

Article

Tuning Antigen–Adjuvant Interactions by Modulating the Physicochemical Properties of Aluminum Hydroxide Nanoparticles for Improved Antigen Stability

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Abstract

Adjuvants are chemical substances used in vaccines to enhance immunogenicity. Among them, aluminum-based nanoparticles are some of the oldest and most widely employed adjuvants in vaccine formulations. A key function of aluminum adjuvants is thought to involve acting as an antigen depot, enabling slow antigen release and providing sufficient time for effective immune activation. Therefore, understanding antigen–adjuvant interactions is essential, as these interactions influence antigen stability, release kinetics, and overall vaccine performance. In this study, we investigated how the physicochemical properties of aluminum hydroxide nanoparticles modulate antigen–protein interactions and affect protein stability. Nanoparticles synthesized under acidic (pH $\approx$ 5.0) to near-neutral (pH $\approx$ 7.1) conditions exhibited lower crystallinity, reduced hydroxyl density, and higher interfacial hydration, whereas those prepared under basic conditions (pH $\approx$ 9.0) displayed increased crystallinity, enriched surface hydroxyl groups, and markedly reduced hydration. Antigen proteins bound to low-crystallinity aluminum hydroxide nanoparticles showed improved thermal stability, while those associated with highly crystalline nanoparticles exhibited reduced thermal stability. Complementary ITC study further suggests that these stability differences are accompanied by changes in their interaction behavior. These findings indicate that the structural and interfacial properties of aluminum hydroxide nanoparticles strongly influence their interactions with antigen proteins and the resulting physical stability. Together, our results demonstrate that the balance among crystallinity, hydroxyl organization, and interfacial hydration governs the thermal behavior of antigen proteins adsorbed onto aluminum hydroxide. This work provides a rational design principle for engineering aluminum-based adjuvants that optimize antigen–protein stability in vaccine formulations.



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Keywords: aluminum adjuvant; aluminum hydroxide nanoparticles; antigen–adjuvant interaction; morphology; protein stability; surface hydration; hydroxyl content; ordered crystalline structure; vaccine formulation

1. Introduction

Aluminum salts, primarily aluminum hydroxide and aluminum phosphate, represent the most extensively utilized adjuvants in human vaccines [1–3]. The immunostimulatory potential of aluminum-based adjuvants was first observed nearly a century ago by Alexander Glenny, who reported that the addition of aluminum salts to diphtheria and

tetanus toxoids significantly enhanced the antibody response in animal models [4]. This discovery led to the widespread inclusion of aluminum adjuvants in human vaccines, and, to date, they remain one of the integral components in several licensed vaccine formulations. Although a variety of alternative adjuvant systems such as oil-in-water emulsions, liposomes, and Toll-like receptor agonists have been developed, aluminum-based adjuvants remain the predominant choice globally. Their continued use is largely attributed to a well-documented safety profile and a robust capacity to induce durable humoral immune responses [5–8]. Consequently, numerous licensed vaccines, including diphtheria, tetanus, pertussis (DTaP/Tdap), hepatitis A and B, human papillomavirus (HPV), and pneumococcal conjugate vaccines, are routinely formulated with aluminum adjuvants to enhance both the magnitude and duration of immune protection [9–16]. Although aluminum adjuvants were introduced as early as 1926, their precise mechanisms of action remain incompletely understood [17,18]. Historically, the “depot theory” was proposed to explain their functionality, suggesting that aluminum adjuvants form antigen depots at the injection site that enable sustained antigen release and prolonged immune stimulation [19,20]. This controlled release is thought to facilitate the recruitment and activation of antigen-presenting cells (APCs), thereby enhancing antigen uptake, processing, and presentation to the immune system [21,22]. Beyond this classical view, more recent studies have revealed that aluminum adjuvants also modulate the innate immune response through pathways involving local inflammation, complement activation, and inflammasome signaling [23–25].

In recent years, increasing attention has been directed toward understanding how the physicochemical characteristics of aluminum adjuvants govern their immunological performance [12,26–28]. Parameters such as particle size, morphology, and crystallinity of aluminum adjuvants have been shown to influence cellular uptake and antibody production [29–32]. It was found that smaller particles and needle-like morphologies are often associated with enhanced antigen presentation and stronger immune responses, while amorphous or poorly crystalline aluminum hydroxide tends to exhibit greater immunogenicity than its more crystalline counterpart [12,26–28]. These findings underscore that the physicochemical properties of aluminum adjuvants play a central role in determining vaccine potency. While these physicochemical parameters have been correlated with immunological outcomes, their impact on the structural stability of bound antigens remains much less understood. The stability of antigens adsorbed onto aluminum adjuvants is influenced by the local microenvironment near the adjuvant surface. Factors such as pH gradients, surface charge, and interfacial hydration can promote protein conformational changes or chemical degradation [33–36]. Yet, results from existing studies are inconsistent. D’Souza et al. reported the deamidation of recombinant protective antigen when adsorbed on aluminum hydroxide adjuvant in anthrax vaccine formulations [37], whereas Colaprico et al. suggested that antigen proteins of Meningococcal Serogroup B vaccine are stabilized upon binding with aluminum hydroxide adjuvants [38]. Furthermore, studies using the same model antigen, ovalbumin, adsorbed to aluminum adjuvants reported conflicting outcomes, ranging from preservation of native structure to reduced thermal stability indicative of partial unfolding [35,39]. These discrepancies highlight that the underlying physicochemical drivers of antigen stabilization or destabilization are not yet clearly defined. Addressing this gap is essential to establish a mechanistic link between adjuvant structure, antigen conformation, and immunogenic function.

In this study, we aimed to elucidate how aluminum adjuvants interact with model antigens by focusing on three key physicochemical parameters: crystallinity, hydroxyl content, and hydration state, that collectively define the surface chemistry of aluminum hydroxide. Although these features are known to influence immunological performance, the relationships among them, as well as their combined effects on antigen stability require

further investigation [31,35]. In particular, while qualitative trends have been reported (for instance, higher crystallinity is often associated with higher hydroxyl content, and that surfaces rich in well-organized hydroxyl groups can result in lower hydration on metal hydroxide surfaces), a systematic investigation linking these properties to protein adsorption and stability has not been performed [31,40]. To address this gap, we synthesized aluminum hydroxide nanoparticles under various pH conditions, producing materials with systematically varied crystallinity, hydroxyl density, and hydration level. The nanoparticles were characterized using transmission electron microscopy (TEM), X-ray diffraction (XRD), and synchrotron X-ray diffraction (SXRD) in solution to establish correlations among these physicochemical features. Differential scanning calorimetry (DSC) was then employed to assess the thermal stability of model antigens following adsorption, providing direct insight into how specific surface characteristics influence protein stability upon binding. In parallel, isothermal titration calorimetry (ITC) was employed to quantify the thermodynamics of antigen adjuvant interactions, enabling assessment of the enthalpic and entropic contributions governing protein adsorption as a function of surface chemistry and hydration. Our results reveal that synthesis pH governs both the interplay among hydroxyl content, hydration, and crystallinity of aluminum hydroxide nanoparticles, and their collective impact on protein stability. Acidic conditions produced highly hydrated, poorly crystalline particles with lower hydroxyl density, whereas basic synthesis yielded more crystalline, hydroxyl-rich particles with reduced hydration. These structural differences were directly reflected in antigen behavior: more hydrated, amorphous particles better preserved protein thermal stability, while highly crystalline, hydroxyl-dense surfaces promoted destabilization. Together, these findings establish, for the first time, a quantitative relationship linking the intrinsic structural chemistry of aluminum adjuvants to their effects on antigen stability. This work provides molecular-level insight into the origin of variability observed in prior studies and offers a rational framework for designing aluminum-based adjuvants with optimized physicochemical properties for balanced antigen preservation and immune activation.

