Electronic Supplementary Material (ESI) for Biomaterials Science.
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Supplemental Information

Table S1. PNP Synthesis Details

Step	Small	Medium	Large
Ethanol addition	1 mL/min	1 mL/min	7 mL/min
Centrifugation	7000 g for 5 mins followed by resuspension in PBS	none	none
Crosslinking	Aqueous, 0.82 mM DTSSP, stirring for 1 hour	In 80% ethanol, 0.82 mM DTSSP, stirring for 1 hour	In 80% ethanol, 0.82 mM DTSSP, stirring for 1 hour
Centrifugation	18,000 g for 32 mins	7000 g for 5 minutes	7000 g for 5 minutes
Resuspension	Sonication in 1 mL PBS or coat solution	Sonication in 1 mL PBS or coat solution	Sonication in 1 mL PBS or coat solution
Coating	Two hours at 4°C	Two hours at 4°C	Two hours at 4°C
Second Centrifugation	18,000 g for 32 mins	18,000 g for 32 mins	7,600 g for 5 mins
Second Crosslinking	Aqueous 6.2 mM, stirring for 2 hours	Aqueous 6.2 mM, stirring for 2 hours	Aqueous 6.2 mM, stirring for 2 hours
Quenching	50 mM Tris base, stirring 15 mins.	50 mM Tris base, stirring 15 mins.	50 mM Tris base, stirring 15 mins.
Third Centrifugation after coating and second crosslinking	18,000 g for 32 mins	18,000 g for 32 mins	7,600 g for 5 mins
Resuspension	Sonication in 0.5 mL PBS	Sonication in 0.5 mL PBS	Sonication in 0.5 mL PBS

Table S1. Protocol details for creating OVA PNPs of different sizes. Coat solution was 6.2 mg/mL OVA in PBS.

Table S2. PNP Physical Characterization

Particle Type	**Average Diameter**	**Average PdI**	**Zeta Potential (mV)**	**Yield (%)**
Small	277 nm	0.113	-21.7 ± 3.0	79.7 ± 6.0
Small Coated	271 nm	0.094	-21.5 ± 3.5	93.5 ± 7.5
Medium	365 nm	0.116	-21.8 ± 3.5	90.1 ± 3.7
Medium Coated	357 nm	0.135	-20.5 ± 3.0	99.4 ± 21
Large	567 nm	0.055	-22.1 ± 2.7	81.5 ± 3.8
Large Coated	559 nm	0.050	-21.4 ± 2.0	100.3 ± 11

Table S2. Size, polydispersity, zeta potential, and yield of PNPs of different sizes and coatings. Yield was defined as final mass over initial desolvated mass. Since extra mass was adsorbed through the coating step, yield for those particles was higher, and it was possible to exceed 100% yield.

