

## **Shape-dependent adjuvanticity of nanoparticle-conjugated RNA adjuvants for intranasal inactivated influenza vaccines**

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## Methods

### AuNP-PIC preparation

Cetyltrimethylammonium (CTA)-stabilized AuNPs were synthesized by a seeding growth method.<sup>[1,2]</sup> After 48 mL of CTA-Sphere20, CTA-Sphere40, CTA-Rod30 and CTA-Rod40 solutions were centrifuged twice and concentrated to c.a. 2 mL, 5 mg of MTAB was added to the AuNP solutions. Solutions were incubated overnight at 42°C. Excess MTAB molecules were removed by centrifugation 4 times to produce MTAB-functionalized AuNPs (MTAB-AuNPs). The concentrations of AuNPs were determined by extinction spectrometry.<sup>[3]</sup>

Scanning transmission electron microscopic (STEM) images were obtained using a STEM HD-2000 system (Hitachi High-Tech Manufacturing & Service Co., Ltd., Japan) with 200 kV accelerating voltage. Extinction spectra were measured with a UV-vis spectrometer (UV-2600; Shimadzu Corporation, Japan). Zeta potentials were measured using a Delsa Nano HC Particle Analyzer (Beckman Coulter, USA). Hydrodynamic diameters were measured using ELSZ-2000 (Otsuka Electronics, Japan)

MTAB-AuNPs were added to a uPIC(40:400) solution reconstituted in distilled water ( $V_{\text{MTAB-AuNPs}}/V_{\text{uPIC(40:400)}} = 1:1$ ) and mixed gently. The concentration varied according to the experiment. As a typical example, a MTAB-AuNP aqueous solution (particle conc. 8.0 nM) was added to the uPIC(40:400) solution (200  $\mu\text{g/mL}$ ) for preparation of AuNP-PICs (Final sample conc.: 4.0 nM MTAB-AuNPs and 100  $\mu\text{g/mL}$  uPIC(40:400)). Mixtures were incubated overnight at 4°C. Prepared solutions were used for characterization, vaccination and cell studies without purification. Zeta potential measurements were carried out in MilliQ water at concentrations of 0.4 nM for MTAB-AuNPs and 10  $\mu\text{g/mL}$  for uPIC(40:400). For the calculation of the number of uPIC(40:400) molecules immobilized on a single AuNP, AuNP-PICs solutions were centrifuged (14,100g, 50 min) to precipitate AuNPs completely and the concentration of unbound uPIC(40:400) in the supernatant was determined by UV-Vis absorption at 250 nm.

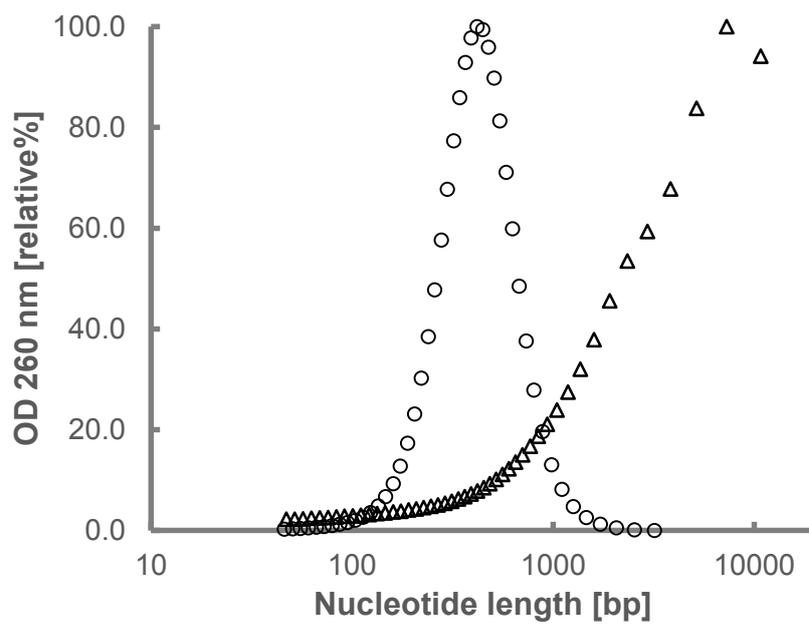
### **Preparation of nasal tissue for TEM observation**

Sphere40-PICs or Rod40-PICs containing 2.0 nM AuNPs and 1.0 mg/mL uPIC(40:400) in 10  $\mu$ L were administrated intranasally to mice. The mice were sacrificed by exposure to excess isoflurane and exsanguinated (via the heart) 6 h after inoculation. After perfusion fixation, the heads of the mice were harvested and immersed in a formalin solution for 2 or 3 days. After fixation, the tissues were decalcified in a buffered EDTA 2Na solution for several days in a refrigerator. After decalcification, heads were transferred into 0.1 M phosphate buffer (PB) (pH: 7.4) containing 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd, UK).

