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Mechanistic understanding of the aspect ratio-dependent adjuvanticity of engineered aluminum oxyhydroxide nanorods in prophylactic vaccines

Zhihui Liang\textsuperscript{a,b}, Xin Wang\textsuperscript{a,b}, Ge Yu\textsuperscript{a,b}, Min Li\textsuperscript{a,b}, Shuting Shi\textsuperscript{a,b}, Hang Bao\textsuperscript{c}, Chen Chen\textsuperscript{c}, Duo Fu\textsuperscript{a,c}, Wei Ma\textsuperscript{a}, Changying Xue\textsuperscript{c}, Bingbing Sun\textsuperscript{a,b,\textsuperscript{⁎}}

\textsuperscript{a} State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, 116024 Dalian, China
\textsuperscript{b} School of Chemical Engineering, Dalian University of Technology, 2 Linggong Road, 116024 Dalian, China
\textsuperscript{c} School of Bioengineering, Dalian University of Technology, 2 Linggong Road, 116024 Dalian, China

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Aluminum oxyhydroxide (AlOOH) adjuvants are widely used in human vaccines. However, the interaction mechanisms at the material-bio interface, and further understandings on physicochemical property-dependent modulation of the immune responses still remain uncertain. Herein, a library of AlOOH nanorods with well-defined aspect ratios is designed to explore the mechanisms of adjuvanticity. The aspect ratios of AlOOH nanorods were demonstrated to be intrinsically modulated by the hydroxide supersaturation level during crystal growth, leading to the differences in surface free energy (SFE). As a result, higher aspect ratio AlOOH nanoadjuvants with lower SFE exhibited more hydrophobic surface, resulting in more membrane depolarization, cellular uptake and dendritic cell (DC) activation. By using hepatitis B surface antigen (HBsAg) virus-like particles (VLPs) or SARS-CoV-2 spike protein receptor-binding domain (RBD) as model antigens, AlOOH nanorods with higher aspect ratio were determined to elicit more potent humoral immune responses, which could be attributed to the enhanced DC activation and the efficient antigen trafficking to the draining lymph nodes. Our findings highlight the critical role of aspect ratio of AlOOH nanorods in modulating adjuvanticity, and further provide a design strategy for engineered nanoadjuvants for prophylactic vaccines.

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Introduction

Aluminum oxyhydroxide (AlOOH) is the most widely used adjuvant and has been formulated in diphtheria, hepatitis A, hepatitis B, and human papillomavirus vaccines [1–4]. As one of the adjuvants in authorized human vaccines, it is also acting as the most desirable choice in prompting immune responses against infectious diseases in pre-clinical and clinical studies, including COVID-19 [5], Ebola [6], and malaria [7], etc. However, current vaccine formulations are heavily relying on the commercially available adjuvants, and there is no specific biological understanding on AlOOH-mediated adjuvant effects, which further limits the development of novel adjuvants and their clinical translations.

The interaction between materials and the biological systems plays a critical role in directing the adjuvant activity of AlOOH. For example, Flach et al. has demonstrated that AlOOH nanoparticles (NPs) could interact with the lipids in the plasma membrane of dendritic cells (DCs), leading to lipid sorting and the activation of DCs [8]. Antunez et al. showed that AlOOH NPs were capable of re-organizing phospholipid domains and further reducing monolayer compressibility [9]. Particle-lipid interaction in antigen-presenting cells (APCs) was critical for the initiation of the adjuvant effects. However, these studies focus on aluminum adjuvant (Alum)-cell lipid interactions, further mechanistic studies at the nano-bio interface are desirable. The commercially available adjuvant, i.e., Al-hydrogel®, has been characterized to consist of the aggregates of AlOOH nanorods at a microscopic level [10]. The rod-like nanoparticles have been confirmed to be more effective in mediating the adjuvant effects compared to particles with other shapes [11,12], and the hydroxyl contents and surface functionalization have been considered as critical factors affecting the adjuvant activity of AlOOH nanorods [13]. However, there are insufficient systematic understandings on how the intrinsic characteristics of AlOOH nanorods affect the immunological responses. Further identification of the key physicochemical properties of nanomaterials that impact the
biological interface will be expected to provide a new perspective for in-depth understanding the adjuvanticity of AlOOH nanorods. In this study, we demonstrated the synthesis of AlOOH nanorods with well-controlled aspect ratios. Due to the modulation of crystal growth orientation and the differences in exhibited surface free energy (SFE), the formation of AlOOH nanorods with different aspect ratios was demonstrated to determine their surface hydrophobicity, which further affected the interaction of AlOOH with DC membranes, thus leading to the different levels of cellular uptake as well as DC activation. When formulated in HBsAg VLP or SARS-CoV-2 RBD vaccines, AlOOH nanorods with higher aspect ratio were shown to elicit more robust humoral responses. Moreover, mechanism study demonstrated that AlOOH nanorods promoted the RBD trafficking to draining lymph nodes and the activation of DCs (Scheme 1). All these data provide insights in understanding the mechanism of adjuvanticity, and further design of more effective engineered nanomaterial-based adjuvants.