Figure S1. Soluble and PNP Ratiometric Fluorescent Standard Curves

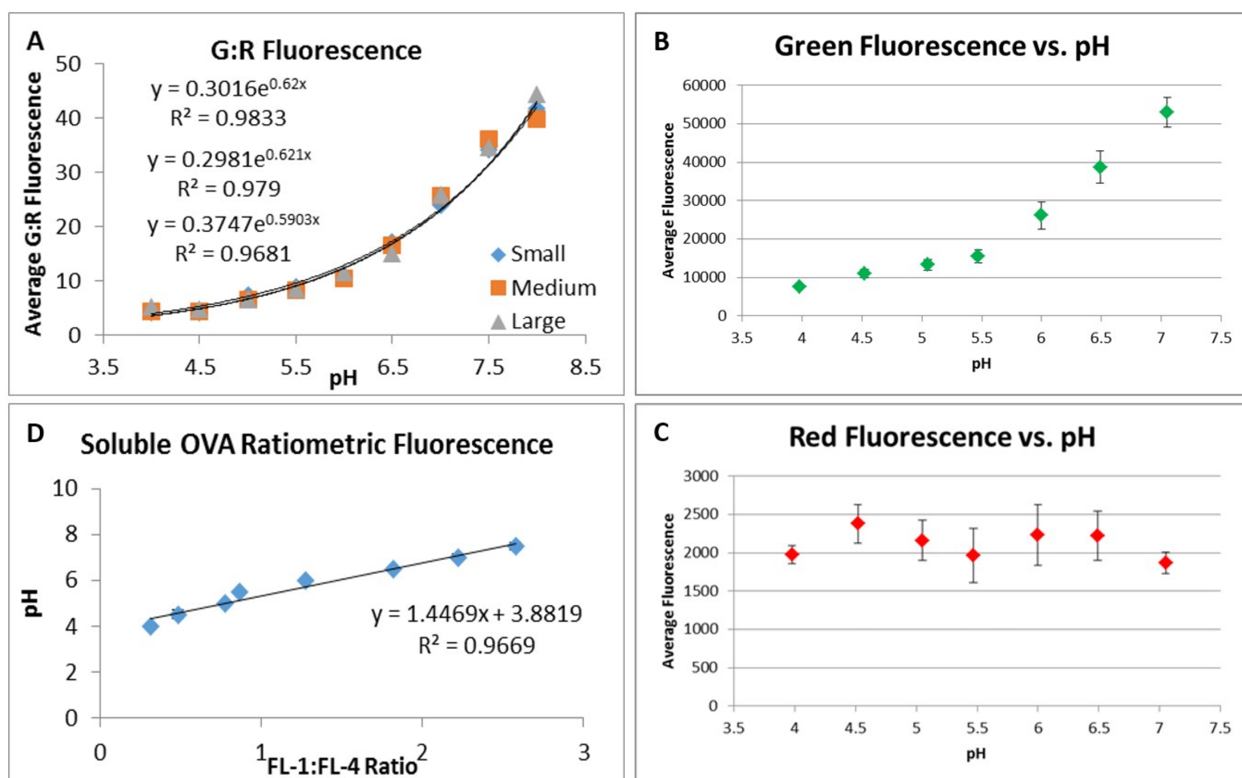

Fig S1. Standard curves of ratiometric fluorescence vs. pH were made from DCs incubated with PNPs and buffered to various pHs (A). Green fluorescence decreases with pH (B) while red fluorescence does not (C). Standard curve for soluble OVA was measured in DCs by flow cytometry (D).

Figure S2. Fluorescent and Non-Fluorescent PNP size

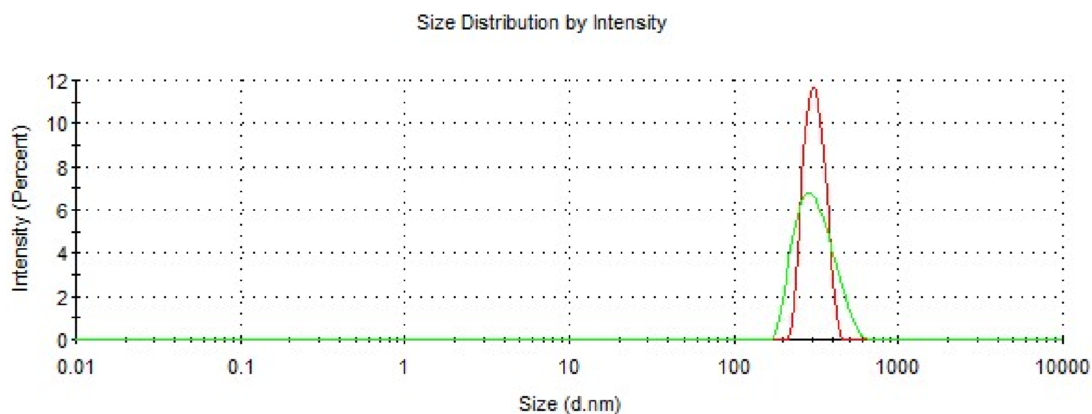

Fig S2. Representative size distributions of medium non-fluorescent (red) and fluorescent (green) PNPs.