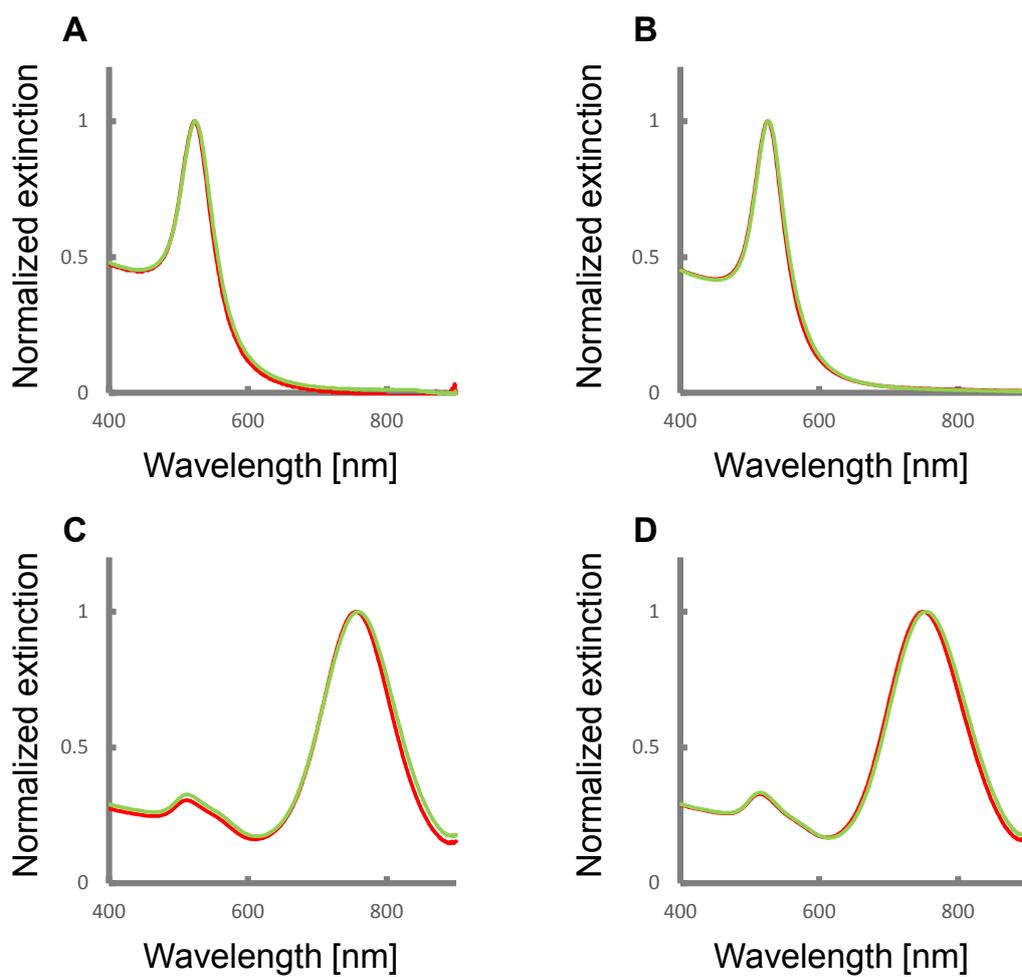
Nasal tissues were sliced and postfixed with an aqueous solution of 1 w/w%  $\text{OsO}_4$  and 1.5 w/w%  $\text{K}_3[\text{Fe}(\text{CN})_6]$  at room temperature for 2 h. The fixed tissues were washed with PB three times, then dehydrated by sequential soaking in 50, 70, 90, 95 and 100% ethanol. The half volume of the soaking solution was gradually replaced by propylene oxide. Finally, the half volume of the solution was replaced by epoxy resin. After mixing, the tissues in the propylene oxide/epoxy resin were incubated at 4  $^\circ\text{C}$  for 1 h. This resin-replacement procedure was repeated four times and the tissues were incubated in epoxy resin at 4  $^\circ\text{C}$  overnight. As epoxy resin, we used the mixture of epon812, DDSA, MNA and DMP-30 (TAAB, England) at a volume ratio of 23:13:14:1. After de-gassing under a vacuum, tissues were embedded in epoxy resin in a mold and the resin was cured by incubation at 37 $^\circ\text{C}$  for 1 day and 60 $^\circ\text{C}$  for 3 days. Ultrathin sections were then cut on a Leica Ultramicrotome. The sections were stained with uranyl acetate followed by lead citrate and examined at 80 kV with a JEM transmission electron microscope (JEOL, Japan).