2. Materials and Methods

2.1. Materials

Alhydrogel[®], a commercially available aluminum hydroxide adjuvant, was obtained from InvivoGen (San Diego, CA, USA) (Lot No. 0002260678). For the synthesis of aluminum hydroxide nanoparticles, aluminum nitrate nonahydrate (analytical grade, $\geq 98\%$ purity) was employed as the aluminum precursor and was purchased from STEM Chemicals (Newburyport, MA, USA) (Lot No. LOO432111). Ethylene diamine (EDA), used as a pH modifying agent, was sourced from JT Baker Avantor (Radnor, PA, USA) (Lot No. S8314547311). Potassium hydroxide (KOH), required for potentiometric titration, was procured from VWR Chemicals (Radnor, PA, USA) (Lot No. 20F0456942). Bovine serum albumin (BSA) (Lot No. 231396) was obtained from Fisher Bioreagents (Pittsburgh, PA, USA) while beta lactoglobulin (BLG) (Lot No. 1003364229) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). All reagents were of analytical grade and used without further purification unless otherwise specified.

2.2. Synthesize of Aluminum Hydroxide Nanoparticles

Aluminum nitrate nonahydrate was used as the precursor for the synthesis of aluminum hydroxide nanoparticles. A total of 5.5 g of aluminum nitrate nonahydrate was first dissolved in ultrapure water. Ethylenediamine was then added dropwise to adjust the solution pH to 5 (acidic), 7 (neutral), or 9 (basic). After vigorous stirring for 20 min, the reaction mixture was transferred to a 100 mL Teflon-lined hydrothermal reactor and

heated in an electric oven at 150 °C for 24 h. Upon completion of the reaction, the reactor was immediately cooled under running tap water. The resulting suspension was buffer exchanged into 10 mM HEPES at pH 7.4. Aluminum hydroxide nanoparticles prepared under acidic, neutral, and basic conditions are denoted as AH1, AH1-N, and AH2, respectively, throughout the manuscript for brevity. Commercial Alhydrogel adjuvant is referred to as AH.

2.3. Transmission Electron Microscopy (TEM)

TEM was used to investigate the morphology of aluminum hydroxide nanoparticles synthesized under varying pH conditions. Imaging was conducted using a JEOL 1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 120 KeV. Prior to sample loading, standard carbon-coated copper TEM grids (300 mesh) were subjected to glow discharge treatment using a LEICA EM ACE600 (Leica Microsystems, Wetzlar, Germany) glow discharge system (60 s, 10 mA). This surface modification was performed to enhance hydrophilicity and minimize aggregation of the nanoparticles by promoting uniform dispersion across the grid. For sample preparation, a dilute aqueous suspension of the nanoparticle dispersion (0.001 mg/mL aluminum concentration) was carefully drop-cast (3 µL) onto the glow discharged grid and allowed to rest for 1 min to facilitate adsorption of particles onto the carbon film. Excess liquid was wiped off using filter paper, and the grid was then dried in a convection oven at 40 °C for 10 min to remove residual moisture without altering nanoparticle morphology. The dried grids were subsequently transferred to the microscope for imaging. Multiple areas of each grid were scanned to ensure representative morphological data, and images were captured using a CCD camera at varying magnifications.

2.4. Synchrotron X-Ray Diffraction (SXRD) Analysis

USAXS instrument at the 12-ID-E beamline of the Advanced Photon Source (APS), Argonne National Laboratory (ANL) was used to collect X-ray diffraction data using X-rays with an energy of 21 keV [41,42]. The scattering vector q is defined as:

$$q = \frac{4\pi \sin \theta}{d} \quad (1)$$

Samples were suspended in 10 mM HEPES buffer (pH 7.4) and loaded into quartz capillaries for measurement. The intensity profiles, $I(q)$, were obtained by beamline provided data reduction software tools. The scattering contribution from the buffer was subtracted from the total scattering of the nanoparticle suspensions to obtain the nanoparticle-specific profiles. Buffer subtraction, data reduction, and data processing were performed using the combination of Nika and Irena packages, built in Igor Pro software (version 9) [43,44]. To complement the synchrotron XRD measurements, diffraction patterns of dried aluminum hydroxide nanoparticles were collected in the powder state. Nanoparticles were heated at 40 °C to remove mobile water while minimizing changes to the hydration layer, gently ground, and mounted on low background sample holders to reduce preferred orientation effects. Powder diffraction data were collected on a Bruker D8 Duo diffractometer (Bruker Corporation, Karlsruhe, Germany) using CuK α radiation (λ = 1.5406 Å) over a 2 θ range of 5–60° with a step size of 0.05° and a total scan time of 1 h per sample. The resulting diffraction profiles, denoted as PXRD, were converted to q to enable direct comparison with the synchrotron XRD (SXRD) data.

2.5. Hydroxyl Content of the Aluminum Hydroxide Nanoparticles Measured by Potentiometric Titrations

To determine the surface hydroxyl content of aluminum hydroxide nanoparticles, a potentiometric titration method was employed. A total of 200 mg of dried hydroxide nanoparticle sample was dispersed in 35 mL of ultra-pure water under continuous magnetic stirring to ensure the formation of a homogenous suspension. To eliminate any potential background contributions, a blank titration using 35 mL of ultra-pure water without nanoparticles was also performed under identical conditions. The nanoparticle suspension was titrated with 0.05 M potassium hydroxide (KOH) using a calibrated pH electrode to monitor the pH changes throughout the titration. The titration curve typically displayed a sigmoidal profile, and the equivalence point was determined by identifying the maximum in the first derivative of the titration curve. The number of moles of KOH consumed at the equivalence point was taken as stoichiometrically equivalent to the number of acidic hydroxyl groups present on the surface of the nanoparticles. Therefore, the total surface hydroxyl content, was calculated as the number of moles of KOH added at the equivalent point. The final surface hydroxyl content was reported as millimoles of hydroxyl groups per gram of aluminum hydroxide (mmol/g).

