

Pollen-Mimetic Metal–Organic Frameworks with Tunable Spike-Like Nanostructures That Promote Cell Interactions to Improve Antigen-Specific Humoral Immunity

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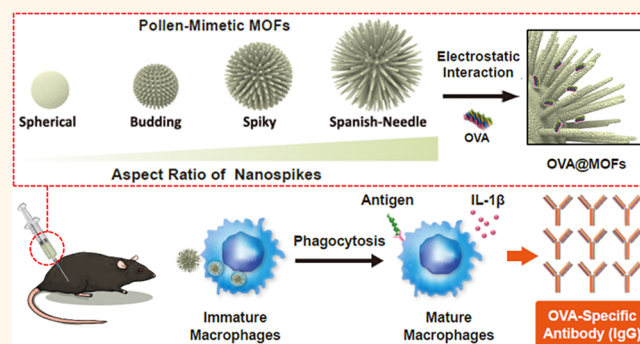
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ABSTRACT: The exine capsules of pollen particles exhibit a variety of characteristic surface morphologies that promote their cell interactions; their use as antigen carriers for vaccination has been proposed. However, the allergy-causing substances in pollen particles may not all be removed, even by vigorous chemical treatments. To resolve this issue, this work develops systemic approaches for synthesizing pollen-mimetic metal–organic frameworks (MOFs), which comprise aluminum (Al) ions and an organic linker (2-aminoterephthalic acid), with tunable spike-like nanostructures on their surfaces. The as-synthesized MOFs act not only as a delivery vehicle that carries a model antigen (ovalbumin, OVA) but also as an adjuvant (AI). Scanning and transmission electron microscopies images reveal that the aspect ratio of the nanospikes that are grown on the MOFs can be controlled. A higher aspect ratio of the nanospikes on the MOFs is associated with greater cell attachment and faster and more efficient phagocytosis in cells, which results in greater expressions of pro-inflammatory cytokines. Consequently, a more robust immune response against the antigen of interest is elicited. These findings have broad implications for the rational design of the future antigen/adjuvant-presenting particles for vaccination

KEYWORDS: vaccination, antigen, adjuvant, nanospikes, biomimetics



Vaccination can induce pathogen-specific immune responses, providing potent and long-term protection against many forms of infectious diseases. Subunit vaccines that consist of well-defined protein antigens that are derived from pathogens have become favored because the safety profiles of such vaccines are better than those of conventional whole pathogen-based vaccines, which are associated with a risk of incomplete inactivation.^{1,2} Moreover, subunit vaccines can be designed to specifically target and neutralize epitopes, preventing any antibody-dependent enhancement of the disease against which they are being used.^{3–5} However, low immunogenicity is often observed following vaccination with highly purified subunit antigens because they lack many intrinsic pathogenic characteristics.⁶ To optimize their immune responses, an adjuvant is commonly coadministered with the

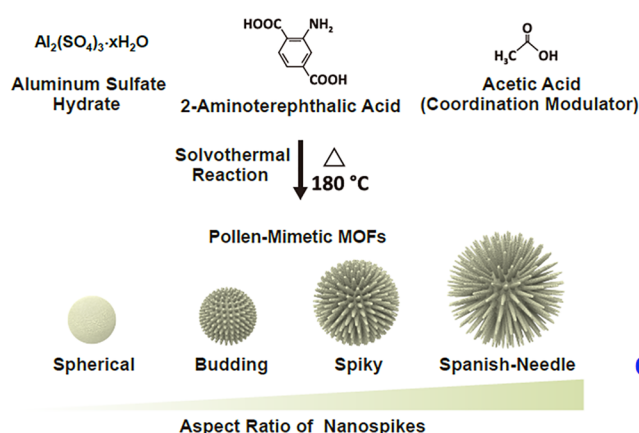
purified subunit vaccines, using a carrier system that can efficiently deliver them to antigen-presenting cells (APCs).^{1,2,6,7}

Pollen is a powdery material of micron-sized particles, which have a range of highly characteristic surface morphologies that are thought to be crucial in their reproduction process.⁸ On account of these surface morphologies, the use of exine capsules of pollen particles as a delivery vehicle for various pharmaceutical agents, including antigens, has been pro-

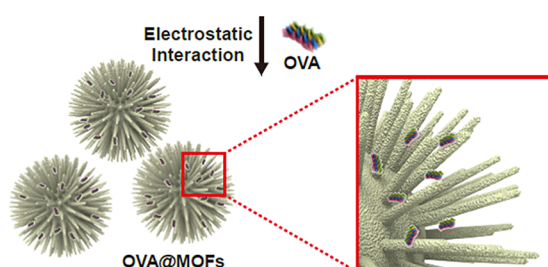
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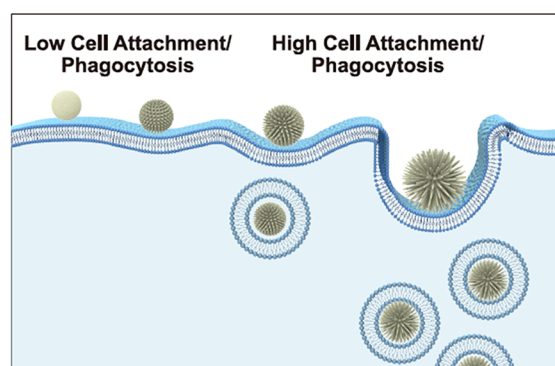
a. Composition/Structures of Pollen-Mimetic MOFs with Tunable Spike-Like Nanostructures



b. Adsorb OVA on External Surfaces of MOFs



c. Enhance Cell Attachment and Phagocytosis



d. Improve Antigen-Specific Humoral Immunity

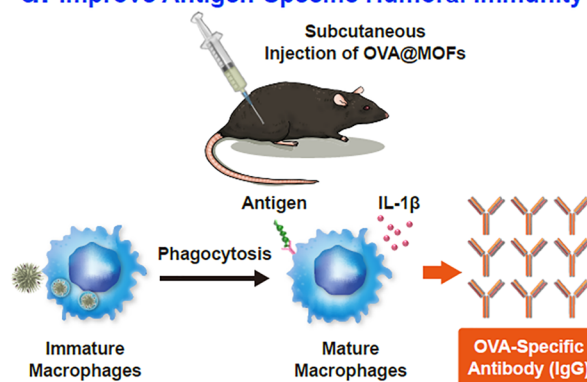


Figure 1. Composition and structures of as-proposed pollen-mimetic MOFs with tunable spike-like nanostructures that carry a model antigen (OVA) and their *in vivo* mechanism in vaccination. (a) Synthesis of pollen-mimetic MOFs with nanospikes with tunable aspect ratios in a solvothermal reaction of aluminum sulfate hydrate, 2-aminoterephthalic acid, and acetic acid at 180 °C. (b) Adsorption of OVA onto as-prepared MOFs by electrostatic interaction. (c) Enhancement of cell attachment and phagocytosis via physical interaction between spike-like nanostructures on MOFs and cell membranes. (d) Antigen-specific humoral immune response upon subcutaneous injection of OVA@MOFs in mice.

posed.^{9,10} However, pollen particles contain many proteins and lipids, which may cause allergic reactions.^{11–13} To ensure their safety, allergen components that are associated with pollen particles must be removed before they are used, typically under extremely harsh conditions that involve various organic solvents, bases, and acids at elevated temperatures.^{9,14} Following such vigorous and prolonged chemical treatments, the surface morphologies of the thus obtained exine capsules may be significantly altered, and not all of their allergy-causing components may be removed.^{9,14}

The surface morphology of pollen particles is responsible for their efficient recognition of, and tight adherence to, the stigma, increasing the likelihood of pollination.^{8,15} This natural pollination process has inspired the proposed use of pollen-mimetic metal–organic frameworks (MOFs) with tunable spike-like nanostructures that can effectively adhere to, and be phagocytosed by, APCs for vaccination. MOFs are organic–inorganic hybrid compounds that consist of metal ions and organic linkers and have tunable morphological sizes and structures.^{16,17} Numerous MOFs have been synthesized for biomedical applications, including bioimaging,¹⁸ biosensing,¹⁶ biocatalysis,¹⁹ and the storage and release of metal ions for therapeutic purposes.^{20,21} Furthermore, the potential use of MOFs in drug delivery has been extensively studied owing to their highly porous structure, which improves their loading capacity for therapeutic molecules.^{16,17,22,23}

Figure 1 presents the as-proposed pollen-mimetic MOFs that contain aluminum (Al) ions and an organic linker of 2-aminoterephthalic acid with tunable spike-like nanostructures that carry a model subunit protein antigen, ovalbumin (OVA); their *in vivo* working mechanism in vaccination is also shown. Al salts are commonly used as an adjuvant in veterinary and human vaccines.^{24,25} The as-prepared MOFs herein can thus act as not only an antigen carrier but also an adjuvant. Because these MOFs are made of synthetic materials, they are anticipated to have no allergenic side effects.