### **BMDC Preparation**

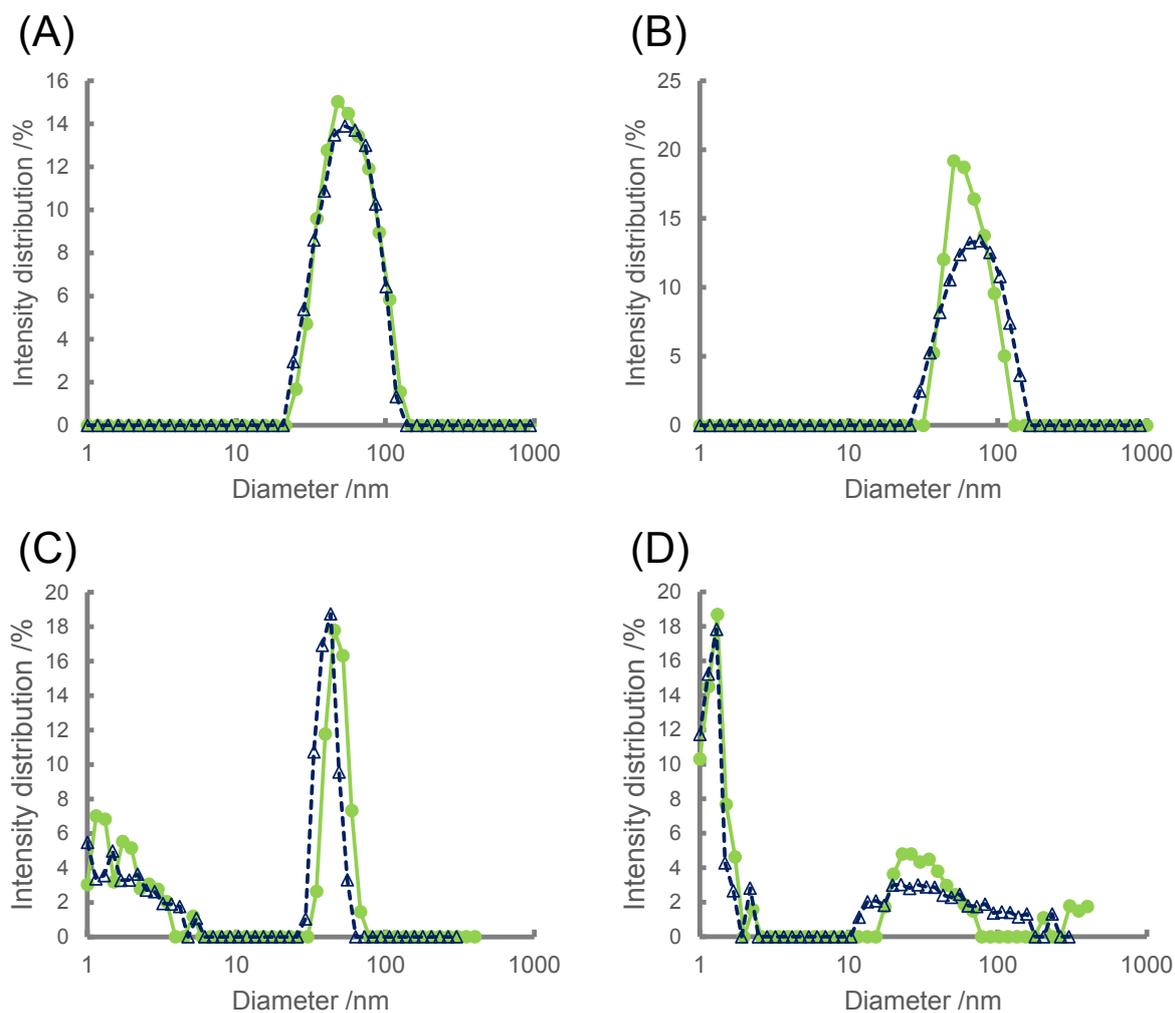
Murine bone marrow-derived dendritic cells (BMDCs) were prepared according to a previously reported method.<sup>4</sup> BMDCs were generated from C3H/HeNJcl (female, 6 weeks old) mice. In brief, bone marrow cells were collected from the femur and tibia. Culture medium containing  $2 \times 10^6$  cells in 10 mL were seeded to TC-treated 100 mm dishes. Cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 25 mM HEPES-KOH (pH: 7.4), 50  $\mu$ M 2-mercaptoethanol and 10 ng/mL GM-CSF for 11 days. Ten mL of culture medium was added on day 3, and one half of the medium was changed for fresh medium on day 6, 8 and 10. After cultivation, floating cells were collected. These animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hokkaido University and approved by the Animal Ethics Committee of Hokkaido University.