Results and discussion

Preparation and characterization of AlOOH nanorods

A library of aluminum oxyhydroxide (AlOOH) nanorods was prepared via a hydrothermal method. The $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$ cations could be formed when $\text{Al(NO}_3)_2\cdot 9\text{H}_2\text{O}$ is dissolved in deionized water [14]. The addition of precipitant, ethylenediamine (EDA), promotes the formation of highly charged Al$^{3+}$ cation and polarization of O-H bonds. As a result, sequential deporation of $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$ cations promotes the formation of aluminum monomers, e.g., $[\text{Al(OH)}^\cdot (\text{H}_2\text{O})_3]^{3+}$ and $[\text{Al(OH)}^\cdot (\text{H}_2\text{O})_2]^3+$, which finally lead to the precipitation of solid Al(OH)$_3$ [14]. With the thermolysis taking place under hydrothermal conditions, the pre-formed precursor, amorphous Al(OH)$_3$ colloid, dissolves rapidly into gibbsite fragments and is converted to form $\gamma$-AlOOH double layers through deporation or hydrolysis [15]. It should be noted that all the synthesis process was under an acidic condition, in which the existing of $\text{H}^+$ could react with hydroxyl ions, and break the original hydrogen bonds between the lamellae [16]. Furthermore, the metastable lamellae precursor grows to form nanorods through the process of sticking and scrolling (Fig. 1a) under a thermodynamically driven condition [15].

TEM analysis showed that AlOOH nanorods with uniform size and morphology were obtained (Fig. 1b). The primary size measurement based on TEM images showed that diameters of nanorods were around 10 nm, and the lengths of nanorods ranged from 20 to 600 nm (Fig. 1b-c). The aspect ratios of as-prepared AlOOH nanorods were determined based on TEM images, and they were 51 ± 14, 23 ± 5, and 7 ± 2 for Rod 1, Rod 2 and Rod 3, respectively (Table 1). The different aspect ratios of the AlOOH nanorods could be attributed to the change of hydroxide (OH$^-$) supersaturation level controlled by the initial synthesis conditions, which determines the growth rate in the longitudinal direction. At lower OH$^-$ supersaturation level, the rate of AlOOH crystal growth was faster than nucleation, resulting in longer crystals formation. While at higher OH$^-$ supersaturation level, nucleation dominates crystal growth, leading to the formation of AlOOH nanorods with lower aspect ratios [17]. Dynamic light scattering (DLS) analysis showed that the hydrodynamic sizes of AlOOH nanorods in water were in the range of 100–300 nm. Zeta potential measurement showed that AlOOH nanorods carried positive charges, and they were ranging from 44 to 51 mV in water (Table 1). For comparison, the characterization of a commercial adjuvant, Alhydrogel®, was also shown (Fig. 1b-c and Table 1). It was demonstrated that they exhibited similar rod-like morphology and carried positive charges in water. It should be noted that the commercial Alhydrogel® exhibited a relative larger hydrodynamic size, i.e., 503 ± 9 nm, due to the tendency to form aggregation in aqueous suspensions [18]. Furthermore, FTIR analysis showed the bands at 1067 cm$^{-1}$ and 478 cm$^{-1}$, which were assigned to asymmetric Al-OH stretching vibration ($\nu_3$ Al-OH) and Al-O deformation vibration bands ($\delta_3$ Al-O) [19], respectively (Fig. S1). The area ratio ($\nu_3$ Al-OH/$\delta_3$ Al-O) was used to represent the relative amount of hydroxyl groups. It was shown that nanorods with different aspect ratios exhibited similar levels of hydroxyl contents, which was further verified by potentiometric titration of OH$^-$ content (Table S1) [20].