When the as-proposed MOFs are administered subcutaneously in mice, their spike-like nanostructures can cause them to interact effectively with the resident APCs, such as macrophages, and be rapidly phagocytosed. The APCs are then activated by their ingested antigen (OVA) and adjuvant (Al) to secrete pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and IL-6, eliciting an antigen-specific antibody response. Most importantly, with the colocalization of OVA and Al in the same APCs, this antigen-specific humoral immunity can be significantly increased.

RESULTS AND DISCUSSION

Many pollen particles have spike-like nanostructures on their surfaces, favoring their interaction with insects or stigma.^{8,15} To facilitate their interaction with APCs, pollen-mimetic MOFs that have nanospikes at tunable aspect ratios (ratio of height to width at midheight) were synthesized. Studies have shown that

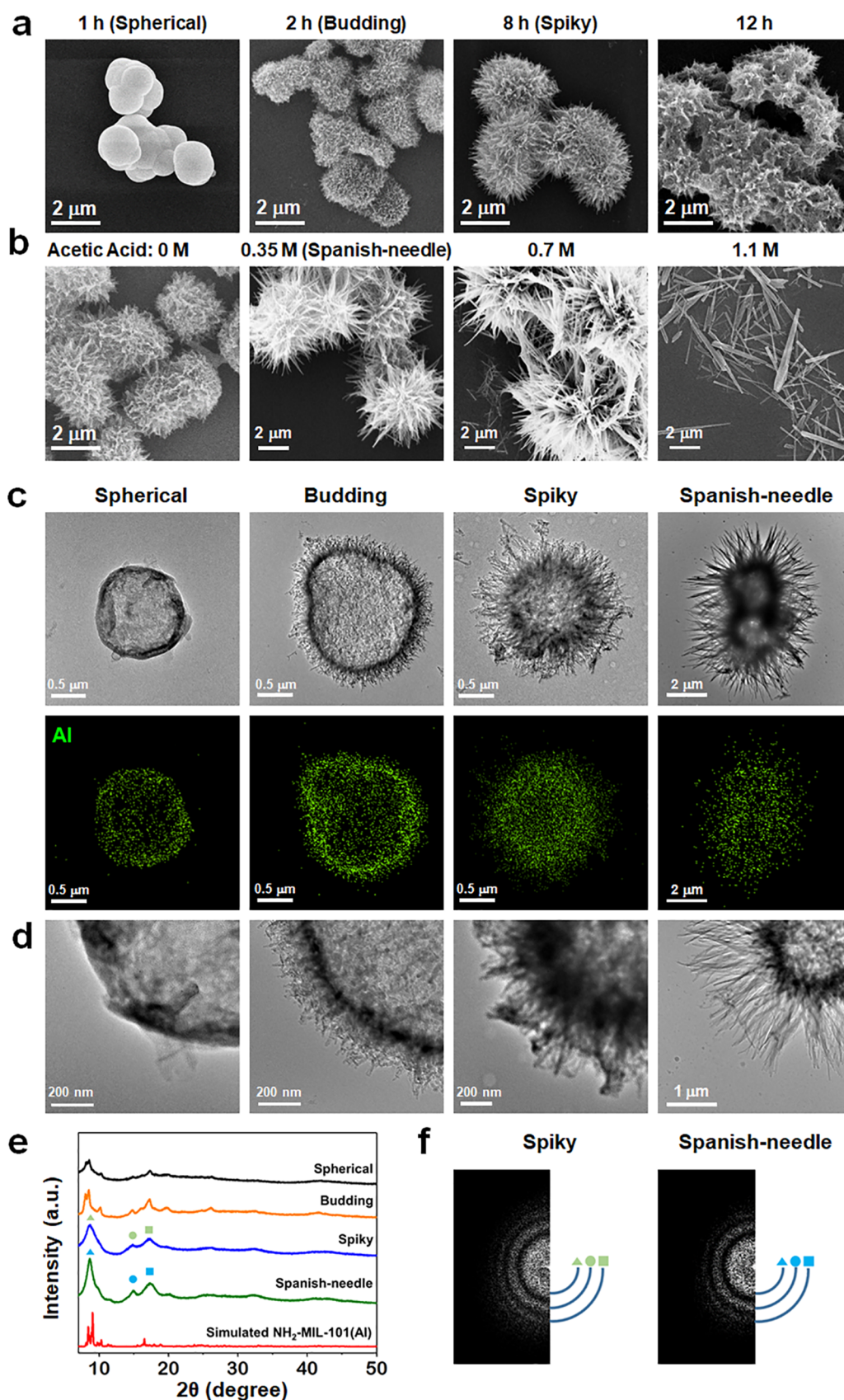


Figure 2. SEM images of (a) MOFs that were synthesized at 180 °C for various periods and (b) those that were synthesized at 180 °C for 8 h with various concentrations of acetic acid. TEM micrographs and elemental mapping images of (c) spherical, budding, spiky, and Spanish-needle MOFs and (d) their nanospikes. (e) PXRD patterns of spherical, budding, spiky, and Spanish-needle MOFs and simulated $\text{NH}_2\text{-MIL-101(Al)}$. (f) Electron diffraction patterns of nanospikes that protruded from spiky or Spanish-needle MOFs.

the surface morphology of particles is critical to their cell attachment and internalization.^{26–28}

The controlled synthesis of MOFs with specific and particularly complex surface morphologies has always been a challenge for scientists. The synthesis process and its reaction conditions dictate the morphological structures of the synthesized MOFs and therefore the characteristics of their cellular interaction. In this work, pollen-like MOFs with diverse surface morphologies were prepared by a solvothermal reaction between aluminum sulfate hydrate and 2-aminoterephthalic acid at 180 °C. To elucidate the possible process that is involved in their formation of surface nanospikes with tunable aspect ratios, time-dependent experiments were carried out. The solvothermal process was monitored by examining changes in the morphological structures of the synthesized MOFs by scanning (SEM) and transmission (TEM) electron microscopies.

On the basis of the obtained SEM images (Figure 2a), the as-synthesized MOFs had cores (1 to 2 μm in diameter), from which spike-like nanostructures of various lengths protruded, which depended on their reaction time. The MOFs that were harvested at 1 h after the solvothermal reaction were approximately spherical and had relatively smooth surfaces (spherical MOFs). Careful examination of the SEM images that were obtained at 2 h revealed that the surface of the spherical MOFs had begun to exhibit bud-like roughness (budding MOFs). Uniform spike-like nanostructures were ultimately observed on MOF surfaces after 8 h of solvothermal treatment (spiky MOFs). Further increasing the reaction time to 12 h caused no significant change in their surface morphology but caused the synthesized MOFs to aggregate.

Recent studies have reported that coordination modulators can regulate the synthesis of MOFs by modulating their ligand–metal interactions.^{17,29} During MOF growth, coordination modulators that are monotopic nonbridging ligands (with a single functional group) compete with polytopic linker ligands (with two or more functional groups) for available metal coordination sites, influencing the surface morphology of the MOFs.

To study the effect of coordination modulators on the surface morphology of the synthesized MOFs, acetic acid (a coordination modulator) was added to the reaction mixture to compete with 2-aminoterephthalic acid (a linker ligand) for coordination to Al ions because they have the same carboxylate functional groups. The reaction was conducted under the conditions that had been used to synthesize the spiky MOFs (at 180 °C for 8 h) but with various concentrations of acetic acid. As shown in Figure 2b, increasing the concentration of acetic acid from 0 to 0.35 M increased the aspect ratio of the nanospikes that grew on the MOFs, whose surface morphology thus became similar to that of Spanish needle (*Bidens Pilosa*) seeds (Spanish-needle MOFs). The as-synthesized Spanish-needle MOFs had core diameters of 1 to 2 μm , and their protruding nanospikes appeared to be much longer than their counterparts that had grown on the spiky MOFs. Further increasing the concentration of acetic acid produced aggregates of needle-like rods. These findings suggest that coordination modulation by acetic acid favored the growth of MOFs in the direction of their nanospikes.

