Electronic Supplementary Information

Transcutaneous immunization by a solid-in-oil nanodispersion

Yoshiro Tahara[a], Kenichi Namatsu[a], Noriho Kamiya[a,b], Masayori Hagimori[c], Seitaro Kamiya[c], Masayuki Arakawa[c,d] and Masahiro Goto*[a,b,d]

[a]Department of Applied Chemistry, Graduate School of Engineering, and [b]Center for Future Chemistry, Kyushu University, 744 Moto-oka, Fukuoka 819-0395, Japan.

[c]Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan.

[d]ASPION Corporation, 1-22 Suketobashi, Tokushima 770-8015, Japan.

Correspondence should be addressed to Marahiro Goto (E-mail: m-goto@mail.cstm.kyushu-u.ac.jp)

Experimental details

1. Preparation and particle size analysis of solid-in-oil nanodispersion

Solid-in-oil (S/O) nanodispersion containing ovalbumin (OVA; Sigma-Aldrich) was prepared as described previously[1]. The sample for mouse immunization was prepared as follows: 2.0 mL of OVA aqueous solution (1.0 mg/mL) and 4.0 mL of sucrose erucate (ER-290; Mitsubishi-Kagaku Foods) cyclohexane solution (25 mg/mL) were mixed with a polytron homogenizer (Kinematica) at 26,000 rpm for 2 minutes to form a water-in-oil (W/O) emulsion. The resulting emulsion was frozen rapidly in liquid nitrogen for 20 minutes, and lyophilized using a freeze dryer (EYELA) overnight. The resulting viscous material was employed as ER-290-OVA complex. Finally, approximately 1.0 mL isopropyl myristate (IPM; Tokyo Kasei) was added to this complex to adjust the OVA concentration to 2.0 μg/μL. The sample for guinea pig immunization was prepared as follows: the W/O emulsion was prepared by mixing a 5.0 mL of OVA aqueous solution (4.0 mg/mL) and a 10 mL of ER-290 cyclohexane solution (100 mg/mL), frozen and lyophilized as described above. Next, IPM was added to the resultant complex to adjust the OVA concentration to 5.0 mg/mL. The particle size in appropriately diluted S/O nanodispersion was evaluated with a Zetasizer Nano ZS (Malvern). The viscosity and refractive index of each formulation were measured using an automatic microviscometer (Anton Parr GmbH) and a refractive-index detector (Kyoto Electronics Manufacturing), respectively.

2. Animals

Male 7-week-old BALB/c mice (Kyudo, Co., Ltd, Saga, Japan) of average weight 20–30 g were used in immunization studies on mouse ear skin. Experiments using mouse were carried out in accordance with the Guidelines of the Animal Care and Use Committee, Kyushu University. Male 8-week-old guinea pigs (Kyudo, Co., Ltd, Saga, Japan) of average weight 450–500 g were used in immunization studies on guinea pig back skin. Experiments using guinea pigs were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Nagasaki International University. All animals were each housed in a cage with free access to food and water.

3. Immunization study on mouse ear skin

A total of 100 μg/animal of OVA was either applied to the native auricle skin of mice transcutaneously or subcutaneously to the abdomen, on two occasions with a one-week interval. Sera collection was then conducted two weeks after the initial immunization. The detailed protocol was as follows: on the first day of immunization, the mice were anaesthetized by intraperitoneal injection of sodium pentobarbital (Nacalai Tesque) prior to each immunization, to prevent grooming. The tissue paper (5 mm × 10 mm) interfused with 25 μL of the samples (either 2.0 μg/μL of OVA in phosphate-buffered saline (PBS), a W/O emulsion, consisting of 5 % (v/v) aqueous solution containing 40 μg/μL of OVA and 95 % (v/v) IPM solution containing 100 μg of ER-290 in 0.95 μL of IPM, or a S/O nanodispersion) were applied on each intact mouse ear. Mice were applied with 50 μL of sample, resulting in a total dose of 100 μg of OVA on each occasion. The tissue papers were covered with an adhesive plaster (Nichiban) on vinyl tape (Fig. S4). The other mice belonging to the positive control group were subcutaneously injected into the abdomen with 50 μL of PBS solution containing 2.0 μg/μL of OVA. After 24 hours, the cutaneously applied samples were removed. The above procedures of both transcutaneous immunization and subcutaneous injection were repeated on the 8-9th day. On the 15th day, sera were drawn from the eye grounds of the anaesthetized mice and all mice were euthanized.