X-Ray Diffraction (XRD) was used to determine the crystal structures of AlOOH nanorods (Fig. 2a). XRD pattern exhibited six typical reflections that correspond to crystalline orthorhombic boehmite [21,22] and the sharp reflection peaks were indexed as $\gamma$-AlOOH [21]. During crystal growth, the exposed face, (010), was considered to exhibit the lowest surface energy that indicated the most stable basal surface [23,24]. For Rod 1, they exhibited the most degree of crystal growth along [100] direction [25], resulting in an increased preferentially orientation towards the (010) basal plane during the rolling process after the cleavage of hydrogen bonds between the boehmite lamellae (Fig. 2a). Thus, Rod 1 with higher aspect ratio could exhibit a lower overall surface free energy. Based on a reported optical method [26], the surface free energy of AlOOH nanorods was estimated (Fig. S2). It was shown that AlOOH nanorods with higher aspect ratio exhibited lower surface energy as expected (Fig. 2b). This experimental result is also consistent with the prediction of an Ab initio molecular-dynamics calculation, which suggested that AlOOH rods grown in the [100] direction are more stable as a result of the coordinately saturated Al ions [27].

The polarity of the material surface is proportional to the SFE. Thus, the interfacial energy between the material and water increases as the surface polarity decreases, which can result in a more hydrophobic surface [28]. Therefore, AlOOH nanorods with lower SFE are expected to be more hydrophobic. A hydrophobic dye, Rose Bengal (RB), which shows an increased adsorption to particle surface with increasing hydrophobicity [29], was selected to determine the hydrophobicity of AlOOH nanorods. The slope of the partitioning quotient (PQ) against to total surface area indicated a prominent hydrophobicity of Rod 1 (Fig. 2c and S3). In comparison, the PQ slopes for Rod 2, Rod 3 and Alhydrogel did not exceed 0.1, indicating their relatively lower hydrophobicity. Taken together, these data suggest that the crystal structure of AlOOH nanorods (Fig. 2d) affects their SFE, ultimately resulting in the differences in the hydrophobicity.
AlOOH nanorod-cell interactions and activation of BMDCs

Surface hydrophobicity could mediate the nano-bio interactions [30], including the affinity of NPs to the cell membrane [31] and translocation behaviors across lipid bilayers [32], etc. Thus, the interaction between AlOOH nanorods and cell membrane was evaluated. As a sensitive indicator of nanoparticle-membrane interaction, membrane depolarization was measured using a fluorescence-based membrane potential sensitive dye, DiBAC\textsubscript{4}(3). Rod 1 showed a significant enhancement in the increase of membrane potential than that of Rod 3 or Alhydrogel\textsuperscript{®}, indicating an aspect ratio-dependent level of cell membrane depolarization (Fig. 3a). Additionally, the interaction kinetics of phospholipid membranes with nanorods were determined by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). Supported lipid bilayers (SLBs) were constructed using 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) (Table S2). After DMPA bilayers were formed through a surface-mediated vesicle fusion [33] (Fig. S4), the equilibrium adsorption and kinetics of AlOOH nanorods on the SLBs were observed to be dependent on the aspect ratios (Fig. 3b). The frequency change before the equilibrium signal due to adhesion of Rod 1 on

Table 1
The aspect ratios, hydrodynamic sizes and zeta potentials of AlOOH nanorods and Alhydrogel\textsuperscript{®} in water.

| Sample ID | Length (nm) | Aspect Ratio | Hydrodynamic Size in Water (nm) | Zeta Potential in Water (mV) |
|-----------|-------------|--------------|---------------------------------|-----------------------------|
| Rod 1     | 342 ± 98    | 51 ± 14      | 271 ± 3                         | 44 ± 1                      |
| Rod 2     | 277 ± 43    | 23 ± 5       | 254 ± 8                         | 55 ± 1                      |
| Rod 3     | 47 ± 11     | 7 ± 2        | 125 ± 2                         | 51 ± 2                      |
| Alhydrogel\textsuperscript{®} | 41 ± 7 | 6 ± 1 | 503 ± 9 | 27 ± 2 |

