfate(Indian Pharmacopoeia, 2010).

Particle size and distribution in aluminium phosphate gel are most influential causes which affect the settlement rate of aluminium phosphate gel. Aluminium phosphate gel is a turbid liquid and the mineral carrier upon keeping may gradually settle down (Indian Pharmacopoeia,2010)

Method of preparation and concentration of chemicals during preparation of gel along with experimental parameters such as temperature and pH highly influence the size and distribution of gel particles in aluminium phosphate gel which finally decides the quality and settlement rate of gel. Lesser the particle size means longer settlement time and less settlement of particles. Particle size and particle distribution in aluminium phosphate gel can be determined using liquid particle counter. The functioning of liquid particle counter is based on principle of light scattering/laser diffraction. They quantify the amount of light scattered by particles when particles passing through the viewing volume. Other methods for doing the same are X-ray diffraction and infrared spectroscopy. Aluminium phosphate gel settlement rate is determined by keeping the aluminium phosphate gel and measure the length of settling area with respect to time by using Vernier calipers.

Particle size of aluminium phosphate gel particles is a critical factor during vaccine formulation because adjuvanticity of adjuvant is directly associated with their capability to convert soluble antigens into their particulate form (Muthurania et al., 2015).Aluminium phosphate gel having larger particles is not as much effective in vaccine delivery system as aluminium phosphate gel with smaller particles. Large particles, having size more than 2microns are not able to access and drain into the lymphatic system and may be not be phagocytosed by antigen presenting cells (APCs) until they go through degradation process to obtain sufficiently smaller size, whereas, very large particles (>10 microns) are difficult to process by antigen presenting cells. Smaller micro-particles of gel (<5micron) can easily access lymphatic system and are readily phagocytosed by APCs. Because of the mentioned reasons, investigation of physicochemical properties of aluminium phosphate gel adjuvant before formulation process is very important.

In present research, aluminium phosphate gels prepared by different methods were compared for their physical characteristics to find an effective method which leads to preparation of aluminium phosphate gel having smaller particle size with longer settlement time.

## Material And Methods

### *Preparation of Aluminium Phosphate Gel*

Aluminium phosphate gel was prepared by four different methods.

#### *Method-1*

Tri-sodium orthophosphate 12 hydrated was weighed and dissolved in 420 ml hot WFI (85˚C). Separately potassium aluminum phosphate 12hydratedwas weighed and dissolved in 420 ml of hot WFI (85˚C).Both the solutions were poured slowly and simultaneously in vessel containing 1.6 L purified water with the help of two peristaltic pumps with constant stirring for 25 minutes. pH of the solution was adjusted to 5 with 1N NaOH or 1N HCL either solution. The solution was kept overnight to settle down. The clear supernatant was decanted and the remaining part of the solution was centrifuged at 4000 rpm for 20 minutes. The sediment was collected after centrifugation and homogenized on vortex for half an hour. The homogenized solution was re-suspended in 1.725 L of normal saline and kept overnight to settle down. Again the clear supernatant was decanted and the remaining part of the solution was centrifuged at 4000 rpm for 20 minutes. Supernatant was decanted and sediment was collected and homogenized on vortex for half an hour. Final volume was adjusted to 900 ml with normal saline and the pH was adjusted to 6.5 with 10% NaOH. The gel was filtered through eight layers of nylon cloth sterilized at 121˚C for 30 minutes and the concentration of aluminum phosphate was estimated.

#### *Method -2*

Potassium aluminum phosphate 12 hydrated was weighed and dissolved in warm distilled water. Tri-sodium orthophosphate was weighed and dissolved in warm distilled water. The above solutions were mixed slowly and stirred simultaneously. The gel was allowed to settle for two days. The supernatant solution was decanted and the collected gel solution was centrifuged for 15 minutes at 3000 rpm. The supernatant was decanted and the sediment was resuspended in 1 L of normal saline. The solution was mixed well and left for overnight. The gel was centrifuged at 3000 rpm for 10 minutes. Supernatant was discarded and the sediment was re-suspended in normal saline. The final volume was made up to 1 L. pH was adjusted to 6.0 with 50% sodium hydroxide. The solution was homogenized for 4 hrs and filtered through double layer of nylon cloth.