Figure S3. Confocal Uptake Images

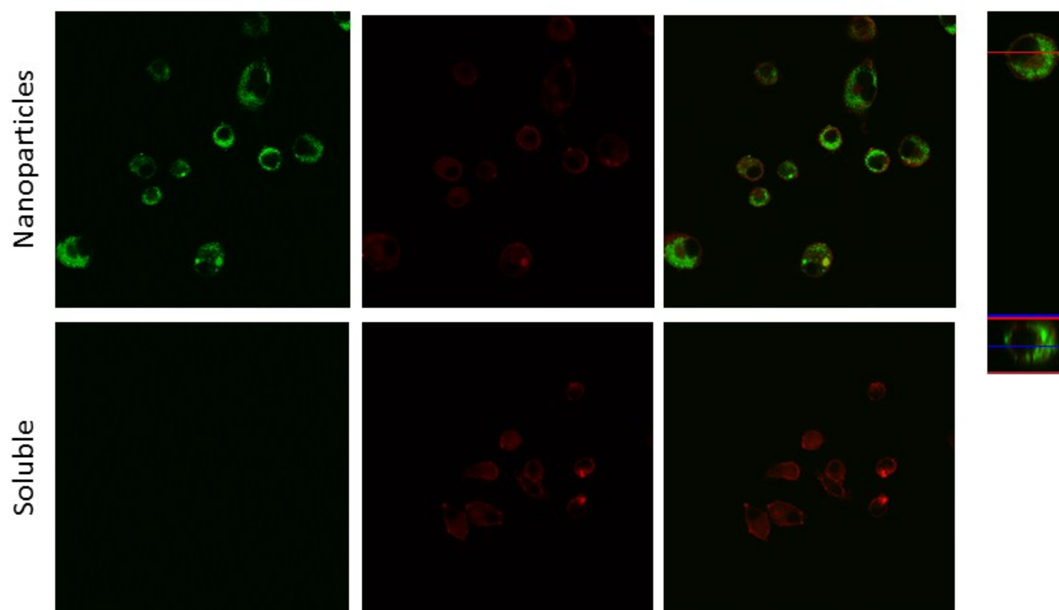

Figure S3. Jaws II Dendritic cells were incubated with 100 µg/mL small, uncoated, green-fluorescent PNPs for 6 hours. Cells were then washed with PBS, permeablized with 1% Triton-X in PBS, and washed twice more. Rhodamine-phalloidin (1:40) was used to stain the actin cytoskeleton, and incubated with the cells for 15 minutes at room temperature. After 3 more PBS washes, the cells were imaged using a Zeiss confocal laser-scanning microscope.

Green-fluorescent PNPs were found to be enclosed by the actin cytoskeleton in all z-stacks (right), indicating uptake rather than adsorption of PNPs. Uptake of soluble protein was barely detectable.

Figure S4. Volume to Circumference Calculations

$$\frac{Volume}{Circumference}\,\alpha\, r^2$$

$$\frac{\frac{V_{PNP}}{C_{PNP}}}{\frac{V_{sol}}{C_{sol}}}=\frac{r_{PNP}^{2}}{r_{sol}^{2}}$$

$$\frac{150\ nm^2}{5\ nm^2} = 900$$

Volume to circumference ratios of PNPs are much greater than those of soluble proteins, suggesting PNPs deliver more antigen to DCs than soluble protein does for a given amount of actin polymerization. This would explain why PNP internalization is not as strongly inhibited by cytochalasin D as soluble OVA is.

Graphical abstract OVA image adapted from PDB ID 1OVA: Stein PE, Leslie AG, Finch JT, Carrell RW. "Crystal structure of uncleaved ovalbumin at 1.95A resolution." J Mol Biol. 1991 Oct 5;221(3):941-59.