Fig. 1. Schematic illustration of the synthetic chemistry and TEM analysis of AlOOH nanorods. (a) Schematic representation of the crystal growth of AlOOH nanorods under acidic conditions via a hydrothermal method. (b) Representative TEM images and (c) length distribution analysis of AlOOH nanorods prepared with initial pH at 4.0, 5.0, and 6.0, respectively. They were noted as Rod 1, Rod 2, and Rod 3. All synthesis of engineered AlOOH nanorods were conducted at 160 °C for 16 h. At least 150 nanorods were randomly selected to determine the length distribution. Alhydrogel\textsuperscript{®} adjuvant was used as a control.
lipid bilayers was much higher than that of Rod 3 or Alhydrogel®, demonstrating the most significant capture of Rod 1 by SLBs. In addition, the steep slope of frequency change of Rod 1 also indicated the strongest interaction with the constructed membrane bilayers.

Considering the fact that the interaction of nanoparticles with cell membrane is key determinant of particle uptake, confocal microscopy was performed to determine the intracellular FITC-labeled AlOOH nanorods in bone marrow-derived dendritic cell (BMDCs). AlOOH nanorods showed an aspect ratio-dependent cellular uptake, with Rod 1 exhibiting the highest uptake level (Fig. 3c). The cellular uptake was also quantitatively confirmed by flow cytometry analysis (Fig. 3d). This demonstrated that the increase in AlOOH nanorods’ hydrophobicity enhanced the affinity of AlOOH nanorods to cell membrane and therefore induced more significant endocytosis (Movie S1). In addition, when RBD was used as a model antigen, AlOOH nanorods could substantially increase antigen uptake than that for pure antigen (Fig. 3e). All these results indicated that the hydrophobic effect could regulate the cellular uptake process of AlOOH nanorods, which is consistent with the existing studies [32–34]. It was reported that hydrophobic NPs could penetrate the bilayers,

Fig. 2. XRD and hydrophobicity analysis of AlOOH nanorods. (a) XRD patterns and (b) surface free energy of AlOOH nanorods. (c) Relative hydrophobicity was determined by adsorption of Rose Bengal (40 μg/mL) on AlOOH nanorods (0–1.2 mg/mL). Alhydrogel® was used as a control. (d) Schematic diagram of the crystal growth of AlOOH nanorods under the hydrothermal conditions. *p < 0.05 and **p < 0.01.
driven by the hydrophobic interactions between NPs and the lipid tails [32]. In addition, study by Ji et al. has demonstrated that high aspect ratio CeO$_2$ nanorods tended to bundle together that was more conducive to contact with the cell surface [17], which may be another reason why Rod 1 showed a significant enhancement on the cellular uptake in BMDCs (Fig. 3c-d). However, using a coarse-grained molecular dynamics (CGMD) model, Huang et al. has indicated that although a spherocylindrical NP with a higher aspect ratio was conducive to be internalized, the aspect ratio does not play a major decisive role in the completion of the cellular uptake [35].
Thus, more studies are necessary to explore the cellular uptake of AlOOH nanorods at the molecular level, including the effect of "protein crown" on the cellular uptake of NPs [34].

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The activation of antigen-presenting cells (APC) is essential for the priming of immunity [36], thus, flow cytometry analysis was performed to examine the ability of AlOOH nanorods to promote DC maturation [11]. After exposed to BMDCs for 12 h, AlOOH nanorods significantly upregulated the expression of costimulatory molecules on CD11c+ cells (Fig. 3f-g). Rod 1 showed the highest level of CD80 and CD86 expressions, with an increase of 30% and 10% compared to Alhydrogel®, respectively. In addition, the ability of AlOOH nanorods to induce the cytokine productions in BMDCs was assessed. Rod 1 notably showed increased interleukin-1β (IL-1β) secretion compared to other nanorods (Fig. 3h). The significant higher section of IL-1β by Rod 1 may be associated with the higher level of lysosomal damage which is more likely to be caused by materials with higher aspect ratio [17]. In addition, AlOOH nanorods induced an aspect ratio-dependent IL-6 production (Fig. 3i). IL-6 has been identified as a signal that directs the magnitude of Th2 immunity [37,38]. However, there was no significant IFN-γ production in