2.6. Hydration Level Characterization

Two methods were used to characterize the hydration level of various aluminum hydroxide nanoparticles, including mass loss measurements as well as thermogravimetric analysis (TGA). For mass loss experiments, we quantified the hydration level of aluminum hydroxide nanoparticles by measuring the weight difference using a stepwise drying procedure, assuming that unbound residual water becomes more easily removed following this stepwise drying procedure. Aluminum nanoparticles synthesized under different pH conditions were aliquoted into pre-weighed microcentrifuge tubes. Each aliquot was centrifuged at $14,000\times g$ for 20 min to separate nanoparticle aggregates from the suspension medium, and the supernatant was carefully removed without disturbing the pellet. To distinguish between mobile water and adjuvant-bound water within the aggregates, stepwise drying procedure was employed. First, the pellets were dried at 40 °C to remove loosely associated, mobile water molecules without disrupting the structural integrity of the nanoparticles. This mild dehydration step ensured selective removal of unbound surface water. The mass recorded at this stage was denoted as $M_{\text{hydrated pellet}}$, representing the sample with residual surface water still present. Subsequently, the partially dried pellets were heated to 100 °C to remove more strongly bound, adjuvant-associated water. The mass recorded after this step was denoted as $M_{\text{dried pellet}}$, corresponding to the fully dehydrated state of the nanoparticles. This sequential drying approach enabled differentiation between distinct hydration states within the adjuvant aggregates. The mass of each sample was recorded after each drying step until a constant weight was achieved, confirming complete water removal. The hydration level of aluminum nanoparticles was quantified as the percentage of mass loss using the following expression:

$$\text{Percentage of mass loss (\%)} = \frac{M_{\text{hydrated pellet}} - M_{\text{dried pellet}}}{M_{\text{hydrated pellet}}} \quad (2)$$

Thermogravimetric analysis (TGA) was also performed using Discovery TGA550 analyzer (TA Instruments) operated in Hi-Res mode to quantify the water content in aluminum hydroxide nanoparticles. Approximately 20 to 30 mg of each sample was placed in an aluminum pan and heated from 20 to 150 °C under a nitrogen atmosphere at a heating rate of 10 °C/min. In Hi-Res mode, the instrument automatically reduced the effective heating rate in response to significant mass loss events to improve resolution of overlapping

thermal processes. Both thermogravimetric (TG) and derivative thermogravimetric (DTG) curves were recorded. Mass loss below 100 °C was attributed to physically bound water, and the corresponding water content was calculated as a percentage of the initial mass. All measurements were conducted under identical conditions to allow direct comparison across samples. Each measurement was performed in triplicate. Representative datasets are shown for clarity, while quantitative values are reported as the average of three independent runs.

2.7. Differential Scanning Calorimetry Analysis

The thermal transition temperature (T_m) of BSA and BLG, used as model antigens, was measured in both its free (solution) form and adsorbed state onto aluminum hydroxide nanoparticles using Nano DSC (TA Instruments, New Castle, DE, USA). Prior to thermal analysis, complete adsorption of BSA and BLG onto the nanoparticles was ensured by preparing the antigen nanoparticle complexes based on previously established binding isotherms (See Supporting Information). Both BSA and BLG was added to 1 mg of aluminum suspended in 10 mM HEPES buffer (pH 7.4, ionic strength 10 mM) in the following amounts: 4.5 mg for AH, 1.5 mg for AH1, 1 mg for AH1-N, and 0.6 mg for AH2. These concentrations correspond to conditions where BSA and BLG is completely adsorbed onto the respective aluminum nanoparticles (see binding capacity measurements in Supporting Information). Each sample was subjected to a thermal scan from 40 °C to 100 °C at a constant heating rate of 1 °C per minute. To account for baseline variations, appropriate reference scans were subtracted. For DSC measurements of protein solutions, the corresponding buffer scans were subtracted, while for DSC measurements of protein–nanoparticle complexes, nanoparticle-only scans were subtracted to remove baseline contributions. Thermograms were processed and analyzed using Nano Analyzer software (V4.1.0) (TA Instruments).

2.8. Isothermal Titration Calorimetry Analysis

Isothermal titration calorimetry (ITC) measurements were performed on a Nano ITC calorimeter (TA instruments, USA). Prior to measurements, both protein and aluminum hydroxide nanoparticle suspensions were prepared in 10 mM HEPES buffer at pH 7.4 and thoroughly degassed under vacuum. For each titration, the sample cell was loaded with aluminum hydroxide nanoparticles at a fixed aluminum concentration of 1 mg/mL, while the syringe was filled with protein solution at a concentration of 10 mg/mL. A total of 24 injections were performed, with each injections titrating 2.01 µL of protein solution into the nanoparticle suspension. The time interval between each injection was set to 600 s to allow the system to return to thermal equilibrium. All titrations were performed with a stirring speed of 300 rpm at 20 °C. To exclude the influence of the heat of dilution, control titrations of protein into buffer were performed under identical conditions, and the resulting heats of dilution were subtracted from the raw data. The corrected ITC thermograms were analyzed by Nano Analyzer software (TA instruments).

3. Result and Discussion

3.1. Morphological Characterization of Aluminum Hydroxide Nanoparticles Synthesized Under Various pH Conditions

Among commercially available aluminum-based adjuvants, Alhydrogel[®], a widely used aluminum hydroxide formulation, typically consists of aggregated, needle-like nanoparticles [1]. Because nanoparticle morphology plays a key role in determining the structural and functional properties of aluminum adjuvants, we sought to synthesize aluminum hydroxide nanoparticles with controlled shapes and compare them with the commercial adjuvant. Previous studies have demonstrated that the synthesis pH strongly

governs the morphology of aluminum hydroxide nanoparticles, producing structures that range from elongated rods to rectangular nanoparticles [31,45]. This pH-dependent morphological change originates from the layered crystal structure of γ -phase aluminum oxyhydroxide (γ -AlOOH, boehmite), which consists of weakly bonded octahedral AlO_6 double layers [46]. Under basic conditions, hydrogen bonding between surface hydroxyl groups stabilizes lateral, two-dimensional growth, leading to the formation of plate-like or rectangular particles [31]. In contrast, under acidic conditions, protonation of surface hydroxyls disrupts these hydrogen bonds, favoring anisotropic one-dimensional growth and resulting in rod-like or needle-shaped particles. Following this established approach, we synthesized aluminum hydroxide nanoparticles under three different pH conditions to systematically control their morphology. As shown in Figure 1a, the TEM micrograph of aluminum hydroxide prepared under acidic conditions ($\text{pH} < 7$) reveals elongated, needle-like structures with lengths of approximately 100–200 nm, forming loosely connected aggregates, consistent with the morphology reported for conventional Alhydrogel[®]. Figure 1b shows the sample synthesized at near-neutral pH (≈ 7), which retains a similar needle-like shape but with shorter particle lengths and reduced aggregation. In contrast, Figure 1c presents the sample prepared under basic conditions ($\text{pH} > 8$), where the particles appear rectangular. These observations confirm that the pH during synthesis strongly influences the nucleation and growth dynamics of aluminum hydroxide, resulting in distinct morphological outcomes. Specifically, as the synthesis environment becomes more alkaline, the particle shape transitions from elongated needles to rectangular plates.