The surface morphologies of the spherical, budding, spiky, and Spanish-needle MOFs were confirmed by TEM (Figure 2c). Their corresponding elemental mapping images, obtained by energy-dispersive X-ray (EDX) spectroscopy, revealed that elemental Al was scattered throughout each MOF, including its nanospikes. Notably, prolonged exposure of the spike-like

nanostructures that protruded from the test MOFs to a beam of electrons did not change their morphology, as revealed by TEM, indicating that they were rather stiff. High-magnification TEM images clearly showed rough surfaces on the surfaces of the budding, spiky, and Spanish-needle MOFs (Figure 2d); the heights, widths at midheight, and aspect ratios of the nanospikes that protruded from the particle surfaces were determined and are provided in Table 1. The powder X-ray diffraction (PXRD)

Table 1. Height, Width at Midheight, and Aspect Ratio of Nanospikes That Protruded from Surfaces of Test MOFs^a

	height (nm)	width at midheight (nm)	aspect ratio
spherical MOFs	0	0	N/A
budding MOFs	168 $\pm$ 26	18 $\pm$ 5	10
spiky MOFs	516 $\pm$ 71	22 $\pm$ 4	25
Spanish-needle MOFs	1679 $\pm$ 212	34 $\pm$ 9	50

^aData were obtained from ten nanospikes of each test particle; three representative particles were used, each from a different batch. N/A: data not available.

patterns of experimentally synthesized MOFs were similar to that of the simulated $\text{NH}_2\text{-MIL-101(Al)}$ (Figure 2e).^{30,31} The fast Fourier transform diffraction patterns (FFT-DPs, Figure 2f) that were obtained from the TEM images reveal that the structures of the nanospikes on the surfaces of spiky and Spanish-needle MOFs were identical to those of their main bodies ($\text{NH}_2\text{-MIL-101(Al)}$).

The cytotoxicities of the as-prepared MOFs with various spike-like nanostructures were separately examined by incubating them with J774A.1 cells, a murine macrophage cell line. Untreated cells and Triton-X-100-treated cells served as negative and positive controls, respectively. Relative to the untreated control group, none of the four test MOF formulations exhibited significant cytotoxicity at particle concentrations of up to 300 $\mu\text{g/mL}$ ($P > 0.05$, Figure 3a).

To induce the required immune responses, particulate antigen carriers must be effectively phagocytosed by APCs, such as macrophages. Phagocytosis typically involves two distinct events, which are the attachment of particles to the cells and the subsequent internalization of the attached particles. In this study, J774A.1 macrophages were used to evaluate the capability of each of the pollen-mimetic MOFs, which had been prelabeled with Alexa Fluor 633, to attach to cells and become internalized by them. Studies have shown that macrophages can readily phagocytose particles with diameters of 0.5–5 μm .³² Cell attachment was studied by incubating J774A.1 macrophages with fluorescence-labeled MOFs at 4 °C³³ and analyzing the attachment by confocal laser scanning microscopy (CLSM). The rate of internalization of the fluorescent MOFs was measured at body temperature (37 °C) by flow cytometry.

A fluorescence signal was detected on cell membranes at 4 h into the above process in each case (Figure 3b). The intensity of fluorescence on cells, which indicates the amount of test MOFs that are attached to cell membranes, followed the order Spanish-needle MOFs > spiky MOFs > budding MOFs $\approx$ spherical MOFs. In nature, Spanish needle seeds attach to anything that they brush against as “hitchhikers”.³⁴ According to Figure 3c, the Spanish-needle MOFs also had faster internalization and a higher uptake by macrophages than the other test formulations ($P < 0.0001$). These results probably follow from the fact that the nanospikes of Spanish-needle MOFs had the highest aspect

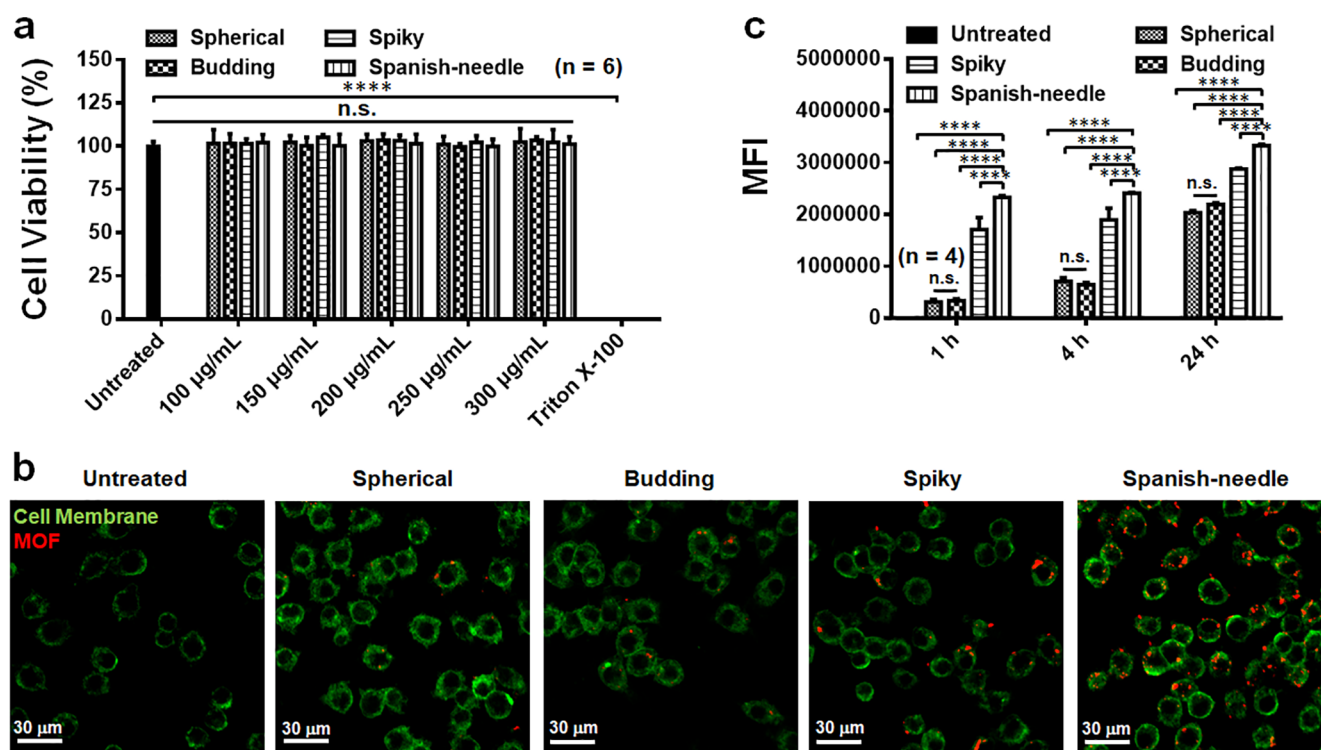


Figure 3. (a) Viability of J774A.1 macrophages following treatment with spherical, budding, spiky, or Spanish-needle MOFs at various concentrations or 0.2 mM Triton X-100. (b) CLSM images of J774A.1 cells (stained with CellMask Green Plasma Membrane Stain) that had been incubated with various fluorescence-labeled test MOFs at 4 °C for 4 h. (c) Results of flow-cytometry analysis of macrophages that had been incubated with various fluorescence-labeled test MOFs at 37 °C for predetermined periods. MFI: mean fluorescence intensity; **** $P < 0.0001$; n.s.: not significant.

ratios among all four studied groups (Table 1), allowing them to interact the most strongly with cell membranes, resulting in the most robust response in phagocytosis.

Particles with a sharp morphology are more favorably taken up by cells.³⁵ Rod-shaped particles can be phagocytosed more effectively than spherical or cubic particles by macrophages.^{36–38} In this study, the nanospikes on the as-synthesized MOFs were normal to their surfaces. A previous study has revealed that the internalization of long nanorods that approach cells perpendicularly requires much less energy than that of those that approach tangentially.³⁸ The above analytical results indicate that the aspect ratio of the spike-like nanostructures on pollen-mimetic MOFs governs their cell attachment and phagocytosis by macrophages. Because the cell attachment by, and internalization of, budding MOFs, which had nanospikes with a minimal aspect ratio, did not differ significantly from those spherical MOFs, which had smooth surfaces, only the spherical MOFs were used in subsequent studies.

Table 2 presents the diameters of pore apertures and external surface areas in the spherical, spiky, and Spanish-needle MOFs, as analyzed by the Horváth–Kawazoe (HK) and *t*-plot methods,³⁹ respectively. Whereas the diameters of the pore

apertures of the three types of MOFs were similar (6–8 Å), the spherical MOFs had a lower external surface area than their spiky and Spanish-needle counterparts. The Al content of all three MOF formulations was determined by inductively coupled plasma–mass spectrometry (ICP-MS) to be 9.6 to 9.9 wt % (Table 2).

The MOFs with controllable OVA amounts, which were determined by the BCA protein assay,⁴⁰ were prepared by varying the feeding weight ratio of the drug (OVA) to the test MOFs. Because the molecular size of OVA ($7.0 \times 3.6 \times 3.0$ nm)⁴¹ greatly exceeded the diameters of the pore apertures in the MOF crystals (6–8 Å, Table 2), the loaded OVA was expected to have been mostly adsorbed onto the external surfaces of MOFs. As shown in Table 3, as the feeding ratio of OVA to MOF increased, the drug loading content (LC) increased, and the drug loading efficiency (LE) decreased. To compare their immune responses fairly, the formulations of the spherical, spiky, and Spanish-needle MOFs that contained approximately equal OVA amounts (ca. 23%) were used in subsequent experiments.