4. Immunization study on guinea pig back skin

A total of 5 mg/kg of OVA was either applied to the shaved back skin of the guinea pig transcutaneously or subcutaneously on two occasions with a one-week interval. Sera collection was then conducted by the same schedule as that used for mouse immunization. The detailed protocol is as follows: on the first day of immunization, sera were collected as pre-immune samples from the pinna. Then, 1 mL/kg of samples containing either 5.0 mg/mL of OVA in phosphate-buffered saline (PBS) or the S/O nanodispersion were applied on the shaved guinea pig back skin. The other guinea pigs belonging to the positive control group were

subcutaneously injected into the back with the 1 mL/kg of PBS solution containing 5 mg/mL of OVA. The above procedures of both transcutaneous immunization and subcutaneous injection were repeated on the 8-9th day. On the 15th day, sera was drawn from the pinna and all guinea pigs were euthanized.

5. Antibody detection

OVA-specific antibody titers were determined using an enzyme-linked immuno-sorbent assay (ELISA). Briefly, 96 well polystyrene plates (Maxisorp, NUNC) were coated with 100 μL/well of OVA antigen aqueous solution (5.0 mg/mL) and then incubated overnight at 4 °C. The plates were washed with PBST (0.1 % Tween-20 in PBS) 5 times for each well, and then blocked with 200 μL/well of bovine serum albumin (BSA; Wako Pure Chemical Industries) in PBS (2.0 wt.%) for 2 hours at 37 °C. Next, the plates were washed again and 20 μL/well of sample, diluted with a PBS solution containing 2.0 wt.% of BSA, were added followed by incubation for 2 hours at 37 °C. After the plates were washed, the 100 μL/well of horseradish peroxidase (HRP) labeled rabbit anti-mouse IgG, IgG1 or IgG2a (Rockland) or anti-guinea pig IgG (Sigma) diluted with a PBS solution containing 2.0 wt.% of BSA were added to each well and the plates were incubated for 2 hours at 37 °C. After washing, 100 μL/well of tetramethyl benzidine solution for ELISA (Sigma or Wako Pure Chemical Industries) was added to each well. After the HRP reaction was complete, 50 μL/well of 0.5 M H2SO4 was added and the end point optical density (O.D.) was measured at 450 nm using a microplate reader (Bio-Tek). The antibody titers were defined as the inverse dilutions at which the O.D. of an immunized animal is equal to that of the control samples. For the BALB/c mice, the control sample used was the 500-fold diluted serum of one non-immunized mouse, and for the guinea pigs, the control sample used was the sera of the pre-immune animals. GraphPad Prism-5 software was used to evaluate approximate curves of dilution for calculation of the titers.

6. Statistical analysis

The immunization results are reported as mean ± standard deviation (S.D.). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. $*p<0.05$ versus PBS solution group. The GraphPad Prizm-5 Software was used for statistical significance analysis and determination of antibody titers by approximate curves of sera dilution.

Reference

1. Y. Tahara, S. Honda, N. Kamiya, H. Piao, A. Hirata, E. Hayakawa, T. Fujii and M. Goto, *J. Control. Release*, 2008, **131**, 14.

