Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Synthetic Skin-Permeable Proteins Enabling Needleless Immunization**

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Supporting Information

Preparation of LMWP-OVA conjugates

LMWP (VSRRRRRRGGRRRR) was prepared by digestion of native protamine using thermolysin according to our previously described method.[1] OVA (Sigma-Aldrich) was conjugated to LMWP via a disulfide bond. In brief, LMWP in PBS (5 mg/ml) was modified using N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP, Pierce) at a reaction ratio of 1:3 to create an active pyridyldisulfide group at the N-terminal. The reaction was conducted at room temperature (RT) for 2 h, and then excess SPDP was removed by using a heparin affinity column (HiTrap, GE Healthcare). Additionally, a sulfhydryl group was introduced to OVA utilizing the Traut's reagent (Pierce). OVA in PBS (10 mg/ml) was incubated with a 2-fold molar excess of Traut's reagent for 1 h at RT. Thiolated OVA was separated from the excess reagent using a desalting column. These two activated compounds were mixed (1:1 mol/mol) and incubated for 2 h at RT to produce LMWP-OVA via the formation of a disulfide bond. The residual LMWP was removed by dialysis. The LMWP-OVA conjugates were purified using a heparin affinity column and eluted with 1M NaCl solution, while the non-conjugated OVA did not bind with heparin column and was directly washed away. The final product was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Typically, the conjugates possessed a 1:1 molar ratio of LMWP : OVA (**Figure S2**). Prior to use, the conjugates were stored at 4 °C in PBS containing 5 %(wt) Pluronic F-127 as a stabilizer to prevent precipitation.

[1] L. Chang, H. Lee, Z. Yang, V.C. Yang, AAPS J. 2001, 3, 7–14

Preparation of LMWP-lysozyme and LMWP-BSA

The chemical conjugation procedures described above were also applicable to the preparation of LMWP-lysozyme and LMWP-BSA.

In vitro uptake in keratinocytes

Human keratinocytes (CCD 1106 KERTr, ATCC) were cultured as described in the Product Information Sheet from ATCC. Briefly, cells were cultured in KFM medium (Gibco, Invitrogen) in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded onto a 24-well plate at a density of 1×10^5 cells per well and grown to 70–80% confluence. Various fluorescent dye labeled proteins and their LMWP-linked conjugates were then added to cells and incubated for 30 min. After thorough washing, cells were imaged using a fluorescence microscope (Olympus BX51).

Percutaneous protein delivery in mice

All the presented animal studies were approved by the University of Michigan Committee on Use and Care of Animals. LMWP-linked lysozyme, OVA, or BSA was applied to the Balb/c mouse skin. An area of dorsal skin was clipped free of hair 24 h prior to the topical application. After anesthetization, the clipped skin area was moistened with wet gauze for 10 min and extra water wiped off. The LMWP-linked protein conjugates were then applied to the clipped area, and after a 2-hour drug exposure the area was washed thoroughly. The antigen-applied skin was then harvested and processed by cryosection. Slides were imaged using a fluorescence microscope (Olympus BX51). Lysozyme (MW 14 kDa) was labeled with Rhodamine B, whereas OVA (MW 44 kDa) and BSA (MW 66 kDa) were labeled with FITC.

Transcutaneous immunization study in mice

Female Balb/c mice (6–7 weeks old) were immunized with the LMWP-OVA antigen. Animals were prepared as above, and LMWP-OVA antigen was applied to an area of about 2 cm^2. After a 3-hour drug exposure, with application of additional moisture of the exposed area when necessary, the skin was washed thoroughly. Two topical booster administrations of LMWP-OVA at the 2- and 4-week marks were followed. Animals were transcutaneously immunized with 100, 250 or 500 μg of LMWP-OVA in 100 μl solution including 50 μg of cholera toxin (CT, List Biological Laboratories, USA) per mouse, and were designated as low- (TI-L), medium- (TI-M), or high-dose (TI-H), respectively. The control group was given a topical application of solution containing 500 μg of non-conjugated OVA and 50 μg of CT under the same conditions as indicated for the TI groups. Another positive control (i.e. the IM group) was also carried out by intramuscular injection of 50 μg of OVA containing an equal amount of alum adjuvant (Imject® Alum, Pierce), followed by two IM boosters at the 2- and 4-week marks.

Sample collection Blood samples were collected at the 6th week by retro-orbital bleeding, and plasma was obtained by centrifugation and then stored in aliquots at −80 °C. Additionally, mouth and vaginal cavities of the mice were washed with PBS containing 1% BSA (IgG free, Sigma-Aldrich), and the washes were collected and stored in aliquots at −80 °C. Mice were then euthanatized, and spleens were dissected and stored at −80 °C. For cytokine measurements, the spleens were homogenized using a BioMasher homogenizer (BioMasher, USA), centrifuged, and the supernatants were then subject to the ELISA assay.

