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Ionic liquid mediated transcutaneous protein delivery with solid-in-oil nanodispersion†

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As a potentially safe and non-invasive vaccination method, transcutaneous immunization represents an attract[illegible] alternative to conventional vaccine delivery by injection. However, the development of transcutaneous immunization is remained a challenge for a large number of hydrophilic macromolecules including protein and peptide antigens. We rep[illegible] a novel ionic liquid (IL)-mediated transcutaneous vaccine formulation consisting of a solid-in-oil (S/O) nanodispersion o[illegible] antigen coated with pharmaceutically accepted surfactants dispersed in IL-containing oil. The introduction of IL [C_{12}mim][Tf_2N] (1-dodecyl-3-methyl imidazolium bis(trifluromethyl sulfonyl) amide) as a penetration enhancer in the formulation significantly enhanced the skin permeability of ovalbumin (OVA), a model antigen. It was also found that I[illegible] mediated S/O nanodispersion obtained high levels of OVA-specific serum IgG compared with both S/O nanodispersions without IL and PBS control. These finidings clearly indicate that ILs - a potentially attractive "green" and "designer" solvent- could serve as a potential skin penetration enhancer in transcutaneous vaccination for hydrophilic macromolecules.

Introduction

Transcutaneous immunization, a new method of vaccination potentially offers safe and non-invasive drug administration. In comparison to conventional drug delivery routes (*i.e.,* oral, injections), transcutaneous vaccination provides many advantages such as needle free vaccination, avoidance of first pass hepatic elimination, improved patient compliance, and offers an easy and cost-effective strategy for disease prevention.[1] Recently, transcutaneous administration has been demonstrated with a wide variety of protein and DNA antigens.[2] However, effective drug delivery through the skin is inherently difficult because of the stratum corneum (SC), the outermost highly structured skin layer which is a natural transport barrier and limits drug diffusion. To address this issue, a number of approaches including tape-stripping,[3] chemical/lipid enhancers, [4] sonication, [5] electroporation,[6] microneedles,[7] and hydrogel patches [8] have been used t[illegible] enhance drug permeability of skin. However, transcutaneous administration has yet to fully achieve its potential for a lar[illegible] number of molecules, especially hydrophilic macromolecules including protein and peptide antigens.[9] It is therefore desirable to develop a method for effective transcutaneou[illegible] administration of hydrophilic macromolecules.

Recently, we reported a novel transcutaneou[illegible] administration method using a unique solid-in-oil (S/O) nanodispersion containing hydrophilic drug molecules and proteins.[10] To prepare the S/O nanodispersion, the protein[illegible] were coated with surfactant molecules to form nanometer sized surfactant-protein/drug complexes. Such complexes were dispersed in an oil phase in which the nano-ordered partic[illegible] allowed the proteins to penetrate into the skin without a[illegible] adjuvants or skin pretreatment. In fact, oil and surfactants are acted as chemical penetration enhancers to overcome the SC barrier and significantly enhanced the permeation of protein[illegible] into the skin.[10a] However, compared with conventional injection systems, level of permeation using a S/O nanodispersion i[illegible] lower.[10e] Because ionic liquids (ILs) have excellent solvent properties, we have hypothesized that the protein permeati[illegible] of S/O nanoparticles could be enhanced by the addition of ILs.

In recent years, ionic liquids (ILs), which are salts of lo[illegible] melting point and consist only of ions, have been increasingly exploited as solvents and/or (co)solvents and/or reagents in a wide range of biological applications including pharmaceuticals due to their tunable chemical, physical and biologica[illegible]

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†Electronic Supplementary Information (ESI) available: schematic diagram of S/O nanoemulsion formulation, Chemical structure of surfactants, OVA and ILs, Solubility of ILs in IPM, size and size distribution of S/O with and without IL, phosphatidylcholine (PC) solubility in various solvents,release profile of OVA from S/O nanoemulsions and motoring weight change of mice with time. See DOI: 10.1039/x0xx00000x

properties.[11-13] Recently, we reported that ILs can dissolve sparingly soluble drug molecules and serve as carriers for topical and transdermal delivery. [12a,c,d] Williams et al. demonstrated IL- based oral drug delivery in which self-emulsifying IL formulation containing poorly water soluble drugs gave rise to 4.3-fold higher exposure than the crystalline drug and prolonged exposure compared with a lipid formulation.[13b] Very recently, Mitragotri et al. conducted a very interesting study where they found that some ILs have excellent antimicrobial activity, minimal toxicity to epithelial cells as well as skin, and are effective for transdermal drug delivery. [13a] Importantly, some ILs increased transdermal delivery by more than 16-fold without any skin irritation. This finding is very significant in exploiting ILs for transcutaneous immunization because skin irritation generally occurs with topical formulations prepared with polar organic solvents such as DMSO or ethanol. In addition, the customizable properties of ILs can be exploited to further increase formulation potency when employing ILs as penetration enhancers.[12b] Taken together, these features present a compelling case for investigating how TCI with S/O nanodispersion, both *in vitro* and *in vivo*, is influenced by using an IL as a skin penetration enhancer.