#### *Method -3*

Aluminium chloride hexahydrate ($AlCl_3.6H_2O$) was dissolved in sufficient volume of distilled water and the final volume was adjusted to 500mL.Tri-sodium orthophosphate ($Na_3PO_4.12H_2O$) was dissolved in sufficient volume of distilled water and final volume was adjusted to 500mL. Both the solutions were poured slowly and simultaneously in 2L flask with constant stirring on magnetic stirrer. pH of the gel was adjusted to 6.26 by adding 10N NaOH solution followed by stirring for another 1 hour.

***Method -4***

This is a standardized method for preparation of buffered aluminium phosphate gel. For preparation of 1.65 L of aluminium phosphate gel,$AlCl_3.6H_2O$ was dissolved in 500 mL of purified water. Tri-sodium orthophosphate was dissolved in 500 mL purified water followed by addition of 150 mL of purified water in it. Both the solutions were poured and mixed simultaneously by using a magnetic stirrer. pH of the gel was adjusted to 6.3 - 6.5 with 40% NaOH. The gel was then mixed with 0.35 L phosphate buffer saline of pH 6.8. pH of the buffered gel was adjusted to 7.7 to 7.9 by the addition of 2% HCl.

Method 2 and 3 produces gel with the aluminum concentration of approximately 2.0 mg/ml, while method 1 and 4 produces gels with aluminum concentration of approximately 1.0 mg/ml. Thus to compare the specifications of gels with originally produced gel of method 3 (2X or with al. content of ~2.0 mg/ml was also used for studies)

***Determination of aluminium content in prepared gel***

1 ml of Aluminium phosphate gel sample was transferred into a 250 ml conical flask. 1 ml of concentrated Sulphuric acid was added followed by addition of 0.1 mL of concentrated Nitric acid. The solution was heated on hotplate until dense white fumes evolved. If charring was observed, few more drops of concentrated Nitric acid were added. A colorless solution was obtained by further heating thesolution.10 ml of distilled water was added in the solution and boiled again to obtain a clear solution. After cooling, 0.05 mL of methyl orange solution was added in the reaction mixture and the contents were neutralized by adding 10M NaOH solution drop wise, with continuous shaking until the color changed from pink to golden yellow. If precipitate was formed, it was dissolved by addition of 1M sulfuric acid.25 ml of accurately measured EDTA solution was transferred followed by addition of 10 ml acetate buffer solution. The flask was heated over hotplate to boil the solution.1 ml of PAN indicator was added to the reaction mixture and titration of the hot solution was carried out against the 0.02 M $CuSO_4$ solution taken in a burette till the appearance of purplish brown color as end point. Simultaneously, reference blank using water was also titrated, as above.

***Settlement of Aluminium phosphate gel***

Settling of gel is the process by which aluminium phosphate gel particles get settled down at the bottom due to gravitational force. In the present work, we determined the rate of settlement of aluminium phosphate gels prepared by different methods. This gravity driven settlement of gels is associated with the differential sizes of gel particles which is the main factor for the increase in the settling rate of gel. However, more concentrated gel with large particle size showed opposite behavior (tend to settle slower).When the condensed gel was diluted to low concentration, it tend to settle down faster as compared to other gels with same or equivalent particle or bigger particle size. So it was important to determine the size and concentration of particles in gel along with settling rate to decide the quality of gel.

To carry out settlement experiment, 50 mL of each aluminium phosphate gel sample was filled in 100 mL measuring cylinder of diameter 2.86 cm and 14.4 cm in height. Sample 1, 2, 3 and 4 have same concentrations of aluminium while sample 3(2X) has double concentration of aluminium. Samples were observed for the settlement rate in a back lighting for better illumination and accurate monitoring (Obunwo et al., 2014). Settlement area was observed after every 30 minutes up to 3 hours. After that, samples were left for 24 hours to get maximum settlement. After 24 hours, final settled area was recorded.