Supplementary data

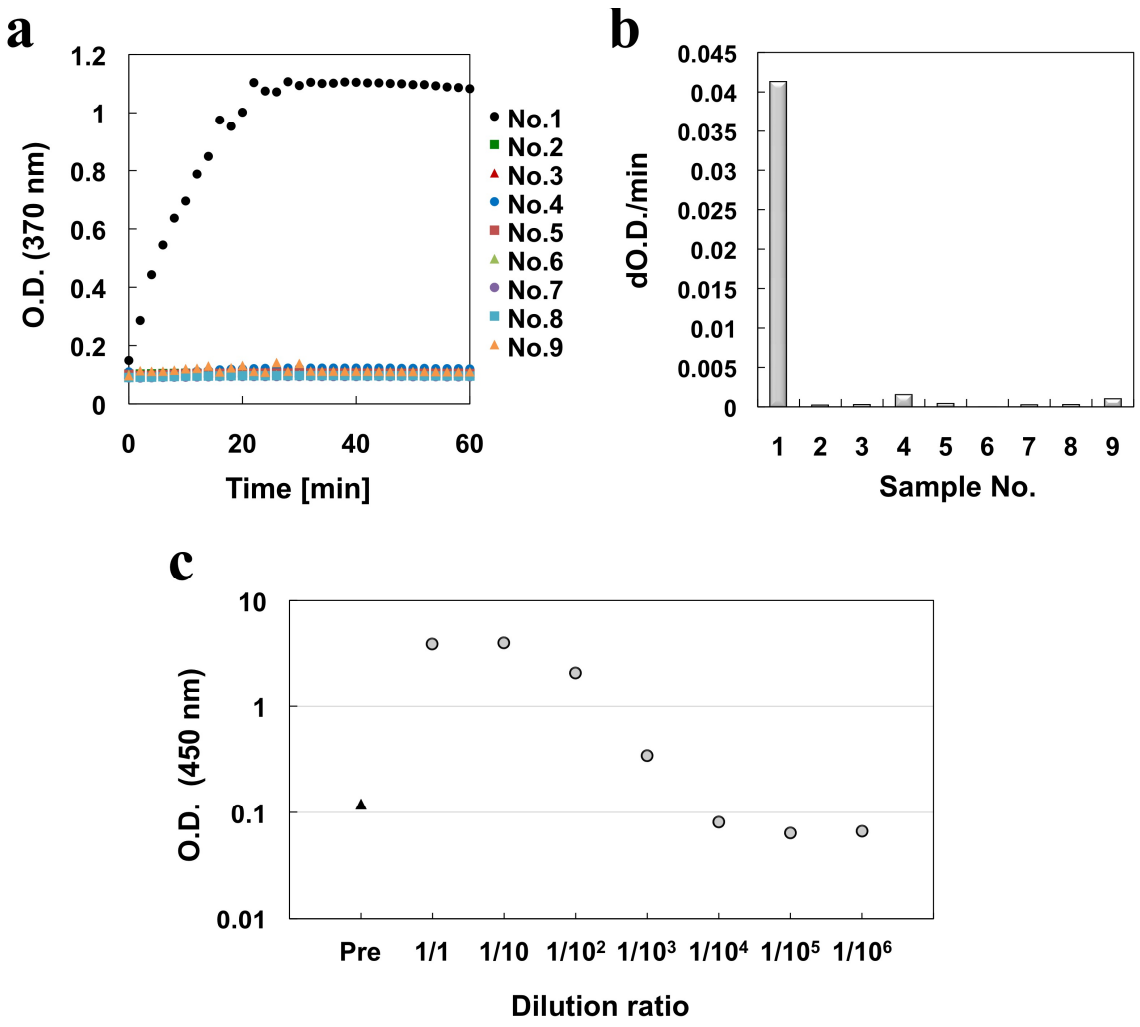

Figure S1 Determination of accuracy of ELISA using the samples shown in Table S2. (a) The optical density (O.D.) of kinetics and (b) the initial velocity by measuring the reaction between tetramethyl benzidine and HRP detected at 370 nm. (c) The titration curve of sera, collected from OVA injected guinea pig, starting with a 1/1 dilution and pre-immunization samples.