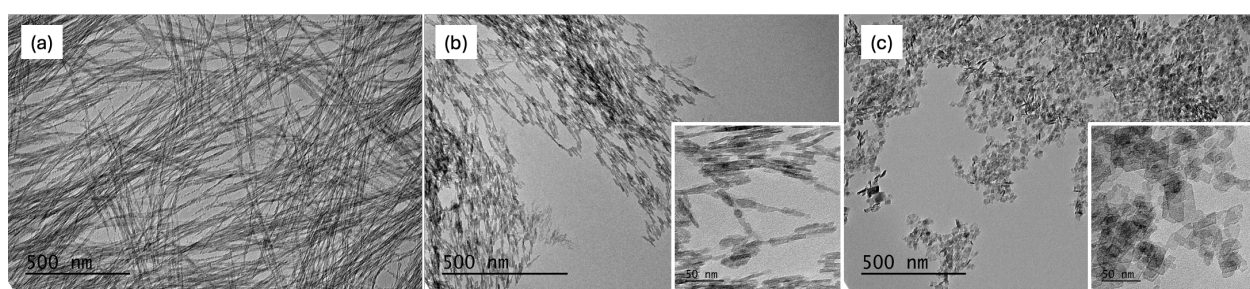


Figure 1. TEM images obtained from aluminum nanoparticles synthesized under acidic, AH1 (a), near-neutral, AH1-N (b) and basic AH2 (c) conditions. Scale bars in the main panels represent 500 nm. The inset reveals the distinct shape of the nanoparticles at increasing pH at higher magnification (Scale bar: 50 nm).

3.2. Crystallinity of the Hydroxide Nanoparticles Synthesized at Different pH

Based on the pH dependent growth mechanism described above, it can be anticipated that variations in synthesis pH may influence not only the external morphology of aluminum hydroxide nanoparticles but also their internal structural ordering. Thus, nanoparticles formed under acidic, near neutral, and basic conditions may exhibit distinct degrees of crystallinity or structural order. This possibility motivates a systematic examination of the crystallinity of the synthesized nanoparticles to assess whether the observed morphological transitions are reflected in their crystallinity. To probe this relationship, we characterized the structural order of the aluminum hydroxide nanoparticles (AH1, AH1-N, and AH2) using SXRD and PXRD, alongside the commercial Alhydrogel (AH) reference sample. The PXRD pattern of the commercial AH sample exhibited a broad, featureless profile (Figure 2), lacking well-defined diffraction peaks. Such diffuse scattering is characteristic of an amorphous or poorly crystalline phase, indicative of the absence of long-range atomic ordering and the presence of highly disordered crystallites [47]. For AH1, the low-intensity reflections correspond to poorly crystalline γ -AlOOH, suggesting significant lattice disorder and limited structural coherence. For AH1-N nanoparticles, the

diffraction peaks became sharper and more intense, indicating that increased synthesis pH facilitated more ordered growth of γ -AlOOH nanocrystals. The basic synthesis of AH2 led to the formation of a highly crystalline phase, as evidenced by well-resolved, narrow reflections consistent with the (020), (120), (031), (051), and (151) planes of γ -AlOOH [35,48] (Figure 2). From this study, formulations prepared at higher pH were found to exhibit a more ordered and crystalline aluminum hydroxide.

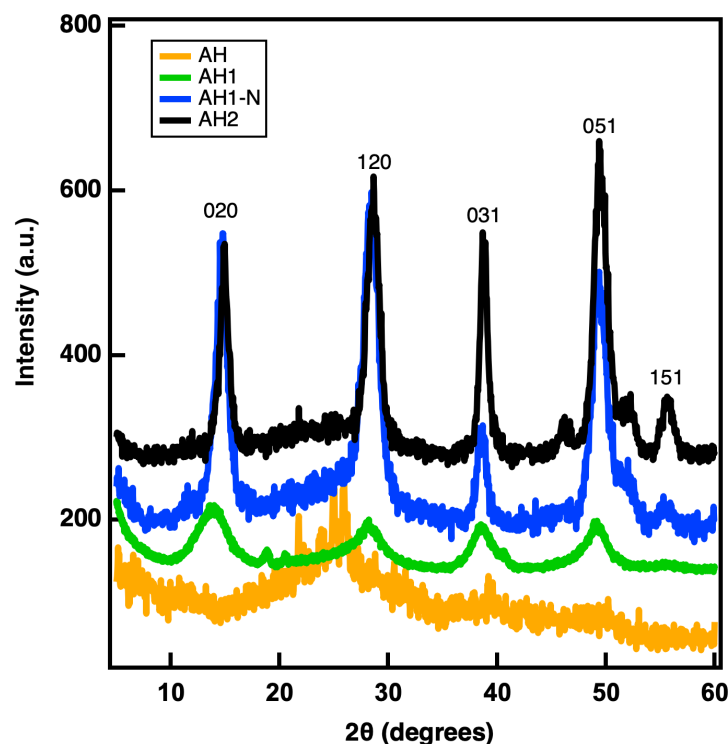


Figure 2. PXRD patterns of aluminum hydroxide nanoparticles AH1, AH1-N, and AH2 compared with commercial AH adjuvant. The synthesized samples exhibit increasing crystallinity with pH, as evidenced by the sharpening and intensification of diffraction peaks corresponding to the (020), (120), (031), (051), and (151) planes of γ -AlOOH. In contrast, the commercial AH shows a broad, featureless pattern, indicating an amorphous or poorly crystalline structure.

To complement the PXRD analysis and gain insight into the structural characteristics of the aluminum hydroxide nanoparticles in their hydrated solution state, SXRD measurements were also performed. SXRD enables in situ characterization making it particularly relevant for evaluating the structural integrity of aluminum adjuvants as used in vaccine formulations, where they remain dispersed in aqueous media [49,50]. The SXRD profiles of aluminum hydroxide samples synthesized under varying pH conditions revealed clear scattering features consistent with the crystalline phases identified by PXRD (Figure 3). Notably, prominent scattering peaks were observed at q values of approximately 1.028, 2.006, 2.673, and 3.446 $\text{\AA}^{-1}$, which directly correspond to the diffraction peaks identified in the PXRD patterns at 2θ values of 14.1°, 27.86°, 38.2°, and 49.1°, respectively. This correlation confirms that the crystalline ordering observed in the solid state is retained in the dispersed phase, reinforcing the conclusion that structural evolution toward a more ordered γ -AlOOH phase occurs with increasing pH. In contrast, the SXRD profile of the commercial AH adjuvant displayed no such distinct features instead a broad, diffuse scattering pattern lacking any sharp maxima is observed consistent with its amorphous or poorly crystalline nature, as previously inferred from its PXRD spectrum. The absence of defined q space peaks in the commercial sample further validates its lack of long-range atomic ordering.

even in their solution state. Together, these results underscore the enhanced crystallinity of laboratory synthesized AH samples prepared with increasing pH.