Zeta potential measurements revealed that the MOFs without OVA were positively charged (Figure 4a). Upon the introduction of OVA (−23 mV) into MOFs, the zeta potential of the MOFs became less positive, suggesting the adsorption of the antigen on the carriers by electrostatic interactions. SEM images revealed that the surface morphologies of all three MOF formulations were not significantly influenced by the adsorbed OVA (Supplementary Figure S1).

The kinetics of the release of OVA from the spherical, spiky, and Spanish-needle OVA@MOFs were studied in phosphate-buffered saline (PBS). According to Figure 4b, ~40% OVA was

Table 2. Diameters of Pore Apertures, External Surface Areas, and Al Content of Test MOFs

	diameter of pore aperture (Å)	external surface area (m ² /g)	Al content (wt%)
spherical MOFs	8.2	114.8	9.9
spiky MOFs	6.0	175.9	9.6
Spanish-needle MOFs	6.2	176.1	9.6

Table 3. Drug Loading Efficiency (LE) and Loading Content (LC) of OVA That Was Adsorbed on Test MOFs That Were Prepared at Various Feeding Weight Ratios of MOF/OVA ($n = 6$ batches)

weight ratio of MOF/OVA		spherical MOFs	spiky MOFs	Spanish-needle MOFs
1.0:0.06	LE (%)	83.0 $\pm$ 11.5	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
	LC (%)	4.9 $\pm$ 0.6	5.9 $\pm$ 0.0	5.9 $\pm$ 0.0
1.0:0.13	LE (%)	57.7 $\pm$ 6.9	95.3 $\pm$ 6.6	92.1 $\pm$ 10.7
	LC (%)	6.7 $\pm$ 0.8	10.6 $\pm$ 0.7	10.3 $\pm$ 1.1
1.0:0.25	LE (%)	37.8 $\pm$ 7.4	84.4 $\pm$ 4.5	82.9 $\pm$ 18.3
	LC (%)	8.6 $\pm$ 1.6	17.4 $\pm$ 0.8	17.1 $\pm$ 3.2
1.0:0.5	LE (%)	32.5 $\pm$ 7.4	57.9 $\pm$ 5.3	60.1 $\pm$ 12.3
	LC (%)	13.9 $\pm$ 2.7	22.4 $\pm$ 1.6	22.9 $\pm$ 3.6
1.0:1.0	LE (%)	30.9 $\pm$ 5.5	50.7 $\pm$ 8.7	49.3 $\pm$ 11.2
	LC (%)	23.5 $\pm$ 3.4	33.5 $\pm$ 3.7	32.7 $\pm$ 5.0

released from each type of test OVA@MOF in the first 8 h; thereafter, no or only a little OVA was released, revealing that most of the adsorbed antigen remained tightly adherent to the top of the test OVA@MOFs.

To elicit a potent antigen-specific humoral immunity, the antigen (OVA) and the adjuvant (Al) must be colocalized in the

same APCs. To evaluate the colocalization of OVA and Al that are delivered by the particulate carriers (MOFs), OVA and MOFs were fluorescently labeled with FITC and Alexa Fluor 633, respectively. The internalization efficiency of the fluorescence-labeled OVA@MOFs in J774A.1 macrophages was obtained by CLSM. The Spanish-needle MOF formulation appeared to produce substantially stronger fluorescence signals of OVA (green) and MOFs (red) in cells than the spherical and spiky MOF formulations after 1, 4, or 24 h of incubation ($P < 0.05$, Figure 4c and Supplementary Figure S2), indicating that the Spanish-needle MOFs can deliver OVA and Al more rapidly and efficiently into macrophages. As shown in Supplementary Figure S3, the uptake of test MOFs increased the size of macrophage cells relative to that of untreated cells, consistent with the literature.^{42,43}

The rate of disintegration of drug carriers in a physiological fluid determines their clinical applications. The disintegration of the spherical, spiky, and Spanish-needle MOFs in PBS, which simulated the intracellular fluid in macrophages, was studied *in vitro* at 37 °C by examining their structural changes at various time points under SEM. As shown in Figure 5a, the structures of the MOFs with all three formulations began to disintegrate on day 3 as a result of the competitive replacement of their organic linkers by phosphate ions in PBS, causing the release of OVA

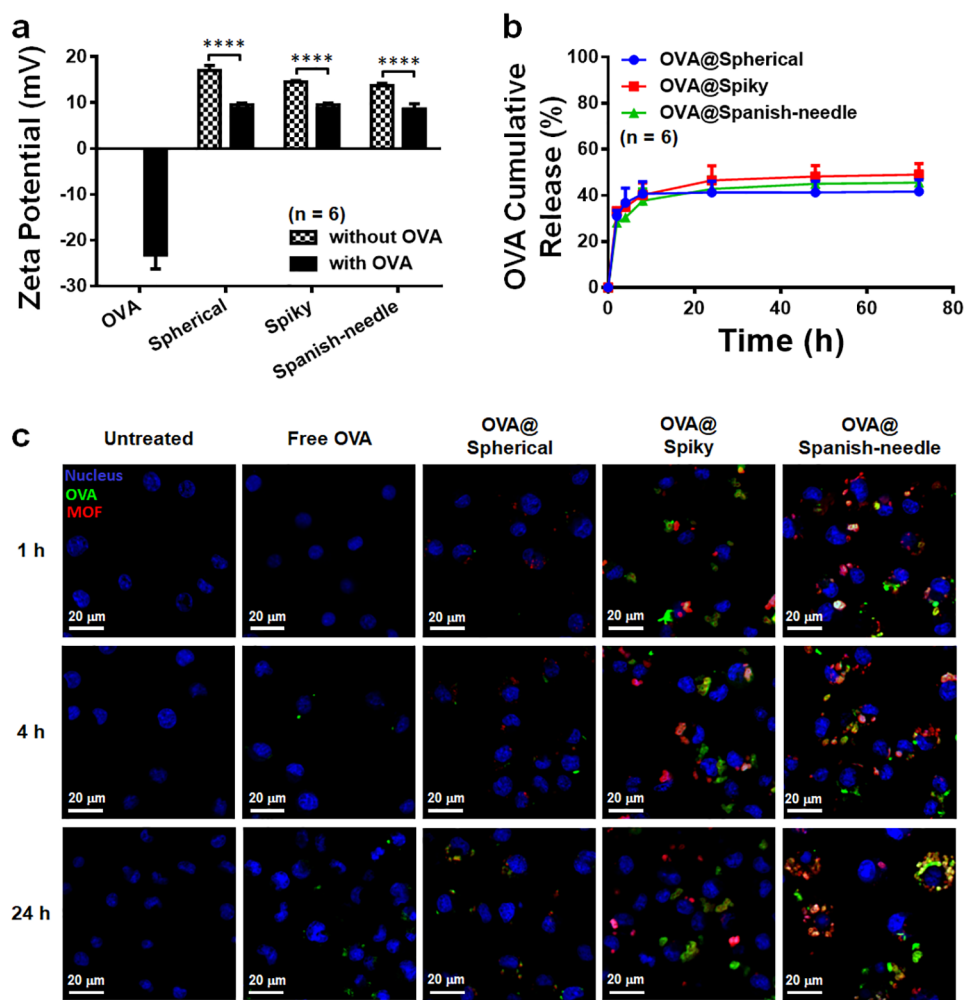


Figure 4. (a) Zeta potentials of spherical, spiky, and Spanish-needle MOFs before and after loading with OVA. **(b)** Cumulative release profiles of OVA from various test OVA@MOFs in PBS at pH 7.4. **(c)** CLSM images of macrophages that had been incubated with free OVA or various fluorescence-labeled test OVA@MOFs at 37 °C for predetermined intervals. **** $P < 0.0001$.