Experimental

Materials

Protein ovalbumin (OVA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Surfactants Sucrose laurate (L-195) and sucrose erucate (ER-290) were kindly provided by Mitsubishi-Kagaku Foods (Tokyo, Japan). Isopropyl myristate (IPM) and cyclohexane were sourced from Tokyo Chemicals (Tokyo, Japan) and Wako (Kyoto, Japan), respectively. All ionic liquids (ILs) including hydrophobic IL [C_{12}mim][Tf_2N] (1-dodecyl-3-methyl imidazolium bis(trifluromethyl sulfonyl) amide) (from Sigma-Aldrich) were purchased from Solvent Innovation GmbH (Germany) and were used as received. Frozen (– 80°C) porcine skin samples, excised from a 5 month old female Yucatan Micropig (YMP), were obtained from Pierce (Rockford, IL USA). All other reagents used in the experiments were analytical grade.

Animals

Female 6-week-old BALB/c mice were purchased from Kyudo (Saga, Japan) and maintained under standardized conditions. Animal experiments were carried out with the approval of the Ethics Committee for Animal Experiments (approval no. A-26-267-0, Kyushu University, Japan) and in accordance with the Guide for the Care and Use of Laboratory Animals (Science Council of Japan).

Preparation of S/O nanodispersions and particle size analysis

Solid-in-oil (S/O) nanodispersion incorporating ovalbumin (OVA) was prepared as described previously with some modification.[10b] A simplified schematic diagram for the formulation of S/O nanodispersion with IL is shown in Fig. 1.

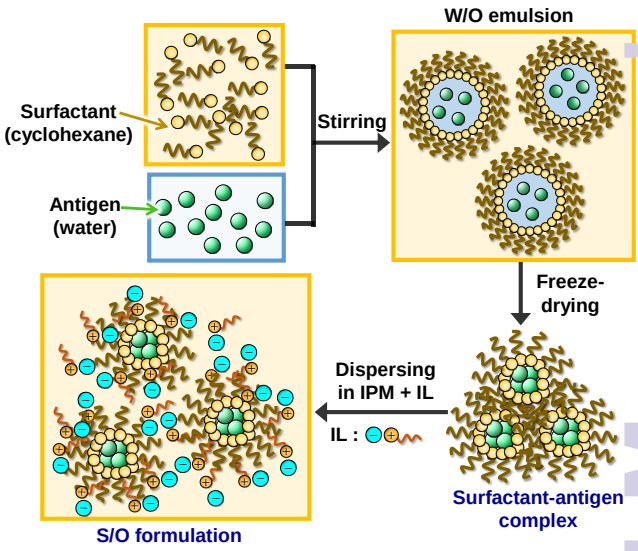

Fig.1 Schematic diagram for the formulation of solid in oil (S/O) nanodispersion in IPM with ionic liquid (IL).

In a typical experiment, 2.0 mL of OVA aqueous solution (1.0 mg/mL) and 4.0 mL of surfactant (ER-290 or L-195) cyclohexane solution (12.5 mg/mL) were mixed with a polytron homogenizer (KinematicaAG, Lucerne, Switzerland) 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, Tokyo, Japan) for overnight. The resulting viscous material was employed as surfactant-OVA complexes. Finally, approximately 1.0 mL isopropyl myristate (IPM) containing different amount of IL was added to yield S/O nanoemulsion. The particle size in appropriately diluted S/O nanodispersion was evaluated with a Zetasizer Nano ZS (Malvern, Worcestershire, UK). 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.

In vitro transcutaneous delivery of OVA with and without IL

The penetration of OVA through full thickness skin pieces of Yucatan hairless micropigs (YMPs) were performed using a hand-made Franz diffusion cells with a diameter of 10 mm. The porcine skin was prepared as we reported previously.[10b] The skin piece was clamped between the donor and receptor compartments of the diffusion cell. The stratum corneum faced the donor side of the cell whereas, the dermal side of the skin was allowed to be in contact with PBS solution (pH 7.4, 5 mL). The buffer solution was maintained at 32.5 ± 0.1°C using a thermostatically controlled water bath and was magnetically stirred at 600 rpm throughout the experiments. To evaluate the transcutaneous delivery of OVA, a fluorescein isothiocyanate (FITC)-labeled OVA S/O nanodispersions or FITC-OVA PBS solution (500 µL each) was applied to the skin surface. After 24

h, samples were removed and the skin surface was carefully washed with an extraction solution (PBS: acetonitrile: methanol = 2:1:1, v:v:v). The skin was minced and immersed in the 500 µL of extraction solution for 3 h with gentle stirring. The concentrations of FITC-labeled OVA in the solutions were quantified by the fluorescence intensities at 523 nm (λ_{ex} = 490 nm) recorded on a LS55C spectrometer (Perkin Elmer, waltham, MA, USA).