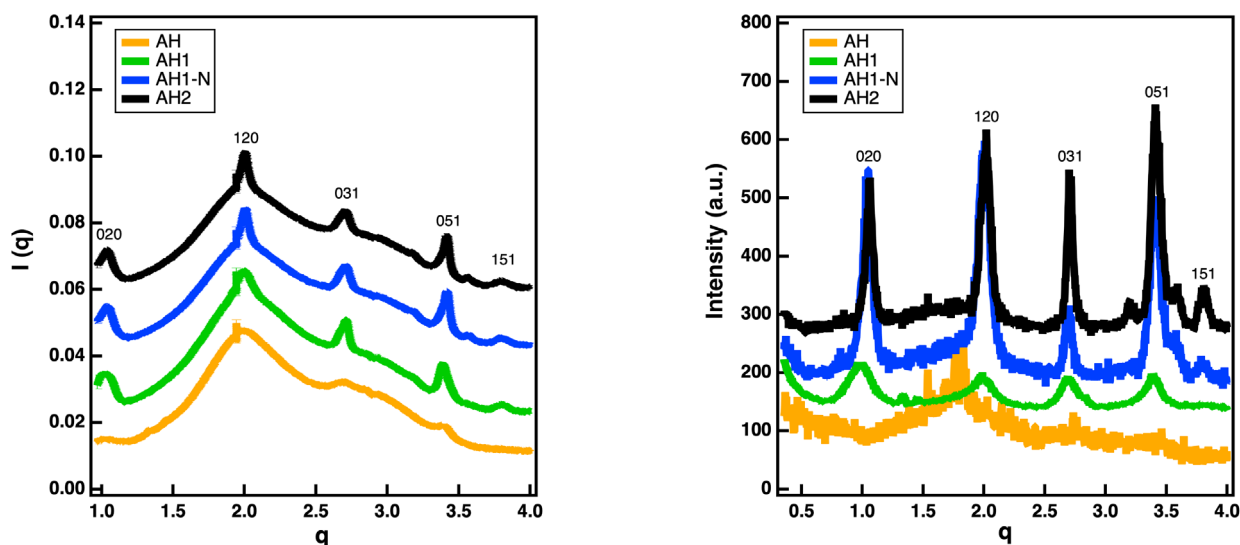


Figure 3. SAXRD (left) and PXRD (right) data of aluminum hydroxide nanoparticles synthesized at different pH (AH1, AH1-N, and AH2) along with commercial AH adjuvant. Datasets are vertically offset to improve visualization.

3.3. Effects of Synthesis pH on Hydroxyl Group Density and Surface Hydration of Aluminum Hydroxide Nanoparticles

To assess whether the pH dependent differences in crystallinity are accompanied by changes in hydroxyl group abundance, the hydroxyl content of aluminum hydroxide nanoparticles was quantified using potentiometric titration and compared with the commercial AH. As shown in Figure 4, a clear trend in hydroxyl content was observed across the synthesized nanoparticles. The AH1, prepared under acidic conditions, exhibited the lowest hydroxyl content (0.144 ± 0.007 mmol/g), consistent with its poorly crystalline structure, while AH1-N displayed an intermediate hydroxyl content (0.214 ± 0.001 mmol/g). The AH2 sample synthesized under basic condition, showed the highest hydroxyl content (0.351 ± 0.003 mmol/g). Commercial AH displayed lowest hydroxyl content (0.116 ± 0.002 mmol/g) reflecting its amorphous or poorly crystalline order. All differences between samples were statistically significant ($p < 0.05$, one-way ANOVA). These results indicate that hydroxyl group density systematically increases with increasing crystalline order which further can be correlated with synthesis pH.

Previous studies on colloidal oxides have shown that the density and spatial arrangement of surface hydroxyl groups critically influence the structure of interfacial water, thereby modulating hydration behavior and biomolecular interactions at the surface [35,40,51]. For example, TiO_2 surfaces with a high density of surface hydroxyls display limited interaction with interfacial water because extensive hydrogen bonding among surface hydroxyls restricts water accessibility. In contrast, surfaces with fewer hydroxyl groups permit more extensive structuring of water at the interface [40]. Building on this understanding, we quantified the extent of hydration of the aluminum hydroxide nanoparticles to establish its relationship with their surface hydroxyl content.

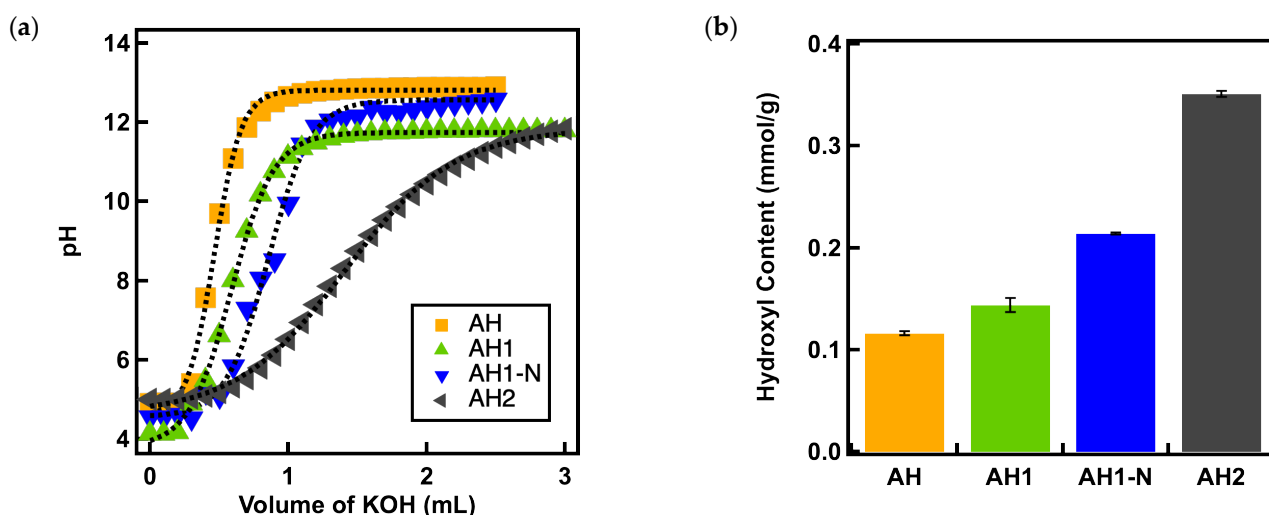


Figure 4. (a) Representative potentiometric titration curves measured from different aluminum hydroxide nanoparticles. Dotted lines are sigmoidal fits to the experimental data. (b) Quantified hydroxyl content of aluminum nanoparticles derived from potentiometric titration. Error bars represent the standard deviation of three different measurements per sample.