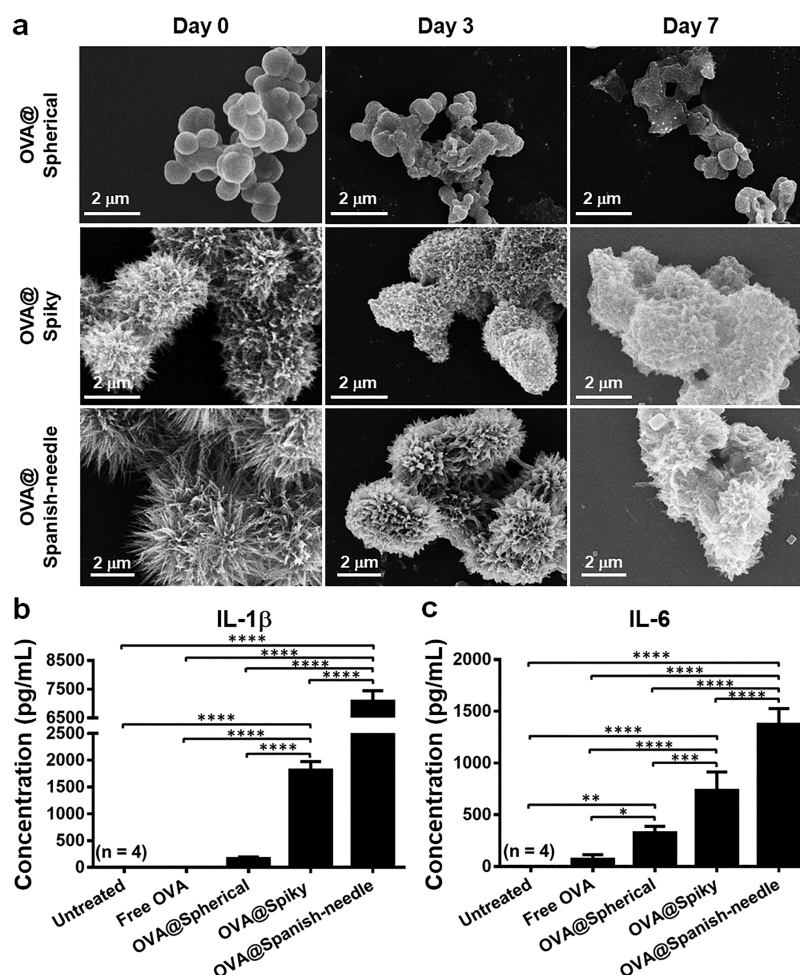


Figure 5. (a) SEM images showing changes in morphology of various test OVA@MOFs that had been disintegrated in PBS for various durations. Levels of (b) IL-1 β and (c) IL-6 cytokines secreted from J774A.1 cells that had been treated with medium alone (untreated), free OVA, or various test OVA@MOFs for 24 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

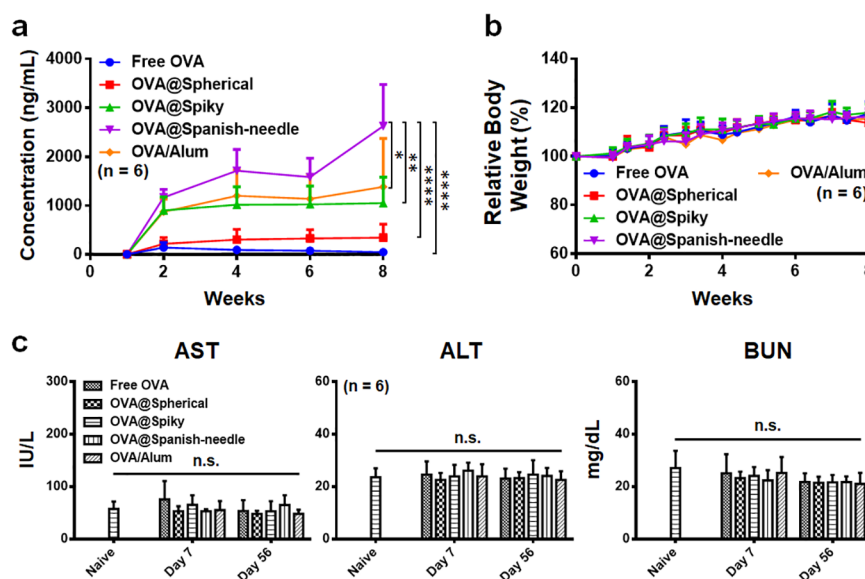


Figure 6. (a) Results of the analysis of OVA-specific IgG in serum at predetermined times following immunization with free OVA, test OVA@MOFs, or OVA/Alum in mice. Changes in (b) relative body weight and (c) blood chemistry. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$; n.s.: not significant.

and Al. Significant structural disintegration had occurred by day 7. Accordingly, the as-proposed pollen-mimetic MOFs are expected to be able to be disintegrated *in vivo* in a physiological fluid.

Studies have reported that particles that are larger than 500 nm depend on active transport by APCs, such as macrophages, to the lymph node.^{44,45} The internalization of a particulate vaccine (OVA@MOFs) by macrophages may cause their maturation and activation and the consequent secretion of pro-inflammatory cytokines, such as IL-1 β and IL-6, which are critical to the mediation of immune responses.^{22,46,47} The effects of OVA-containing spherical, spiky, and Spanish-needle MOFs on the maturation of J774A.1 macrophages were separately investigated; the amounts of their IL-1 β and IL-6 cytokines that were secreted into the media following incubation for 24 h were measured. Macrophages that were untreated or treated with free OVA were controls.

Cells that received OVA-containing MOF formulations exhibited markedly higher expressions of IL-1 β and IL-6 than the untreated control and the cells that received free OVA ($P < 0.05$, Figure 5b,c). Interestingly, among the three MOF formulations, the Spanish-needle MOF formulation induced the highest cytokine expressions, followed by the spiky MOF formulation and the spherical MOF formulation ($P < 0.0001$). Additionally, cells that were treated with the Spanish-needle MOF formulation exhibited significantly higher levels of CD80 and CD86 surface markers than untreated cells and those that were treated with free OVA (Supplementary Figure S4, $P < 0.0001$). These findings are attributable to the fact that a higher aspect ratio of the nanospikes on the MOFs results in greater attachment to, and internalization by, macrophages (Figures 3b,c and 4c). The consequently higher expression levels of cytokines and cell surface markers probably result in stronger antigen-specific antibody responses *in vivo*.

To study the potency of the particulate vaccine on the elicited humoral immune response *in vivo*, OVA-containing spherical, spiky, and Spanish-needle MOFs were separately injected into the subcutaneous tissues of test mice. Free OVA was used as a negative control, whereas the Alum-adjuvant-containing OVA (OVA/Alum) served as a positive control. Alum has been approved by the Food and Drug Administration (FDA) for use as an adjuvant in human vaccines.⁴⁸ The sera of test mice were collected at various times, and their OVA-specific immunoglobulin G (IgG) titers were analyzed using ELISA.

As presented in Figure 6a, the Spanish-needle MOF formulation elicited a significantly stronger antibody response against OVA than did the control formulations, including OVA/Alum ($P < 0.05$). One advantage of the as-proposed particulate vaccine is its codelivery of the adjuvant (Al) with the antigen (OVA) into the same APCs (Figure 4c). The colocalization of the adjuvant and the antigen of interest elicits a prompt and strong immune response against the antigen.^{49,50} These findings reveal that OVA-containing MOFs significantly enhanced the antigen-specific humoral immune response to an extent that depended on the aspect ratio of the nanospikes that protruded from the test MOFs, possibly owing to their enhancement of cell attachment and internalization by APCs. The levels of cytokines (IL-1 β and IL-6) in serum were analyzed and found to be almost undetectable (Figure S5), probably because the test OVA@MOFs were injected subcutaneously and phagocytosed by the resident APCs, reducing the systemic exposure of their encapsulated adjuvant and antigen and thereby attenuating the

induction of systemic cytokines. Similar observations have been made elsewhere.^{50–52}

Additional experiments, involving changes in the body weights and blood chemistry of test mice as well as histological sections of their skin from the injection site and main organs, were performed to evaluate the biosafety of OVA-containing MOF formulations. No obvious loss of body weight was observed in any of the investigated groups ($P > 0.05$, Figure 6b). The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are hepatic function markers, and that of blood urea nitrogen (BUN), which is a renal function marker,⁵³ were measured to be normal ($P > 0.05$, Figure 6c), suggesting no detectable hepatic or renal dysfunction.

Histological evaluation of the tissue sections from the skin and main organs that were stained by hematoxylin and eosin (H&E) revealed no obvious inflammatory infiltration or histological abnormality (Supplementary Figure S6 and S7). The concentration of Al in the spleen, which is the largest secondary lymphoid organ in the body,⁵⁴ was quantified by ICP-MS. As shown in Supplementary Figure S8, the spleens of test mice that were treated with test OVA@MOFs or OVA/Alum did not contain significantly more Al than those of the untreated mice ($P > 0.05$), suggesting that most of the Al-based materials were removed from the body. These findings together showed that the as-proposed OVA-containing spherical, spiky, and Spanish-needle MOF formulations did not exhibit significant systemic toxicity.

CONCLUSIONS

In conclusion, this work provides systemic approaches for synthesizing pollen-mimetic MOFs with spike-like nanostructures with various aspect ratios; these MOFs act not only as antigen carriers but also as an adjuvant. The data that were obtained herein reveal that the aspect ratio of the nanospikes that protruded from the as-synthesized MOFs strongly affected their cell attachment and internalization by macrophages, markedly promoting humoral immune responses.

MATERIALS AND METHODS

Materials. 2-Aminoterephthalic acid (99%) was procured from Alfa Aesar (Tewksbury, MA). Aluminum sulfate hydrate (98%), OVA ($\geq 98\%$ according to agarose gel electrophoresis), and Triton X-100 (laboratory grade) were purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid ($\geq 99.7\%$) was obtained from Echo Chemical (Miaoili, Taiwan). Alexa Fluor 633 NHS ester, CellMask Green Plasma Membrane Stain, and SYTO 60 Red Fluorescent Nucleic Acid Stain were acquired from Thermo Fisher Scientific (Waltham, MA). All other chemicals and reagents were of analytical grade.