***Particle counting***

To determine the size and concentration of gel particles, we used liquid particle counter. The particles in the gel were classified in different size range. The liquid particle counter was able to measure particles from 2µm to >100 µm diameter. The samples were diluted 5 times as saturation caused counting error when the particle concentration is high.1 mL sample volume was used for single analysis. A total number of five samples (sample1, 2, 3, 3{2X} and 4) were used for the determination of particle size.

**Results And Discussion**

Aluminium phosphate gels was prepared by four different methods and basic characteristics of aluminium phosphate gel were determined; such as physical appearance and aluminium concentration in gels along with specified characters of the gels such as settlement rate, particle size and counting & concentration. Aluminium phosphate gel prepared by all the methods had white turbid/colloidal appearance, in which the gel particles settled down after some time.

Aluminium concentration in aluminium phosphate gels were determined by complexometric titration using EDTA. Volume of cupric sulfate consumed was recorded to calculate the aluminium concentration by using the formula as recommended in the pharmacopeia-

$$C = \frac{(Vb-Vs)}{A} \times 0.5396$$

Where,
C= concentration of aluminium content
Vb=volume of cupric sulfate solution (0.02M) consumed in the titration of blank.
Vs= volume of cupric sulfate solution (0.02M) consumed in the titration of sample.
A=Volume of sample
0.5396= Mass aluminium equivalent for 1.0 mL of cupric sulfate solution (0.02M)
The results were expressed in mg/mL (table-1).

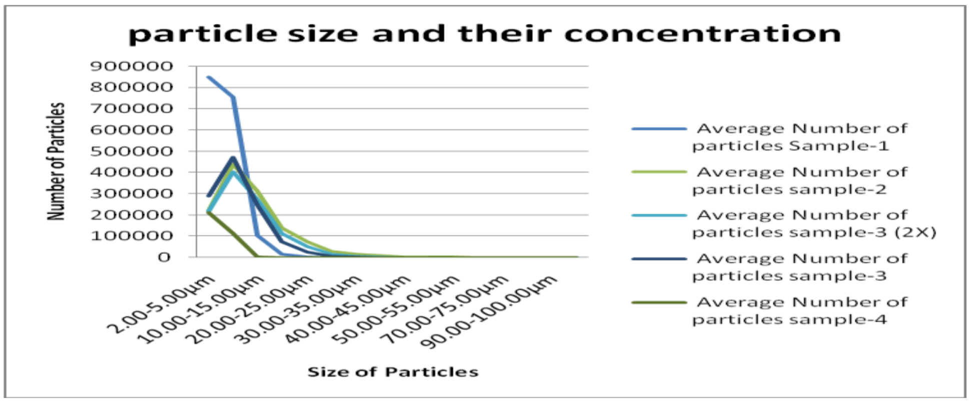



Figure1. Estimation of particle size and their distribution in aluminium phosphate gel prepared by different methods

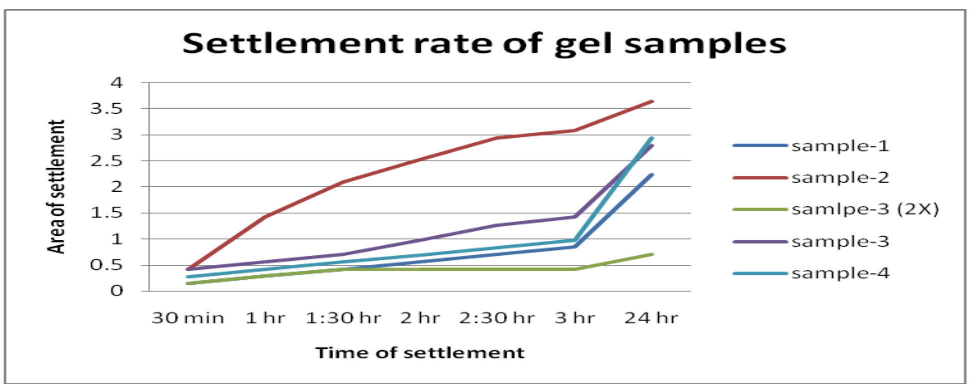



Figure2. Determination of settlement rate of aluminium phosphate gel particles prepared by different methods