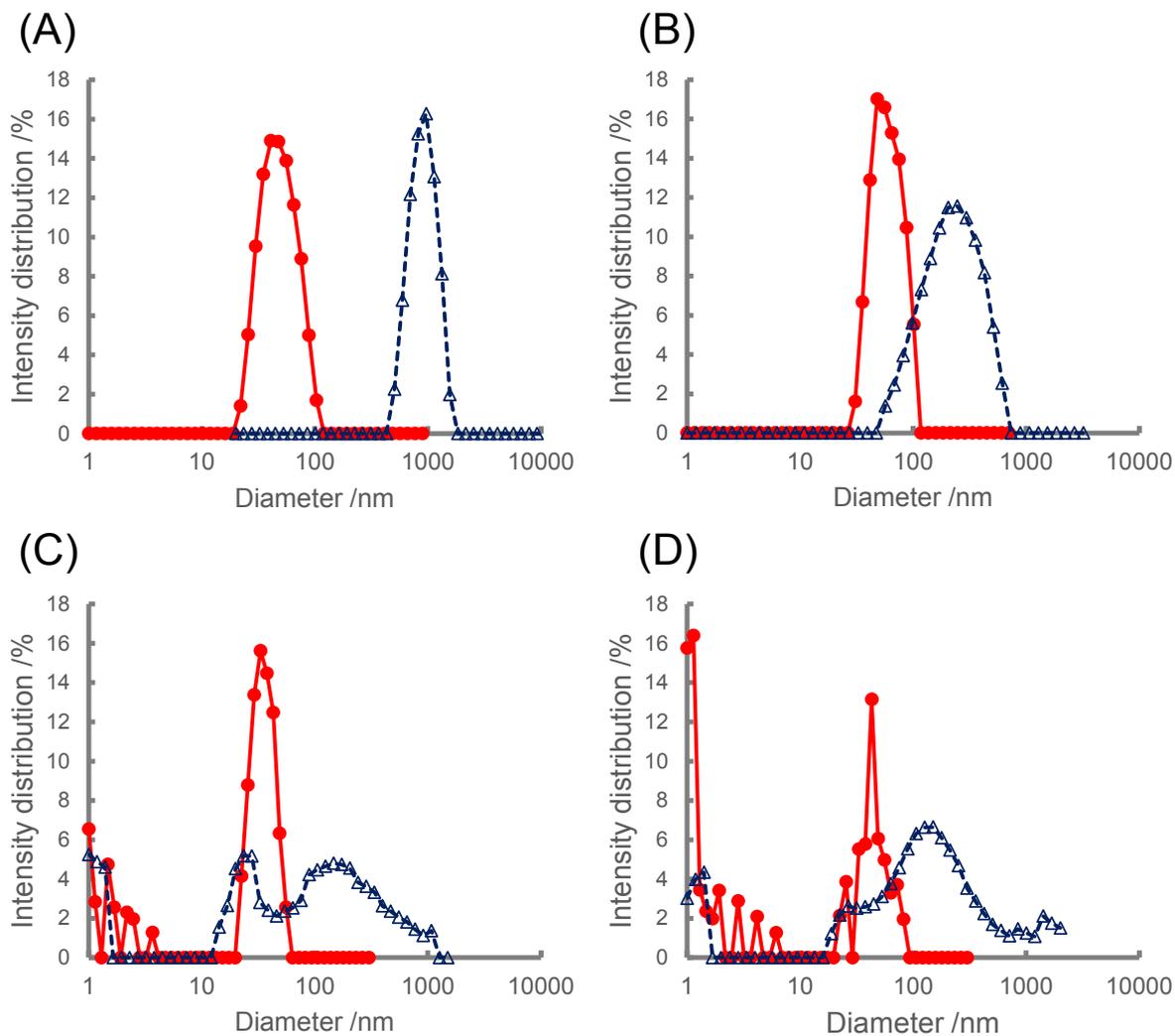
**Figure S1.** Molecular weight distribution of uPIC(40:400) (circles) and commercially available high-molecular-weight poly(I:C) (triangles) used as positive controls in this study.