To evaluate differences in hydration among aluminum hydroxide nanoparticles synthesized under various pH conditions, a controlled drying experiment was conducted to quantify the extent of water association. In this analysis, mass loss upon heating was used as an indirect indicator of hydration, as detailed in the Experimental Section. Sequential drying at 40 °C and 100 °C enabled differentiation between mobile and more tightly bound water. Among all examined sample, AH exhibited the greatest mass loss (7.8%), indicating a higher degree of water association compared with the laboratory-synthesized nanoparticles (Figure 5). AH1 and AH1-N showed comparable mass losses of 3.28% and 2.83%, respectively, with no statistically significant differences ($p > 0.05$), suggesting that their hydration characteristics are similar despite differences in synthesis pH (Figure 5). In contrast, AH2 exhibited a significantly lower mass loss (0.63%) ($p < 0.05$), indicating markedly reduced hydration. This result demonstrates that nanoparticles synthesized under more basic conditions are inherently less hydrated, as evidenced by their minimal water retention after controlled drying.

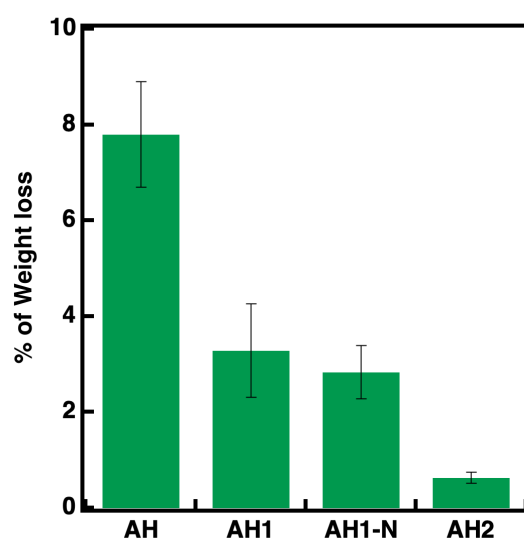


Figure 5. Percentage of mass loss for aluminum hydroxide nanoparticles synthesized under different pH conditions. Error bars represent standard deviation of three independent measurements.

To further validate the complement the mass loss measurements, TGA experiments were also performed to quantify the water content of aluminum hydroxide nanoparticles synthesized under different pH conditions. As shown in Figure 6, all samples exhibit a single dominant mass-loss event below 100 °C, with the corresponding DTG curves displaying one well-defined peak between approximately 20 and 70 °C. This behavior indicates a single-step dehydration process dominated by the water loss, with no evidence of additional thermal degradation events in the temperature range up to 120 °C [52,53]. Quantitative analysis of the mass loss below 100 °C reveals clear differences in hydration among the samples. Commercial AH exhibits the highest water content (89.89%), followed by AH1 (85.14%) and AH1-N (84.10%), which show comparable values ($p > 0.05$), while AH2 displays lower hydration level (75.24%, $p < 0.05$). Both techniques confirm a consistent hydration trend of $AH > AH1 \approx AH1-N > AH2$. These results demonstrate that variations in synthesis pH systematically influence the surface hydration of aluminum hydroxide nanoparticles.

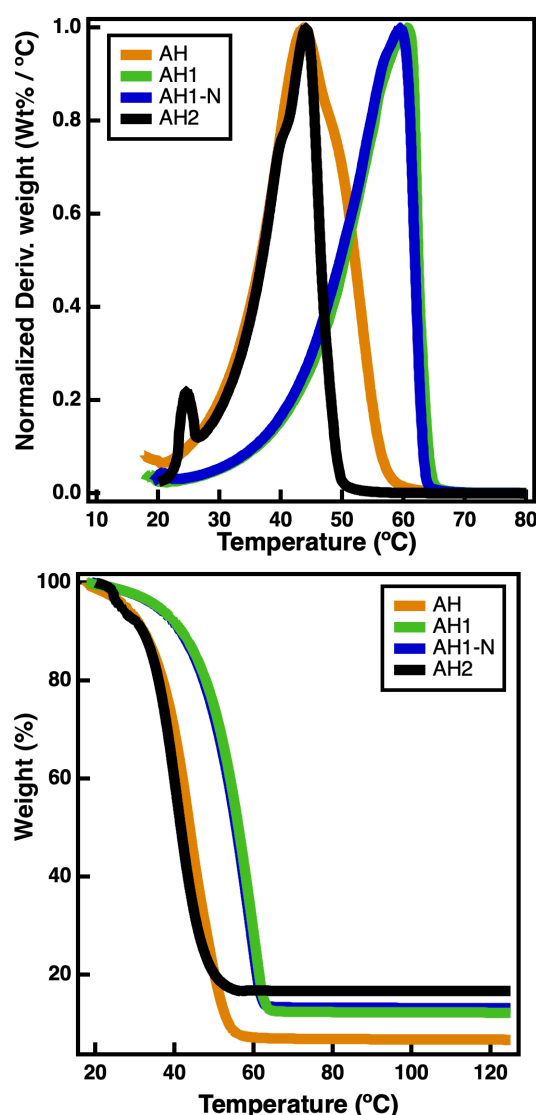


Figure 6. Derivative thermogravimetric (**top**) and thermogravimetric (**bottom**) curves measured from various aluminum hydroxide nanoparticles. Measurements were performed in triplicate, although only one representative curve is shown for clarity.

3.4. Stability of Model Antigens upon Adsorption to Hydroxide Nanoparticles

Our results show that aluminum hydroxide nanoparticles exhibit synthesis-dependent variations in morphology, surface hydroxyl content and surface hydration. In particular, higher hydroxyl density is associated with reduced surface hydration, whereas lower hydroxyl density supports a more extensive interfacial water layer. These differences suggest that antigens may encounter distinct interfacial environments upon adsorption. Therefore, we next examined the stability of protein molecules bound to various aluminum hydroxide nanoparticles. In this study, bovine serum albumin (BSA) and β -lactoglobulin (BLG) were selected as model antigens due to their well-characterized structures and well-documented biophysical properties. Both proteins possess isoelectric points near 5.0 (BSA, $pI \approx 5.0$; BLG, $pI \approx 5.2$) and thus carry net negative charges under physiological conditions. Given the positive surface charge of aluminum hydroxide nanoparticles, electrostatic interactions are expected to contribute to protein adsorption. However, as discussed below, additional interaction mechanisms, including hydration-mediated effects, are also likely to play a role under these conditions [54,55].