Preparation and Characterization of MOFs. Test MOFs were prepared in a solvothermal reaction.⁴⁷ In brief, 2-aminoterephthalic acid (0.5 mmol), aluminum sulfate hydrate (0.25 mmol), and acetic acid (0–70 mmol) were dissolved in *N,N*-dimethylformamide (DMF, $\geq 99.8\%$, 50 mL) at room temperature, transferred to a 50 mL Teflon-lined stainless-steel autoclave, and heated to 180 °C for predetermined periods (1–12 h). The obtained solutions were centrifuged at 7000 rpm for 10 min, and the solid products were washed using deionized (DI) water and then ethanol.

Annular dark-field STEM images, EDX elemental maps, and electron diffraction patterns of MOFs were obtained by TEM (JEOL F200, JEOL Technics, Tokyo, Japan) with a OneView charge-coupled device (CCD). The crystalline structures of MOFs were analyzed by X-ray diffractometry (Bruker D8A25, Bruker, Billerica, MA). The Al content of the test MOFs was determined by ICP-MS (Agilent 7500ce, Agilent Technologies, Santa Clara, CA) with the digested materials in aqueous HNO₃. The diameters of the pore apertures and external of MOFs were

measured using a surface area analyzer (ASAP 2060, Micromeritics, Norcross, GA).

Preparation and Characterization of OVA@MOFs. The as-prepared MOFs (1 mg) were first dispersed in 1 mL of DI water. A predetermined amount of OVA (0.06 to 1 mg) in 1 mL of DI water was then added to the dispersed MOF solution, and the OVA was loaded onto the MOFs by probe sonication (VCX 750 sonicator, Sonics & Materials, Newtown, CT) for 15 min. The obtained OVA@MOFs were centrifuged at 15 000 rpm for 10 min and washed three times using DI water to remove excess OVA. The drug LE and LC of the OVA@MOFs were determined by analyzing the amount of free OVA that remained in the supernatants using a Pierce BCA protein assay kit (Thermo Fisher Scientific).⁴⁰ The drug LE and LC of OVA@MOFs were calculated using the following equations.⁵⁵

$$\text{LE (\%)} = \frac{\text{total amount of OVA added} - \text{free OVA}}{\text{total amount of OVA added}} \times 100\%$$

$$\text{LC (\%)} = \frac{\text{total amount of OVA added} - \text{free OVA}}{\text{weight of OVA@MOFs}} \times 100\%$$

The sizes and morphologies of the test MOFs were determined by SEM (JSM-5600, JEOL Technics), and their surface charges were measured by dynamic light scattering (DLS, Zetasizer, 3000 HS, Malvern Instruments, Worcestershire, U.K.). To examine their degradability, test OVA@MOFs (1 mg) were incubated in PBS (1 mL) at 37 °C. After predetermined durations, samples were taken out for SEM imaging.

Cell Viability Assay. The cytotoxicities of the as-prepared MOFs were evaluated by incubating them at various concentrations (0–300 µg/mL) with J774A.1 macrophages (ATCC TIB-67, 8×10^4 cells/well) in 96-well plates. Cells that were treated with Triton X-100 in culture medium (0.2 mM) served as a positive control.⁵⁶ After 24 h of incubation, cell viability was measured using a CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI).⁵⁷

Attachment of MOFs to Macrophages, Their Uptake, and Activation. To determine the effect of the aspect ratio of the nanospikes on the MOFs on cell attachment, J774A.1 macrophages (2.5×10^5 cells/mL) were stained with CellMask Green Plasma Membrane Stain. The stained cells were incubated for 4 h with Alexa Fluor 633-labeled MOFs at 4 °C, a condition under which internalization does not occur, and then washed twice using PBS.³³ The attachment of particles to the cells was observed using CLSM (LSM 780, Carl Zeiss, Jena, Germany). To evaluate the ability of J774A.1 macrophages to take up the test MOFs, they were incubated for predetermined periods (1, 4, and 24 h) with Alexa-Fluor-633-labeled MOFs at 37 °C, at which internalization of the particles occurs. Cellular uptake was measured using a flow cytometer (BD Accuri C6, BD Biosciences, San Jose, CA), and the data thus obtained were analyzed using FlowJo software (Treestar, Ashland, OR).

To observe the localization of OVA and MOFs in cells and their cell morphology, the J774A.1 cells were incubated with FITC-OVA@Alexa Fluor 633-MOFs. Following incubation for predetermined periods (1–24 h), the cells were washed twice using PBS, incubated with SYTO 60 Red Fluorescent Nucleic Acid Stain for 15 min and then observed using CLSM. To quantify the cellular uptake of MOFs and OVA, Carl Zeiss LSM software (Carl Zeiss) was used to determine the fluorescence intensities from individual cells, with the area within the cells defined as a region of interest (ROI).

To study the activation of macrophages by the test MOFs, the J774A.1 cells were stimulated with the OVA-containing MOFs (250 µg/mL) or free OVA (57.5 µg/mL); 24 h later, the culture supernatants were collected to quantify the IL-6 therein using a Cytometric Bead Array kit (CBA, BD Bioscience).⁵⁸ To measure the secretion of IL-1β by J774A.1 cells, they were primed with lipopolysaccharide (100 ng/mL) before being incubated with the test MOFs. 24 h later, the concentrations of IL-1β in the supernatants were assayed using a Mouse IL-1 beta/IL-1F2 Quantikine ELISA kit (R&D Systems, Minneapolis, MN).⁵⁹ To measure the levels of expressed cell surface markers, the stimulated cells were incubated with antimouse CD16/32 (Fc block, eBioscience, San Diego, CA) and then analyzed by flow cytometry using PE-conjugated antimouse F4/80, APC-conjugated antimouse

CD80, and FITC-conjugated antimouse CD86 antibodies (BioLegend, San Diego, CA).⁶⁰

Experimental Animals. Female C57BL/6 mice (six to 8 weeks old, BioLASCO, Taipei, Taiwan) were used in the animal study. All animal experiments complied with the “Guide for the Care and Use of Laboratory Animals” that was produced by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press in 1996. The Institutional Animal Care and Use Committee of National Tsing Hua University approved the animal handling procedures.

In Vivo OVA-Specific Humoral Immune Responses. Mice were immunized subcutaneously with OVA-containing spherical, spiky, or Spanish-needle MOFs; free OVA and OVA/Alum were used as controls ($n = 6$ for each group). Each formulation contained the same amount of OVA (10 µg). The doses of Al in the OVA-containing MOFs and OVA/Alum that were administered subcutaneously in mice were approximately 3.5 and 173.0 µg, respectively. Serum samples were obtained weekly from test mice and examined for the presence of the OVA-specific IgG antibody using a Mouse Anti-OVA IgG antibody assay kit (Chondrex, Redmond, WA) following the manufacturer's instructions. The body weights of the test mice were measured every 3 to 4 days. The levels of IL-1β and IL-6 cytokines in the serum were obtained using a Mouse IL-1 beta/IL-1F2 Quantikine ELISA kit⁵⁹ and a CBA kit,⁵⁸ respectively.

Histological Analysis, Blood Biochemistry, and ICP-MS. To measure the potential toxicity of the OVA-containing MOFs to the liver and kidney, sera from test mice were collected on days 7 and 56, and their AST, ALT, and BUN levels were measured. To assess the safety of the MOF particles at the injection sites, skin tissues were collected on day 7 and at the end of the experiments. To evaluate the organ-specific toxicity of the MOFs, major organs (spleen, liver, kidney, lung, and heart) were collected at the end of the experiment and fixed in 10% neutral buffered formalin, embedded in paraffin wax, and then sectioned for H&E staining. The Al content in the spleens was obtained by ICP-MS (Agilent 7800 ICP-MS, Agilent Technologies) with the digested tissues in aqueous HNO₃.^{61,62}

Statistical Analysis. All results are presented as mean ± SD. The two-tailed Student's *t*-test was used to compare the means of two groups. To determine variations among three or more groups, one-way analysis of variation (ANOVA) was used, followed by Bonferroni's or Tukey's post hoc test. A *P* value <0.05 was considered to indicate statistical significance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.1c01129>.

Figures S1–S8: SEM images of MOFs, uptake of MOFs by macrophages, morphology of macrophages, CD80 and CD86 expression on macrophages, IL-1β and IL-6 levels in serum of test mice, histopathological photomicrographs of subcutaneous tissue and major organs, concentrations of Al in spleens (PDF)

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Notes

The authors declare no competing financial interest.