Table 1. Aluminium concentration in gel prepared by different methods

| Sample No. | Aluminium concentration (mg/mL) |
|---|---|
| Aluminium Phosphate Gel- 1 | 1.13 |
| Aluminium Phosphate Gel- 2 | 2.26 |
| Aluminium Phosphate Gel- 3 | 2.15 |
| Aluminium Phosphate Gel- 4 | 1.07 |

Gel prepared by method 2 and 3 having approximately double concentration of aluminium as compared to other two methods. Gel prepared by method 2 and 3 were adjusted to same aluminium concentration by dilution method. Sample identification details were shown in table-2.Further experiments were carried out with gels having approximately same concentration (~1.10 mg/ml). Concentrated form of gel prepared by method 3 were also kept in all experiments and were mentioned as Gel 3(2X). Therefore, total five samples were observed for determination of particle size and settlement rate.

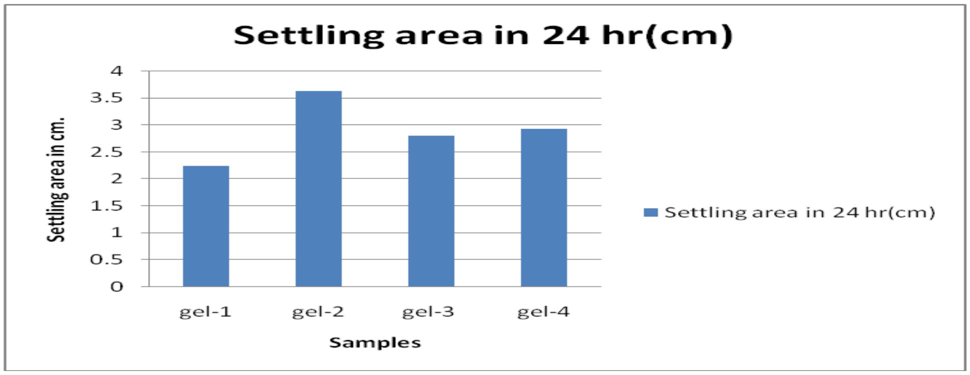
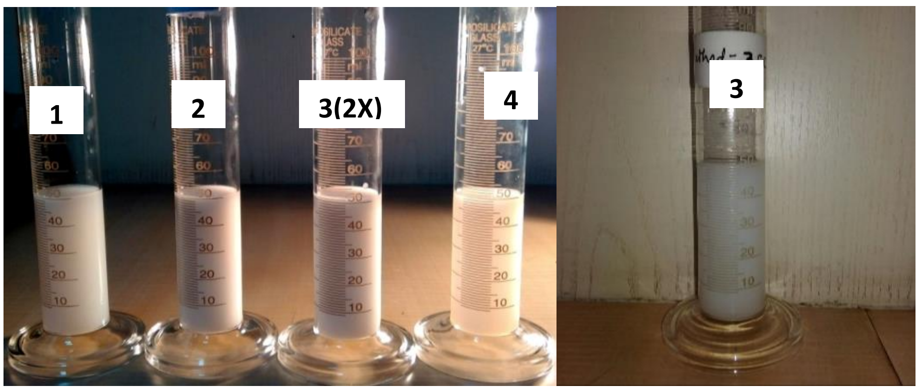
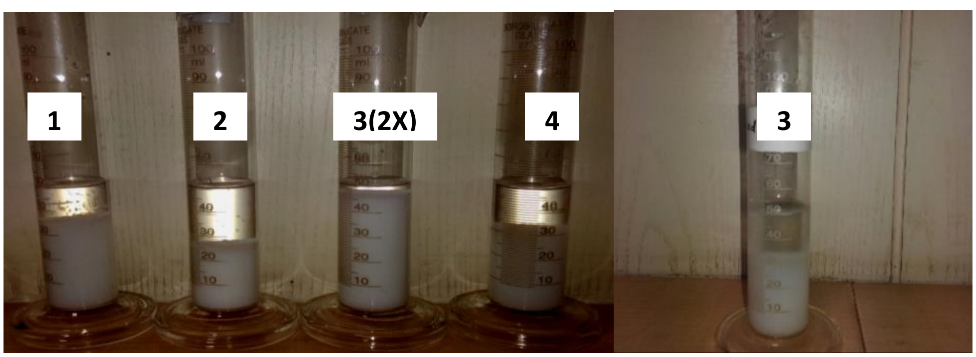