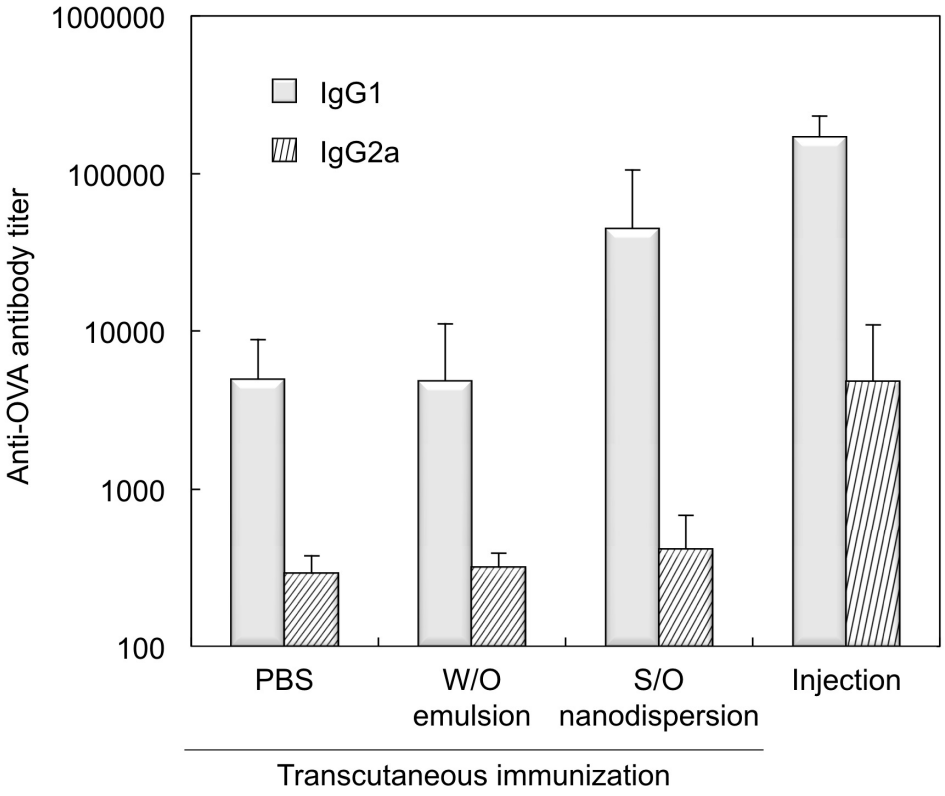

Figure S2 OVA-specific IgG1 or IgG2a responses in BALB/c mice after immunization with OVA. Mice were vaccinated transcutaneously (PBS solution, W/O emulsion or S/O nanodispersion) or subcutaneously (injection) twice with a one-week intervals. One week after the final vaccination, sera were collected and assayed for anti-OVA IgG1 and IgG2a titers by ELISA. Data are expressed as mean ± S.D. of results from 5-6 mice.