Fig. 4. AlOOH nanorods enhanced humoral immune responses and antigen retention in HBsAg vaccination model. (a) Vaccination procedure. 6-week female C57BL/6 mice (n = 6) were vaccinated with HBsAg/AlOOH (2 μg/30 μg Al) intramuscularly (i.m.) on day 0 and day 21. Saline-, HBsAg- and Alhydrogel-immunized mice were used as controls. Serum HBsAg-specific (b) total IgG and (c) IgG1 titers on day 42. *p < 0.05 and **p < 0.01 compared to saline buffer-treated mice; *p < 0.05 and *p < 0.01 compared to HBsAg/Rod 1-immunized mice. (d-e) HBsAg retention at injection sites. AlOOH nanorods were pre-adsorbed with Alexa Flour™ 700-labeled HBsAg. C57BL/6 mice were immunized with different vaccine formulations containing 10 μg of HBsAg and 250 μg of Al by intramuscular administration. The fluorescence intensity at Ex 702/Em 723 nm was recorded by an IVIS optical imaging system (NightOwl II LB 983, Berthold) at the indicated timepoints (0–96 h). IndiGo software was used to quantify the mean fluorescence intensity (MFI) at the injection sites, and the data were normalized by the initial fluorescence intensity. *p < 0.05 and **p < 0.01 compared to HBsAg-treated mice; *p < 0.05 compared to HBsAg/Alhydrogel-immunized mice.
VLP vaccine model (Fig. 4a). For comparison, Alhydrogel® as a control. Chromogenic Limulus Amebocyte Lysate assay evaluate the enhancement of adaptive immune responses in a HBsAg vaccination model which may further instruct their adjuvant activity ratios could affect their immunostimulating potentials in vitro.

Humoral immune responses in AlOOH nanorod-adjuvanted HBsAg VLP vaccination model

Considering their aspect ratio-dependent potential to activate BMDCs in vitro, Rod 1 and Rod 3 were selected to systemically evaluate the enhancement of adaptive immune responses in a HBsAg VLP vaccine model (Fig. 4a). For comparison, Alhydrogel® was used as a control. Chromogenic Limulus Amebocyte Lysate assay confirmed the free of endotoxin (Fig. S7). After HBsAg was formulated with AlOOH nanorods, 6-week female C57BL/6 mice were vaccinated by intramuscular injection (i.m.) on day 0 and 21. Serum was collected on day 28 and 42 (Fig. 4a). On day 28, engineered AlOOH nanorod-adjuvanted mice exhibited comparable levels of HBsAg-specific total IgG and IgG₃ titers compared with those of Alhydrogel®-immunized mice (Fig. S8). However, there was no significant difference on the HBsAg specific antibody titers between Rod 1- and Rod 3-immunized groups. On day 42, increased levels of humoral immune responses elicited by engineered nanorods were observed, and the total IgG titer in the serum of Rod 1-adjuvanted mice was 2.13 × 10⁵, far exceeding than that of Rod 3 and Alhydrogel®, which were 5.03 × 10⁴ and 1.07 × 10⁵, respectively (Fig. 4b). Similarly, HBsAg-specific IgG₃ titers were evaluated by 2.2 and 2.5 times in the serum of the Rod 1-primed mice compared with Rod 3 and Alhydrogel®, respectively (Fig. 4c). Meanwhile, splenocytes were stimulated ex vivo, and the cytokine production was determined. It was shown that IL-6 secretion was significantly increased in Rod 1-adjuvanted mice (Fig. S9a), but not for IFN-γ (Fig. S9b). Moreover, the serum biochemical analysis in AlOOH-adjuvanted mice showed no significant changes in biomarkers for systematic toxicity (Table S3), suggesting the biocompatibility of the engineered nanorods.