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Pollen-Mimetic Metal–Organic Frameworks with Tunable Spike-Like Nanostructures That Promote Cell Interactions to Improve Antigen-Specific Humoral Immunity

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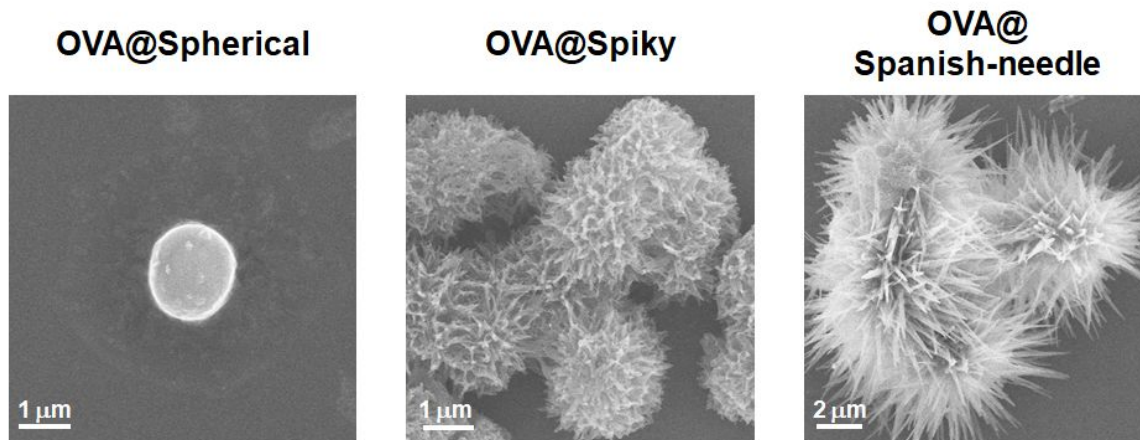


Figure S1. SEM images of spherical, spiky, and Spanish-needle MOFs after loading with OVA.

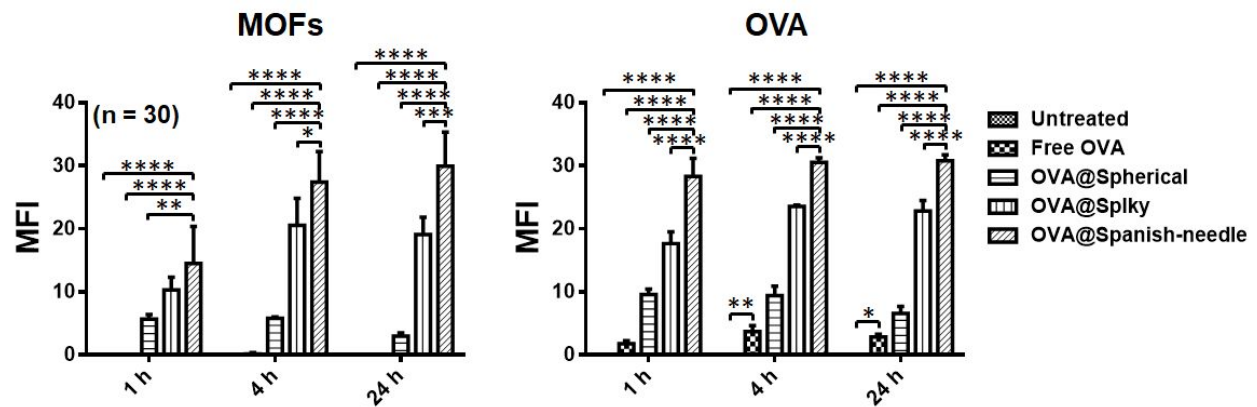


Figure S2. Mean fluorescence intensity (MFI) of macrophages that had been incubated with free OVA or various fluorescence-labeled test OVA@MOFs at 37 °C for predetermined intervals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

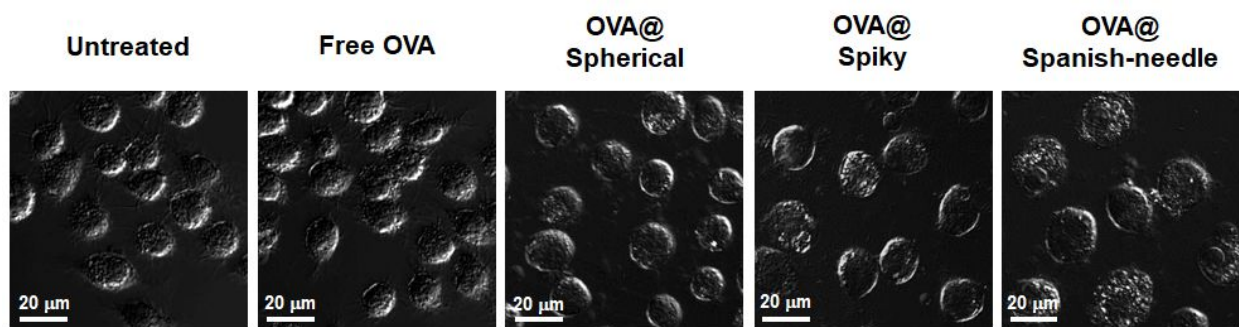


Figure S3. CLSM images of macrophages that had been incubated with various test OVA@MOFs at 37 °C for 24 h.

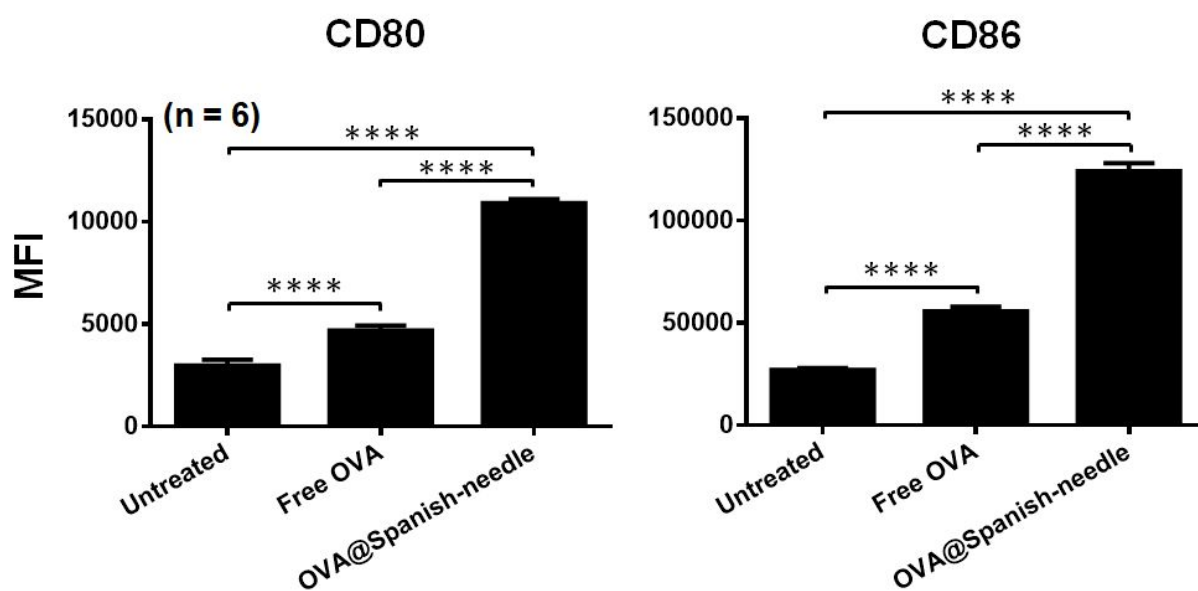


Figure S4. Levels of CD80 and CD86 that were expressed on surfaces of untreated macrophages and those that had been treated with free OVA or OVA@Spanish-needle MOFs for 24 h. **** $P < 0.0001$.

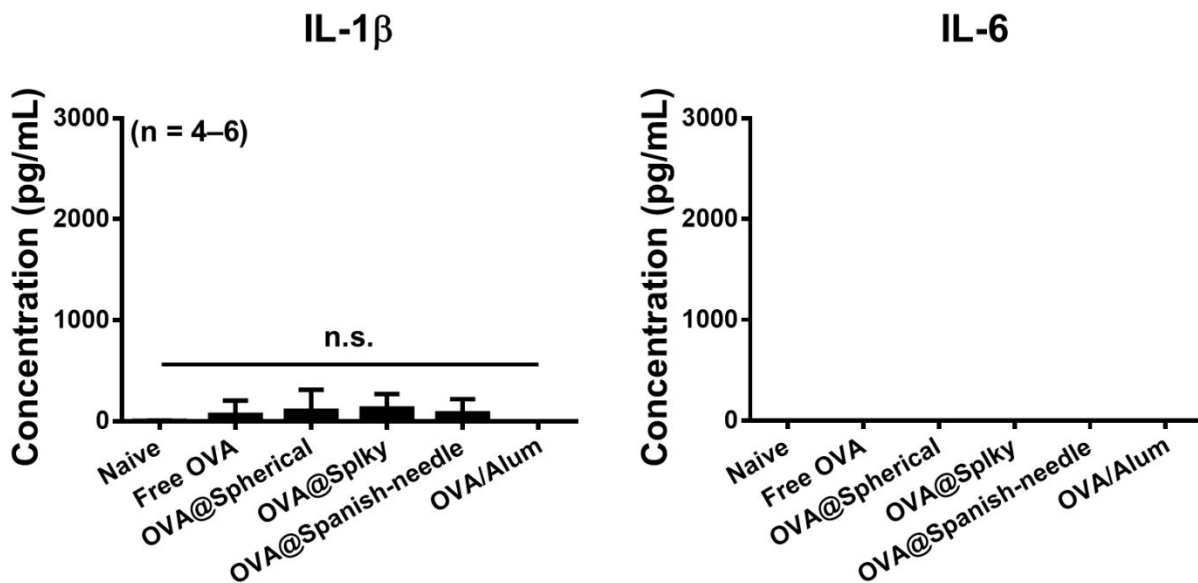


Figure S5. Levels of IL-1 β and IL-6 cytokines in serum of naïve mice or test mice on day 28 following immunization with free OVA, test OVA@MOFs, or OVA/Alum. n.s.: not significant.