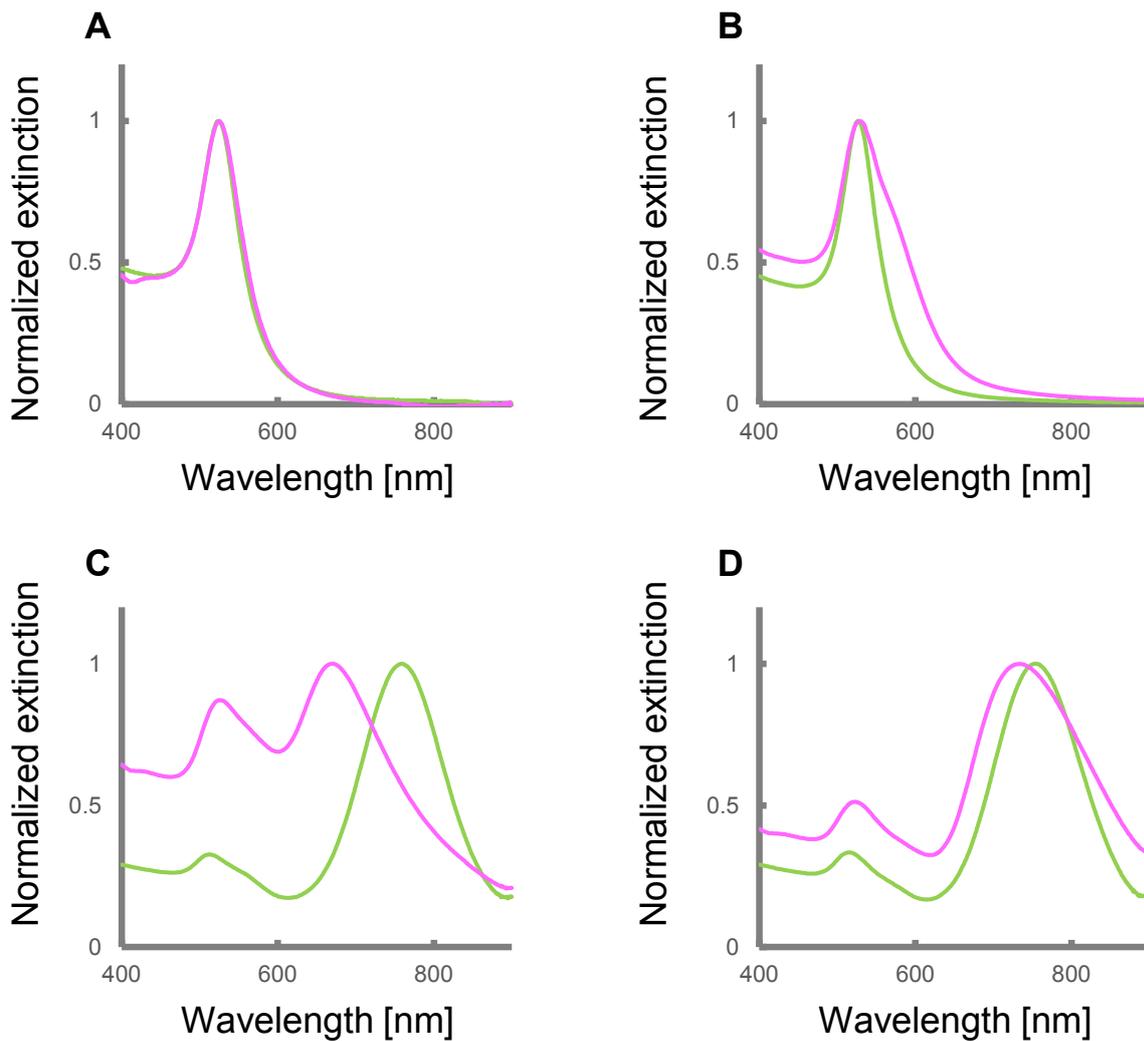
**Figure S2.** Extinction spectra of MTAB-AuNPs (red) and AuNP-PICs (green) in MilliQ water. Maximum extinction was normalized as 1. (A) Sphere20s, (B) Sphere40s, (C) Rod30s and (D) Rod40s.