Figure3. Comparison of aluminium phosphate gel having approx same concentration for their area of clear supernatant after 24 hour




A. Settlement of aluminium phosphate gel(T= 0)




B. Settlement of aluminium phosphate gel(T= 24 hrs.)

Figure4. (A and B) Determination of settlement time of aluminium phosphate gels prepared by different methods (A) At time T=0, (B) T=24 hours

Table 2. Sample identification

| Aluminium phosphate gel Sample ID | Preparation method | Aluminium concentration (mg/mL) |
|---|---|---|
| Sample- 1 | Method-1 | 1.13 |
| Sample- 2 | Method-2 | 1.13 |
| Sample- 3(2X) | Method-3 | 2.15 |
| Sample- 3 | 50% dilution of method-3 | 1.07 |
| Sample- 4 | Method-4 | 1.07 |

Gel samples were adjusted to same aluminium concentration before settlement and particle counting experiment. From the results shown in table3, 4 and 5 aluminium phosphate gel prepared by method-1, had the highest quantity of smaller particles (2 µm- 20 µm) and had considerably very less quantity of larger particle sized >100µm. Aluminium phosphate gel prepared from method-1 remarkably showed highest settlement time along with lesser settlement area (2.24 cm in 24 hours) as compared to other gels. Gel-1 also showed constant settlement velocity at all time checkpoints (T1-T6) that is -0.0046 cm/min, where negative sign indicated downward velocity of solid liquid interface. Fastest settlement rate was shown by aluminium phosphate gel prepared by method-2 that is 3.64 cm in 24 hrs. Gel-2 showed random settlement velocity at all checkpoints. Table-3 reveals that aluminium phosphate gel prepared from method-2 had the highest number of larger particles sized > 20 µm. Gel- 3 (having approximately same aluminium concentration as gel-1, 2 and 4 i.e. ~1.13 mg/mL) also showed random settlement as shown in case of gel-2. Gel-4 showed gradual decrease in settlement velocity from checkpoint T1 to T6.

Table 3. Results of particle counting of aluminium phosphate gel particle counting