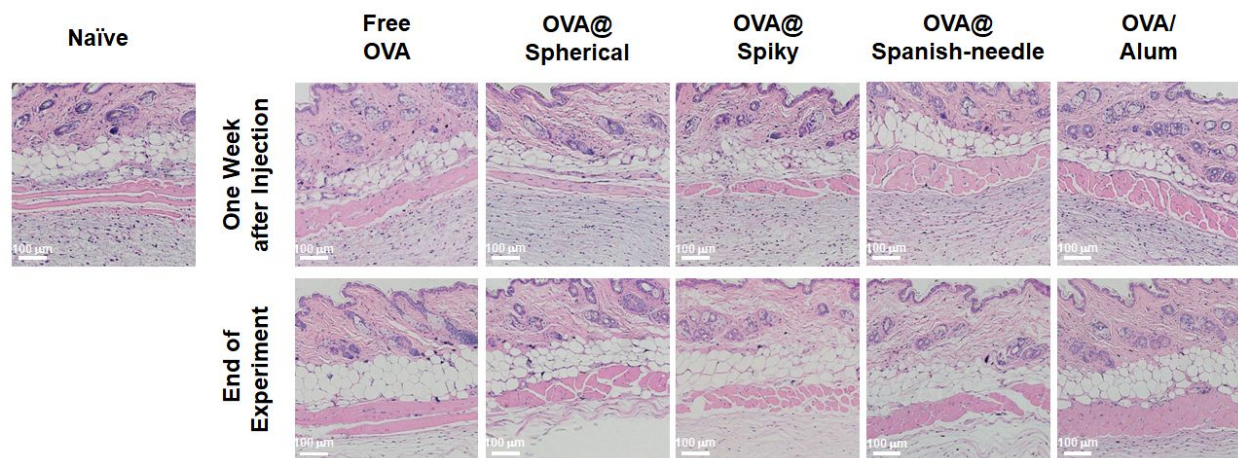


Figure S6. Histopathological photomicrographs of subcutaneous tissue sections at injection sites that were harvested from naïve mice and mice that had been treated with free OVA, test OVA@MOFs, or OVA/Alum.

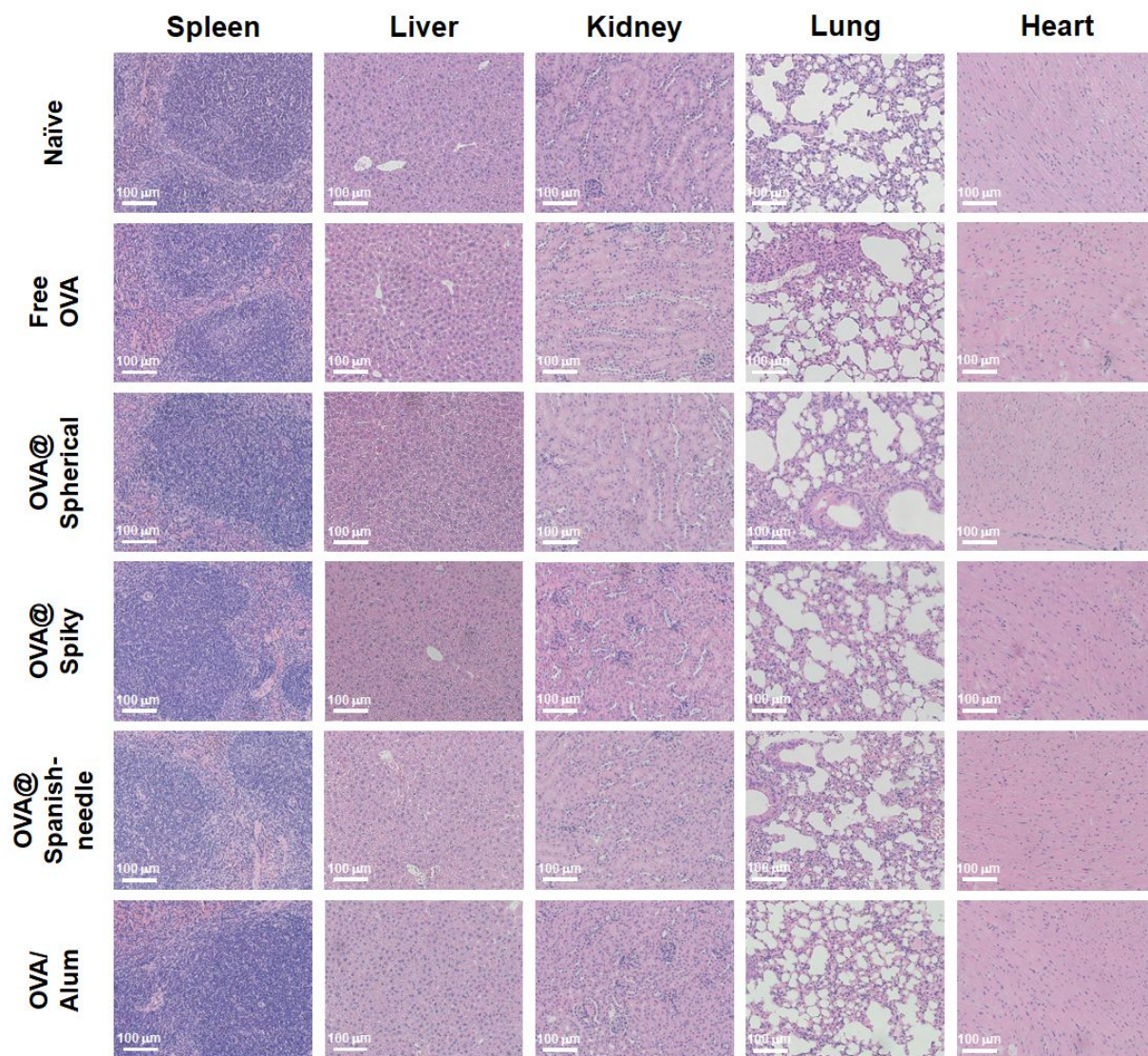


Figure S7. Histopathological photomicrographs of tissue sections of major organs that were harvested from naïve mice and mice that had been treated with free OVA, test OVA@MOFs, or OVA/Alum.

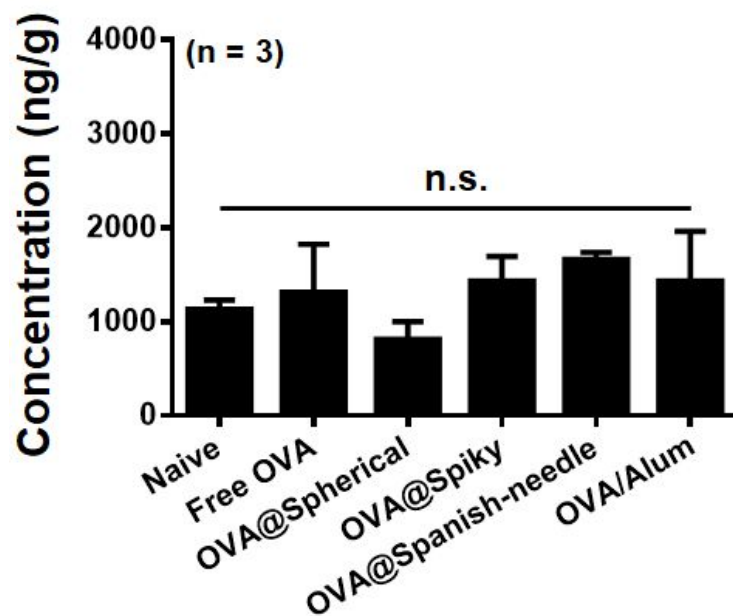


Figure S8. Concentrations of Al in spleens that were harvested from naïve mice and mice that had been treated with free OVA, test OVA@MOFs, or OVA/Alum. n.s.: not significant.