BSA and BLG are well-characterized globular proteins with high structural stability; therefore, adsorption induced structural changes at room temperature could be subtle and difficult to resolve. To enhance the sensitivity of the detection, thermal stress was introduced using DSC, allowing small changes in the interfacial microenvironment to be amplified and reflected in protein unfolding behavior. Although the temperatures accessed during DSC measurements are not physiologically relevant, this approach is commonly used to comparatively assess protein stability under different formulation conditions [56]. Building on this rationale, the thermal stability of both free and nanoparticle adsorbed BSA and BLG was evaluated by DSC. To ensure complete protein adsorption onto the aluminum hydroxide nanoparticles and to confirm that the DSC measurements reflect the thermal stability of bound protein molecules, protein binding isotherms were determined for each aluminum nanoparticle system prior to DSC analysis (see Supporting Information). Protein–nanoparticle complexes were then prepared using stoichiometries within the measured binding capacities of the corresponding protein–nanoparticle pairs. Moreover, samples were centrifuged after protein–nanoparticle complexes were formed, and the supernatant was removed and replaced with an equivalent volume of buffer, such that any unbound protein (if any) was excluded from the DSC measurements. As shown in Figure 7, free BSA exhibited a characteristic T_m at c.a. 65.13 °C, consistent with literature values [57–59]. Upon adsorption onto commercial AH, the T_m increased to 68.26 °C, indicating enhanced thermal stability upon interaction with the adjuvant surface. In contrast, adsorption onto the synthesized nanoparticles produced variable thermal responses: BSA bound to AH1 showed a slight decrease in T_m (64.34 °C) (not statistically significant), while adsorption onto AH1-N and AH2 resulted in progressively lower T_m values of 62.29 °C and 59.90 °C, respectively. Analysis of the unfolding enthalpy further supported these trends. Free BSA exhibited an enthalpy change (ΔH) of 544.2 KJ/mol, which increased to a maximum of 579.7 KJ/mol upon adsorption to commercial AH. In contrast, ΔH decreased to 525.6 KJ/mol for AH1, followed by a further reduction to 522.9 KJ/mol for AH1-N, and reached the lowest value of 502.6 KJ/mol for AH2 (Table 1). The higher enthalpy associated with BSA adsorbed to commercial AH suggests a more energetically stable conformation, requiring greater thermal energy to disrupt [59]. Conversely, the progressive decrease in ΔH from AH1-N to AH2 indicates increasing destabilization of the adsorbed BSA, consistent with weaker structural integrity upon adsorption [59].

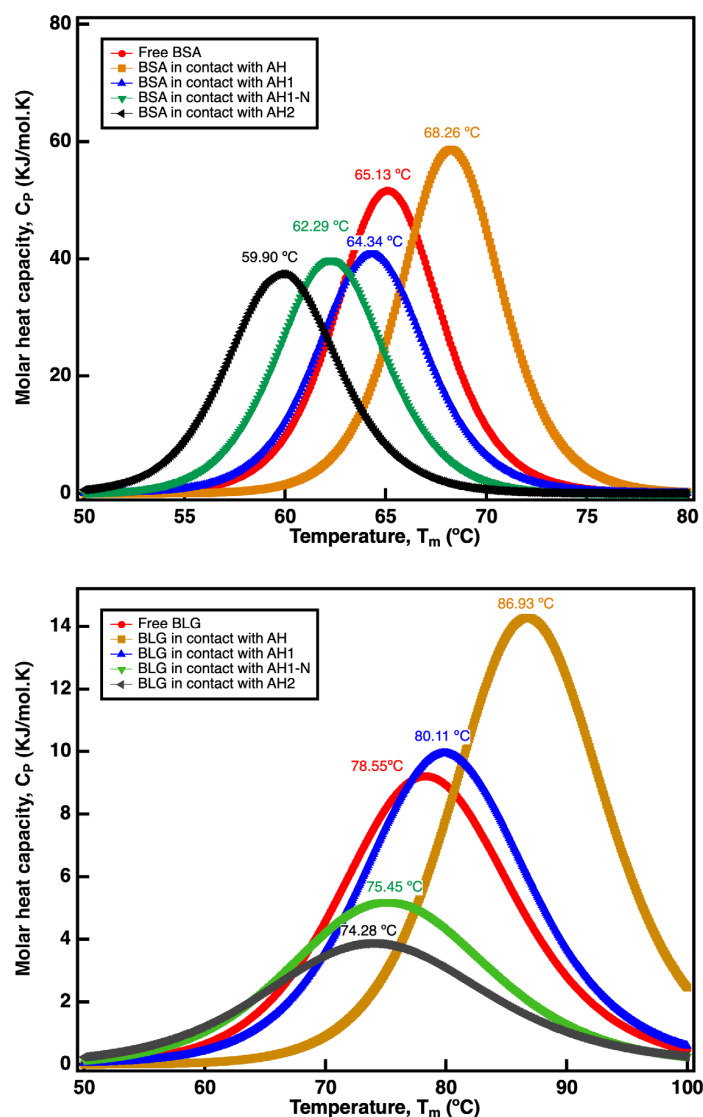


Figure 7. Representative DSC thermograms of protein–nanoparticle complexes prepared according to the binding isotherm. Protein concentrations were 4.5 mg/mL for AH, 1.5 mg/mL for AH1, 1.0 mg/mL for AH1-N, and 0.6 mg/mL for AH2. The free protein sample corresponds to a concentration of 5 mg/mL. Three independent measurements were performed for each sample and yielded comparable thermogram profiles. For clarity, only one representative thermogram is shown.

Table 1. Thermodynamic parameters of BSA and BLG adsorbed onto AH, AH1, AH1-N, and AH2 nanoparticles measured by DSC at 20 °C.

Sample Name	BSA ΔH (KJ/mol)	BLG ΔH (KJ/mol)
Free protein	544.20 ± 5.55	215.55 ± 5.30
AH	579.70 ± 5.30	255.20 ± 3.14
AH1	525.60 ± 2.16	224.35 ± 4.21
AH1-N	522.90 ± 2.19	185.00 ± 5.65
AH2	502.60 ± 1.42	165.00 ± 3.16

A similar trend was observed for BLG (Figure 7). Free BLG exhibited a thermal transition at 78.55 °C ($\Delta H = 215.55$ KJ/mol). Upon adsorption onto commercial AH, the T_m increased markedly to 86.93 °C with a corresponding increase in unfolding enthalpy ($\Delta H = 255.20$ KJ/mol), indicating a substantial stabilization. Adsorption to AH1 resulted in

only modest stabilization ($T_m = 80.11\text{ }^{\circ}\text{C}$, and $\Delta H = 224.35\text{ kJ/mol}$), whereas BLG adsorbed onto AH1-N and AH2 exhibited reduced thermal stability, with T_m values of $75.45\text{ }^{\circ}\text{C}$ ($\Delta H = 185\text{ kJ/mol}$) and $74.28\text{ }^{\circ}\text{C}$ ($\Delta H = 165\text{ kJ/mol}$), respectively. It is also worth noting that the magnitude of T_m shifts depends strongly on the protein. BSA exhibited relatively modest changes in T_m across different nanoparticle formulations, whereas BLG showed more pronounced variations, possibly reflecting a greater sensitivity to the physicochemical properties of the aluminum hydroxide nanoparticle surface. Nevertheless, both proteins followed the same overall trend, exhibiting higher thermal stability upon adsorption to commercial AH and reduced thermal stability on AH2. These observations suggest a relationship between nanoparticle surface hydration and the stability of bound proteins, with more highly hydrated surfaces being associated with improved protein thermal stability. Additional measurements of protein secondary structure would be valuable for assessing potential conformational changes upon nanoparticle interaction and for enabling a more direct interpretation of the DSC results in terms of structure–stability relationships.