Anti-OVA IgG assay Anti-OVA IgG concentrations in plasma samples from immunized mice were determined by the ELISA assay. Briefly, 96-well polystyrene plates (Corning

Costar 9017) were coated with OVA (50 μg ml^{-1} in 0.1 M bicarbonate/carbonate buffer, pH 9.6, 100 μl per well) and incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween-20 (PBST), and were then blocked with 1% BSA/PBST buffer for 2 h at 37 °C. After washing the plates, the test plasma samples were added (100 μl per well at a dilution of 1:100,000), and the plates were incubated at 37 °C for 1 h and then washed. Following addition of dilute peroxidase-conjugated goat anti-mice IgG (H+L) antibody (KPL), the plates were incubated for 30 min at 37 °C and washed thoroughly. One hundred microliters of the TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (KPL) were added to each well, and the plates were further incubated for 15 min at RT. After quenching the reaction by the addition of HCl, the absorbance at detection wavelength of 450 nm and reference wavelength of 570 nm were measured using a microplate reader (Bio-tek).

Anti-OVA sIgA assay ELISA assay was performed according to the same procedures described in the previous section. For determination of the anti-OVA sIgA levels, peroxidase-labeled goat anti-mouse IgA (KPL) was employed.

OVA-specific cytokine assay Plates (Corning Costar 9018) were coated with 100 μl per well of the capture antibody (anti-mouse IFN-γ, XMG1.2, eBioscience) in DPBS buffer (Gibco, Invitrogen) at the concentration of 1 μg ml^{-1} overnight at 4 °C. After washing, the plates were blocked with 1% BSA/PBST buffer for 1 h at RT. Test samples or the dilute standard solutions (recombinant Mouse IFN-γ, ELISA RSG standard, eBioscience) were added to the plates, followed by incubation for 2 h at RT. The plates were washed and added with the detection antibody at 100 μl per well (biotin anti-mouse IFN-γ, R4-6A2, eBioscience) and then incubated for another 1 h at RT. After washing, peroxidase-avidin (eBioscience) was added to the plates, and the reaction mixtures were incubated for 30 min at RT. One hundred microliters of TMB solution were added

to each well, and the plates were incubated for 15 min at RT. After addition of HCl to quench the reaction, absorbance at detection wavelength of 450 nm and reference wavelength of 570 nm was measured using a microplate reader (Bio-tek).

Transcutaneous immunization boosted existing immunity to OVA.

Mice were primed with OVA (50 μg per mouse) by IM injection, followed by booster administration, via topical application, of LMWP-OVA (250 μg per mouse) at the 2- and 4-week marks as described previously in the section above. Anti-OVA levels were determined by the ELISA method above, and the data were shown in **Figure S1**.

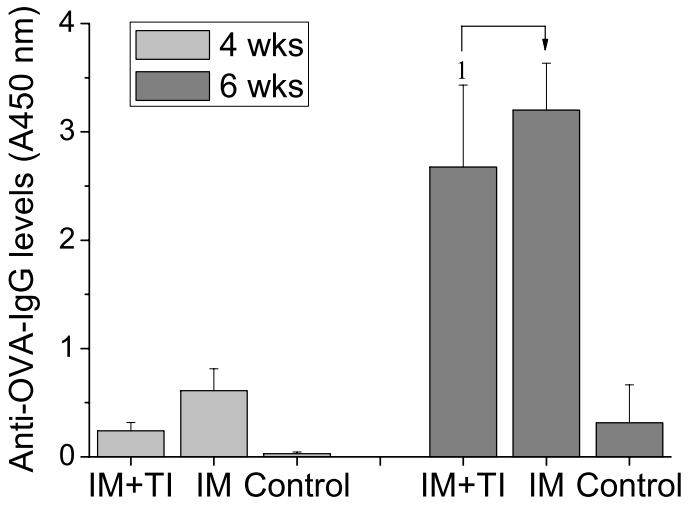

Figure S1 Transcutaneous booster immunization of LMWP-OVA following the primer intramuscular immunization. Results showed that the combined immunization (IM+TI) was able to elicit high anti-OVA IgG responses at the levels statistically comparable to those obtained by the multi-dose intramuscular immunization (IM) (^1p = 0.0823). (n = 10)

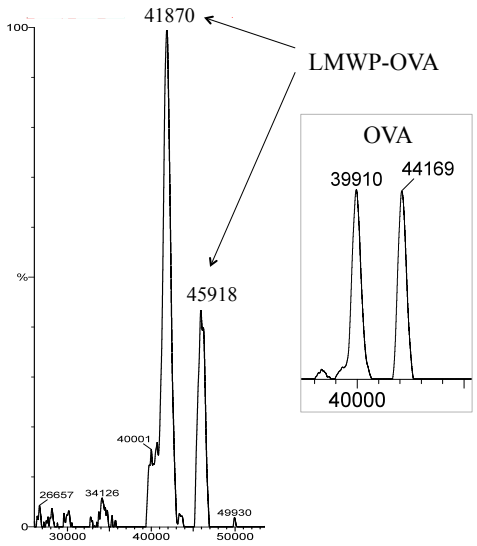

Figure S2 Characterization of LMWP-OVA by MALDI-TOF-MS. The calculated molecular weight of LMWP is 1880. Compared to OVA, the increased size of the LMWP-OVA conjugates indicated the addition of one LMWP.