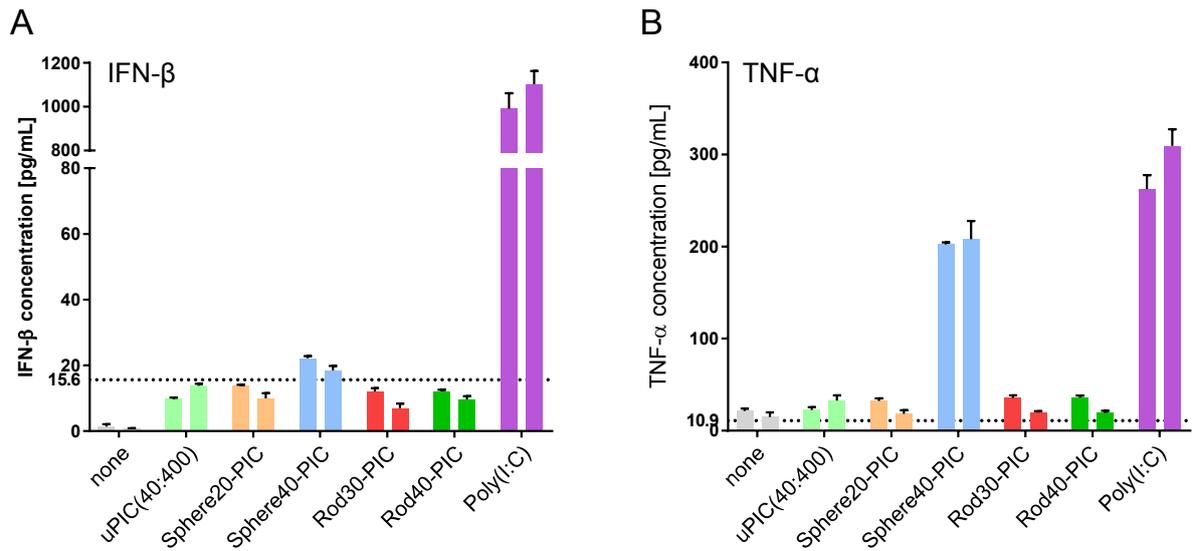
**Figure S3.** Size distribution of (A) Sphere20-PICs, (B) Sphere40-PICs, (C) Rod30-PICs and (D) Rod40-PICs determined from DLS. Green and blue plots indicate AuNP-PICs and AuNP-PICs mixed with HA, respectively. Buffered solution of concentrated HA was added to the aqueous solution of AuNP-PICs. DLS measurements were carried out after incubation for 30 min at RT. Final concentration:  $[\text{AuNP}] = 0.4 \text{ nM}$ ,  $[\text{uPIC}(40:400)] = 10 \text{ }\mu\text{g/mL}$ ,  $[\text{HA}] = 1 \text{ }\mu\text{g/mL}$ .



**Figure S4.** Size distribution of (A) Sphere20s, (B) Sphere40s, (C) Rod30s and (D) Rod40s determined from dynamic light scattering (DLS). Red and blue plots indicate MTAB-functionalized AuNPs and AuNPs mixed with HA, respectively. Buffered solution of concentrated HA was added to the aqueous solution of MTAB-AuNPs. DLS measurements were carried out after incubation for 30 min at RT. Final concentration:  $[\text{AuNP}] = 0.4 \text{ nM}$ ,  $[\text{HA}] = 1 \text{ }\mu\text{g/mL}$ .



**Figure S5.** Extinction spectra of AuNP-PICs in MilliQ water (green) and in culture medium (pink). Maximum extinction was normalized as 1. (A) Sphere20-PICs, (B) Sphere40-PICs, (C) Rod30-PICs and (D) Rod40-PICs.



**Figure S6.** The effects of antigens on (A) IFN- $\beta$  and (B) TNF- $\alpha$  production. BMDCs were stimulated by AuNP-PICs with (Right bar) or without (Left bar) HA. Final concentrations of uPIC(40:400), AuNPs and HA were 100  $\mu\text{g/mL}$ , 100 pM and 1.0  $\mu\text{g/mL}$ , respectively. The detection limit is indicated by the horizontal dotted line.

## References

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