| S.N. | Diameter of particles (µm) | Average Number of particles | | | | |
|---|---|---|---|---|---|---|
| | | Sample-1 | Sample-2 | Sample-3(2X) | Sample-3 | Sample-4 |
| 1 | 2.00-5.00 | 848961.25 | 222050.75 | 216929.75 | 287944 | 211435.56 |
| 2 | 5.00-10.00 | 754802.25 | 438595.5 | 403809.75 | 470981.25 | 115061.4 |
| 3 | 10.00-15.00 | 103690.75 | 312339.5 | 273873.5 | 245503.5 | 3245.4 |
| 4 | 15.00-20.00 | 15470 | 140064.25 | 113914.25 | 74345 | 174.75 |
| 5 | 20.00-25.00 | 4665.25 | 75828.25 | 53037 | 26972.5 | 48.8 |
| 6 | 25.00-30.00 | 1361.75 | 30235.25 | 17259.25 | 7430 | 14.95 |
| 7 | 30.00-35.00 | 617.25 | 15527.5 | 7226.75 | 2919.25 | 4.75 |
| 8 | 35.00-40.00 | 316.5 | 8391 | 3155.25 | 1241.5 | 2.7 |
| 9 | 40.00-45.00 | 155 | 4526 | 1404.75 | 551.75 | 1.2 |
| 10 | 45.00-50.00 | 88.75 | 2342.25 | 624.75 | 246.75 | 0.8 |
| 11 | 50.00-55.00 | 72.25 | 1917.25 | 452.5 | 175.75 | 0.6 |
| 12 | 60.00-65.00 | 22.25 | 551.75 | 106.25 | 45.75 | 0.35 |
| 13 | 70.00-75.00 | 8 | 189.26 | 33 | 17 | 0.15 |
| 14 | 80.00-85.00 | 1.75 | 60 | 9.75 | 8.25 | 0.15 |
| 15 | 90.00-100.00 | 1.5 | 20 | 5.25 | 1 | 0.05 |
| 16 | >100 | 2.5 | 10.75 | 3 | 1 | 0.05 |

Table 4. Results of Settlement of Aluminium Phosphate Gel Samples

| Clear supernatant height in cm (Initial Height-height after time T) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (T) | 30 min(T1) | 1 hr (T2) | 1:30 hr (T3) | 2 hr (T4) | 2:30 hr (T5) | 3 hr (T6) | 24 hr (TF) |
| sample-1 | 0.14 | 0.28 | 0.42 | 0.56 | 0.7 | 0.84 | 2.24 |
| sample-2 | 0.42 | 1.43 | 2.1 | 2.52 | 2.94 | 3.08 | 3.64 |
| samlpe-3(2X) | 0.14 | 0.28 | 0.42 | 0.42 | 0.42 | 0.42 | 0.7 |
| sample-3 | 0.42 | 0.56 | 0.7 | 0.98 | 1.26 | 1.43 | 2.8 |
| sample-4 | 0.28 | 0.42 | 0.56 | 0.7 | 0.84 | 0.98 | 2.94 |

Table 5. Settlement Velocity of gels prepared by different methods at different checkpoints

| S.N. | Sample no. | Settlement velocity at T = height at T- original height/ T (cm/min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | T1 | T2 | T3 | T4 | T5 | T6 |
| 1 | Gel-1 | 0.0046 | 0.0046 | 0.0046 | 0.0046 | 0.0046 | 0.0046 |
| 2 | Gel-2 | 0.014 | 0.023 | 0.023 | 0.021 | 0.019 | 0.017 |
| 3 | Gel-3 (2X) | 0.0046 | 0.0046 | 0.0046 | 0.0039 | 0.0028 | 0.0023 |
| 4 | Gel-3 | 0.014 | 0.0093 | 0.0077 | 0.0081 | 0.0084 | 0.0079 |
| 5 | Gel-4 | 0.0093 | 0.007 | 0.0062 | 0.0058 | 0.0056 | 0.0054 |

## Conclusion

As aluminium compounds are most frequently used adjuvants, so to continue their use in vaccine formulation, it is important to optimize their physicochemical properties. From the present study it can be concluded that aluminium phosphate gel prepared by using method-1 shows highest quantity of smaller particles along with constant settlement velocity and longest settlement time and is therefore suitable for formulation process. Physiochemical properties of aluminium phosphate gel such as pH, aluminium concentration and ionic strength can be further optimized according to type of formulation required.

Improvement in quality of gel can be further achieved by reducing the particle size and conversion of micro particles to sub-microparticle and nano-microparticle through several techniques like homogenization, ultracentrifugation and sonication as they can be more effective immune-stimulators. There is a need of further advancement in these techniques that can be time saving and more practical for vaccine formulation at commercial scale. In vaccine formulation this is one of the most important factor that should be deeply investigated for improvement in vaccine delivery system.

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