Moreover, the role of AlOOH nanorods in the antigen “depot effect” was evaluated. After formulation, mice were immunized with Alexa Flour™ 700-labeled HBsAg or HBsAg/AlOOH intramuscularly, and the antigen deposition at the injection sites was monitored over time. It was demonstrated that the mean fluorescence intensity (MFI) at the injection sites of HBsAg-immunized mice had decreased to less than 50% at 12 h after the immunization, while the residual antigen in AlOOH nanorod-adjuvanted mice still maintain more than 70% (Fig. 4d-e). It should be noted that there was no significant difference in antigen retention between Rod 1- and Rod 3-adjuvanted mice, which could be explained by the comparable level of adsorptive coefficient of HBsAg adsorbed on AlOOH nanorods (Table S4) [40,41]. It indicated that the antigen “depot effect” induced by AlOOH nanorods was not affected by the aspect ratios of the nanoparticles when HBsAg was selected as an antigen model. Instead, it was the cellular uptake and the activation of DCs that could mediate the enhanced HBsAg-specific humoral responses triggered by Rod 1 (Fig. 3c-g).
Potent humoral immune responses in SARS-CoV-2 RBD vaccination model and activation of lymph nodes

SARS-CoV-2 RBD, a subunit soluble antigen, was selected to further confirm the role of aspect ratios of AlOOH nanorods in their immunomodulatory effects. Adsorption study confirmed equal adsorption level of RBD to the different adjuvants (Table S5). Rod 1- or Rod 3-adjuvanted vaccines, were immunized into 6-week female BALB/C mice (Fig. 5a). On day 42 post the first administration, the ROD-adjuvant ed vaccines were immunized further to confirm the role of aspect ratios of AlOOH nanorods in their model and activation of lymph nodes (Fig. 5b-c). It should be noted that Rod 1 elicited comparable levels of RBD-specific IgG and IgG₂ titers with those of Alhydrogel®, showing the robust humoral immune responses. In addition, the immunopathology analysis of the major organs, e.g., the heart, liver, spleen, lung and kidney, indicated the good biological safety of the adjuvants (Fig. 5d).

Antigens are transported from the interstitial space to downstream lymph nodes via lymphatic vessels to initiate the effective adaptive immunity [42]. Since the internalization of RBD in BMDCs induced by AlOOH nanorods had been demonstrated in vitro, we speculate that it could also modulate the antigen presenting process.

To examine the trafficking of RBD into the draining lymph nodes (dLNs), FITC-labeled RBD was formulated with AlOOH nanorods and immunized to female CD1 mice via intramuscular injection. After 48 h, the entry of RBD-internalized DCs to dLNs in Rod 1-treated mice showed an improved level compared to other groups (Fig. 5e and S10a). Moreover, the activation of DCs located in dLNs was determined. Compared with the soluble RBD or Rod 3-adjuvanted groups, Rod 1-immunized group promoted the MHC II molecule expression (Fig. 5f and S10b), as well as the co-stimulation molecule, CD80 (Fig. 5g and S10c), suggesting that the activation of DCs played an important role in AlOOH nanorod-mediated immune responses (Fig. 3f-g).

Conclusion

In this study, a library of AlOOH nanorods with controlled aspect ratios was designed. They were determined to exhibit correlated surface hydrophobicity, and the hydrophobic AlOOH nanorods elicited more membrane depolarization and subsequent cellular uptake in dendritic cells. By using both HBSAg and SARS-CoV-2 RBD vaccine models, AlOOH nanorods with higher aspect ratios exhibited enhanced antigen-specific humoral responses, which could be attributed to the enhanced activation of DCs and efficient antigen trafficking to draining lymph nodes. These findings shed light on the mechanisms of AlOOH nanorods to exert the adjuvant effects, which further provide a scientific basis for the design of more effective engineered nanomaterial-based vaccine adjuvants.

The easy synthesis route and well-controlled properties ensure the reliability of engineered AlOOH nanoadjuvants. For their translational potential in vaccine developments, it still needs further validation in other antigen models to evaluate their adjuvanticity. Regarding the absence of Th1 type immunity, engineered nanomaterial-based combination adjuvants can promise more balanced immune responses. In addition to be formulated in subunit and VLP vaccines, the engineered AlOOH nanorods can potentially be integrated in inactivated and lipid nanoparticle (LNP)-based mRNA vaccines.

CRediT authorship contribution statement

Bingbing Sun and Changying Xue conceived the study and designed the experiments. Zhihui Liang, Xin Wang, Ge Yu, Shuting Shi and Bingbing Sun analyzed the data and contributed to writing of the manuscript. All authors contributed to the manuscript and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2022.101445.

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