3.5. Interaction of BSA with Aluminum Hydroxide Nanoparticles Assessed by ITC

To examine the thermodynamic nature of protein–aluminum hydroxide nanoparticle interactions, isothermal titration calorimetry (ITC) measurements were performed by titrating BSA into suspensions of the different aluminum nanoparticles (Figure 8). Although extraction of quantitative thermodynamic parameters typically requires that binding reaches saturation over the course of the titration, such saturation was not achieved in our ITC experiments. Nevertheless, distinctly different binding behaviors were observed for BSA interacting with the various aluminum nanoparticles. As can be seen from the ITC thermograms shown in Figure 8, interactions between BSA and aluminum nanoparticles exhibit different binding behaviors. The interactions between BSA and commercial AH, as well as nanoparticles synthesized under acidic (AH1) and near-neutral (AH1-N) conditions, were found to be endothermic and predominantly entropy driven (Figure 8). Such favorable entropy change could be resulted from the desolvation of the hydration layers surrounding both the protein and the nanoparticle surface [60]. In contrast, the interaction of BSA with AH2 nanoparticles was exothermic and predominantly enthalpy-driven. This observation is consistent with earlier results showing that the AH2 surface is minimally hydrated. Therefore, desolvation contributions are reduced and direct protein–surface interactions are likely to play a more dominant role.

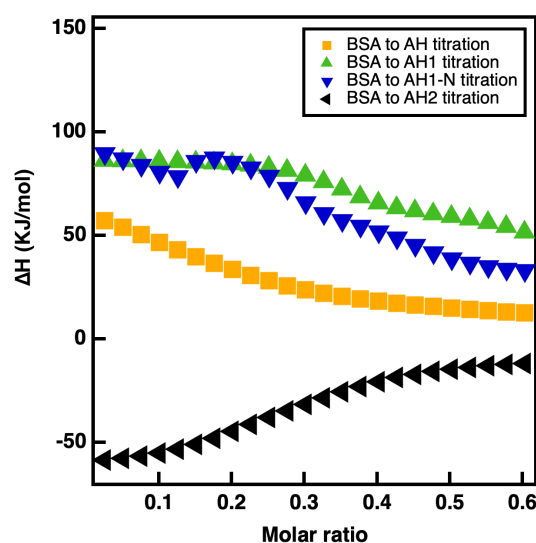


Figure 8. Isothermal titration calorimetry (ITC) profiles for BSA interaction with aluminum hydroxide nanoparticles.

The ITC results led us to speculate possible reasons for the trends observed in the DSC data. It is possible that for aluminum hydroxide nanoparticles with highly hydrated surfaces, such as commercial AH, the strong affinity of the nanoparticle surface for interfacial water may limit direct protein–surface contacts and thereby favor a more compact and thermodynamically stable adsorbed protein state. As the hydration layer at the nanoparticle interface becomes less extensive, direct protein–surface interactions may become more prominent, potentially perturbing intramolecular interactions that help stabilize the native structure of protein. Increased surface engagement may therefore be associated with deviations from the most compact equilibrium structure and reduced conformational stability. This interpretation is consistent with the reduced T_m values observed for BSA when adsorbed on AH2 compared to commercial AH.

This study demonstrates that variations in synthesis pH strongly influence the physicochemical characteristics of aluminum hydroxide nanoparticles, giving rise to distinct interfacial environments for protein adsorption. Rather than evolving independently, particle morphology, crystallinity, surface hydroxyl content, and hydration level appear to change concomitantly as a function of synthesis conditions. These coupled structural and interfacial features collectively dictate how proteins interact with aluminum hydroxide nanoparticles. In the present work, our discussion focuses primarily on interfacial properties, including hydroxyl content, surface hydration, and crystallinity, and on how these parameters relate to the thermodynamic nature of protein adsorption and the thermal stability of bound proteins. This study is motivated by the consideration that, upon adsorption, the nanoparticle surface provides the immediate environment with which protein molecules interact. Therefore, we anticipate that changes in interfacial properties can directly influence the behavior of bound proteins. This emphasis does not imply that nanoparticle morphology plays a negligible role. Rather, morphology is expected to influence protein behavior indirectly, through its effects on nanoparticle packing and the resulting porosity of nanoparticle assemblies.

Our previous results indicate that differences in particle morphology can lead to distinct aggregation behaviors, resulting in aluminum hydroxide networks with varying degrees of porosity [32]. When proteins bind within such porous nanoparticle assemblies, the interior of the network may create a confined environment that differs from the local interfacial chemistry at individual particle surfaces. Confinement effects at this mesoscopic length scale could further influence protein stability and dynamics, in addition to the molecular-scale interactions governed by surface hydration. Eventually, the observed stabilization or destabilization of proteins adsorbed onto aluminum hydroxide nanoparticles could be the outcome of multiple, interconnected factors operating across length scales, ranging from local protein–surface interactions to mesoscale confinement within nanoparticle aggregates. The present study establishes a clear relationship among surface hydroxyl content, hydration level and crystallinity of synthesized aluminum hydroxide nanoparticles, providing a framework for understanding protein stability from the perspective of interfacial chemistry. Future studies examining nanoparticle morphology, aggregation behavior, and porous confinement will be important for developing a more comprehensive understanding of how aluminum-based adjuvants regulate antigen stability.

4. Conclusions

This study highlights how synthesis pH can be used to tune the physicochemical properties and interaction behavior of aluminum hydroxide nanoparticles intended for use as vaccine adjuvants. Variations in synthesis pH govern not only nanoparticle geometry, but also internal crystallinity and surface hydration. Increasing synthesis pH promotes the formation of more crystalline, hydroxyl-rich, and less hydrated nanoparticles, whereas

acidic conditions yield less crystalline and more hydrated structures. Our results further suggest that protein interactions with less hydrated nanoparticles are exothermic and predominantly enthalpy-driven, whereas interactions with highly hydrated nanoparticles are endothermic and entropy-driven. These observations suggest that interfacial water plays an important role in determining protein binding behavior on aluminum hydroxide nanoparticles. The presence of interfacial water may “buffer” direct protein–nanoparticle interactions, thereby potentially reducing interface-induced perturbations to protein conformation and preserving protein thermal stability. Findings of this study underscore the importance of controlling crystallinity and hydration when designing aluminum-based adjuvants to maintain the stability of bound antigen proteins, which is critical for eliciting effective and sustained immune responses.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/colloids10010016/s1>, Figure S1: Binding isotherms of BSA and BLG onto various aluminum nanoparticles; Table S1: Hydrated and dried pellet masses used to calculate percentage of mass loss (%) for various aluminum hydroxide nanoparticle samples; Table S2: Mean water loss and statistical comparison between aluminum hydroxide nanoparticles samples; Table S3: Mean water loss and statistical comparison between aluminum hydroxide nanoparticles samples (TGA method); Table S4: Surface hydroxyl content of aluminum hydroxide nanoparticles.

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