Figure S5. Coating PNPs with BSA

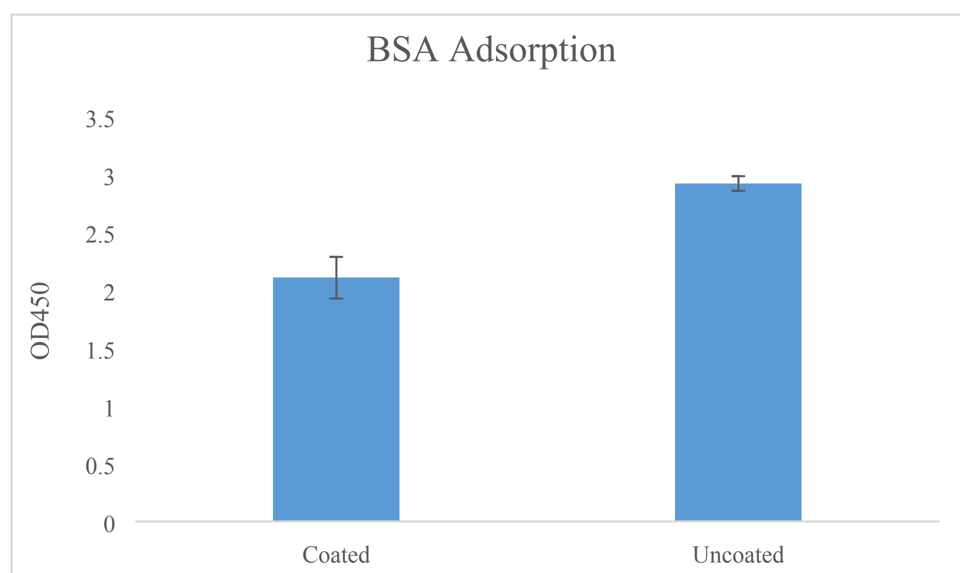

Figure S5. Small coated and uncoated OVA PNPs were incubated in 10 mg/mL bovine serum albumin (BSA) for 2 hours, collected by centrifugation, and crosslinked as described in Table S1. Nanoparticles were then assessed for BSA presence via ELISA. Uncoated OVA PNPs adsorbed significantly more BSA than OVA-coated OVA PNPs did ($p = 0.02$, $n = 2$ per group).

Figure S6. MHC I and MHC II Upregulation

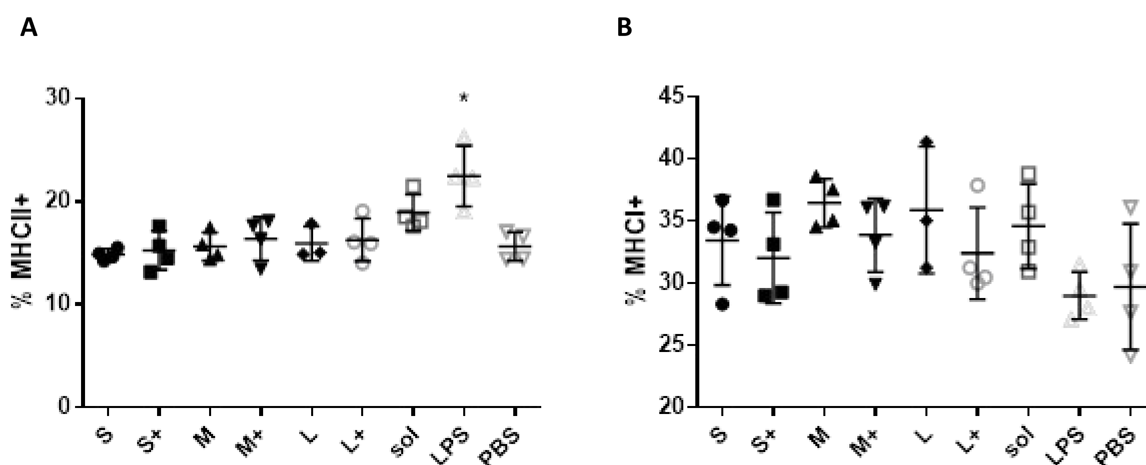

Figure S6. C57BL/6 bone marrow was cultured and matured into BMDCs as described in Section 2.8, and seeded at a density of $3*10^5$ cells/mL in 96-well plates. After 24 hours of stimulation with OVA PNPs, soluble OVA (sol), or soluble OVA + 1 µg/mL LPS (LPS), cells were blocked with anti-CD16/32, and incubated with APC-conjugated anti-H-2K^b-SIINFEKL (MHC I) and FITC-conjugated anti-I-A^b (MHC II) according to the manufacturer's instructions (Biolegend). Upregulation was assessed by flow cytometry, and the percentage of cells expressing high levels of MHC II (A) or MHC I-SIINFEKL (B) are displayed.

LPS-treated BMDCs expressed significantly higher levels of MHC II than OVA PNP types and PBS (left). No significant differences between PNPs, soluble OVA, and PBS were observed. Our observations of humoral responses generated to PNPs in previous *in vivo* studies appears contradictory to the lack of MHC II presentation observed here, although the display of MHC II on LPS-stimulated BMDCs could suggest that different timescales of MHC II presentation are relevant for different types of adjuvants. No significant differences in MHC I-SIINFEKL expression were observed across any groups (B). This may be because PNPs and soluble OVA and OVA+LPS controls contained whole OVA antigen, not the SIINFEKL OVA peptide, which is normally used for MHCI-SIINFEKL assessment.