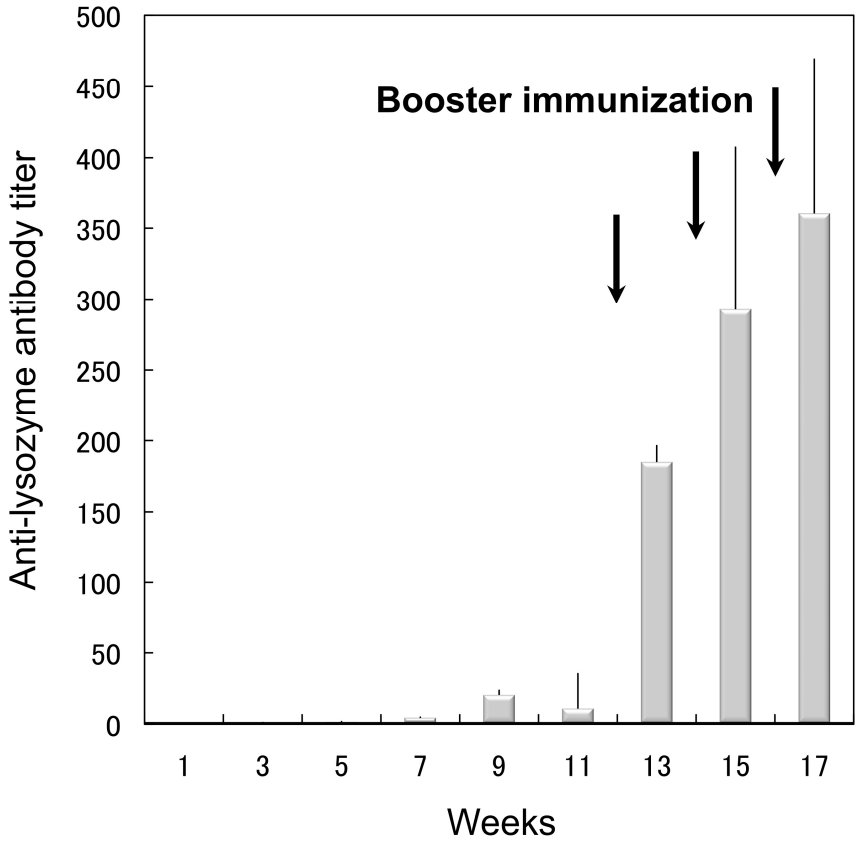

Figure S3 Lysozyme-specific IgG response in male New Zealand rabbits after immunization with hen egg white lysozyme. Rabbits were vaccinated onto ear with S/O nanodispersion transcutaneously and booster immunization with S/O nanodispersion was conducted at 12th, 14th and 16th week. Sera were collected and assayed for anti-lysozyme IgG titers by ELISA. Data are expressed as mean ± S.D. of results from 3 rabbits.

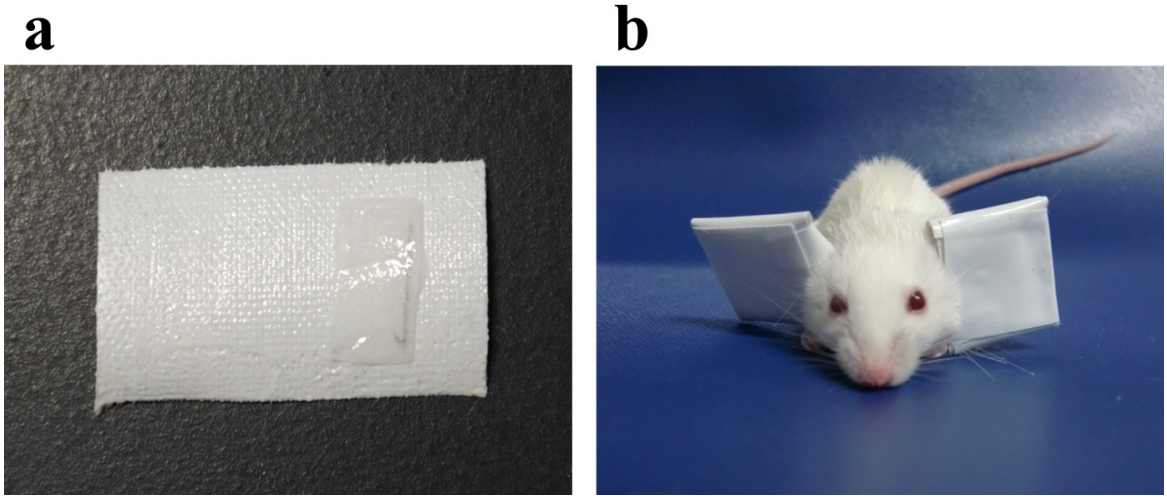

Figure S4 (a) The tissue paper interfused sample on adhesive plaster and (b) transcutaneous vaccinated BALB/c mouse with samples attached to the intact auricle skin.

Table S1 Particle size analysis of S/O nanodispersion (The average mean particle size, PDI and distribution width were measured in triplicate for 4 samples)

Mean particle size [nm]	**PDI**	**Distribution width [nm]**
243 ± 38	0.01 – 0.27	70 ± 11

Mean ± S.D., n=4.

Table S2 Samples for determining accuracy of ELISA

No.	Samples
1	Anti-OVA IgG (from guinea pig) in serum developed in guinea pig by OVA injection
2	Anti-lysozyme IgG (from rabbit) in serum developed in rabbit by lysozyme injection
3	Anti-BSA IgG (from rabbit) in PBS purchased from Sigma
4	Anti-insulin IgG (from guinea pig) in PBS purchased from Sigma
5	Serum developed in non-immunized guinea pig
6	Serum developed in non-immunized rabbit
7	2 % BSA in PBS
8	PBS
9	Distilled water