Ovalbumin (OVA)-release study with and without IL

A 0.1 mL OVA S/O nanodispersions was placed on a 0.4 mL of phosphate buffered saline (PBS) in a glass vial and the vial was kept at 37°C with gentle stirring. Aliquots of the underlying PBS were collected after 1, 3, 6, and 24 h, and the OVA concentrations in the samples were evaluated using a bicinchoninic acid kit (Sigma-Aldrich) according to the manufacturer's protocol.

***In vivo* transcutaneous immunization using S/O nano-dispersions with and without IL**

The BALB/c mice were vaccinated by transdermal immunization with OVA S/O nanodispersions thrice at a one-week interval. Transdermal immunization was carried out by applying OVA S/O nanodispersions onto a native auricle using patches (approximately 0.5 cm x 1.0 cm). After 24 h, the patches were removed. Blood samples were collected from a tail vein, one week after each immunization. The production of OVA-specific serum IgG was evaluated using an enzyme-linked immunosorbent assay (ELISA), as described previously. [10b] The antibody titer was defined by the dilution factor at which the optical density of the sample was equal to that of a non-immunized sample. In this experiment, mice topically immunized with OVA PBS solutions at the base of the earlobe were prepared as controls.

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 using Graph Pad Prism6 (Graph Pad Software, Inc., La Jolla, CA).

Results and discussion

Formulation and characterization of S/O nanodispersions

An S/O nanodispersion containing OVA, a model antigen protein having a molecular weight of 45 kDa was prepared using sucrose erucate (ER-290) or sucrose laurate (L-195) (Fig.S1 in ESI†) as the surfactant and Isopropyl myristate (IPM) as the oil. We selected ER-290 and L-195 as surfactants and IPM as an oil because they have been extensively used in formulating drug delivery carriers. [10 a, b, 14] In this study, the hydrophobic IL [C_{12}mim][Tf_2N] (1-dodecyl-3-methyl imidazolium bis (trifluromethyl sulfonyl) amide) (Fig. S1 in ESI†) was used due to its high solubility in IPM (more than 25% w/w) whereas hydrophilic ILs were not soluble in IPM (Fig. S2 in ESI†). We also checked the solubility of DMSO, a well known chemical enhancer for drug delivery [4a] and found it to be only partially soluble in IPM. We checked whether protein permeability through the skin was increased by using insoluble ILs in IPM and found no significant enhancement (data not shown).

To investigate the potential of nanodispersions as drug carriers, it is important to investigate their microstructure. The sizes and size distribution of OVA-surfactant complexes in IPM with or without IL were characterized by DLS (Table 1). Adding 5% v/v IL to the IPM continuous phase led to an increase in droplet sizes for OVA-surfactant complexes prepared with both ER-290 and L-195. Both systems produce nanoparticles with PDI values below 0.3, indicating a narrow size distribution. It was observed that with increased IL concentrations the mean diameter increased (Fig.S3 in ESI†), possibly because more IL was available at the interface. We have checked the formation of IL aggregates in IPM and found none. This is due to the non-coordinating anion (Tf_2N^-) in the IL, which has a poor ability to form hydrogen bonds with surfactant head groups. However the IL used has a dodecyl group attached to the cation, which can interact easily with the chains of the surfactants via inter digitation.[15] One would expect that the concentration of IL at the interfacial region increases with increasing total IL concentration. Hence, the size of the nanoparticles increased with the addition of IL.

Table 1. Mean particle diameter (mean ± standard error of the mean) of S/O nanodispersion in IPM with[a] and without IL.

S/O samples	Mean diameter (nm)	PDI
S/O ER-290 (IPM)	86.0 ± 3.2	0.212 ± 0.023
S/O ER-290 (IPM + IL[a])	118.2 ± 4.0	0.263 ± 0.049
S/O L-195 (IPM)	129.9 ± 11.8	0.1814 ± 0.019
S/O L-195 (IPM + IL[a])	171.0 ± 7.4	0.211 ± 0.007

[a] IL[C_{12}mim][Tf_2N] was added to IPM at a concentration of 5 % v/v

Skin penetration of S/O nanodispersions with and without IL

The main focus of this study was to show that IL can be used as a penetration enhancer in S/O nanodispersions for transcutaneous immunization. The permeation of OVA from the formulations through YMP skin was evaluated *in vitro* using Franz-type diffusion cells as reported previously.[12a] Fig. 2 shows OVA delivery into the skin using various formulations. The inclusion of IL in S/O nanodispersions significantly enhanced OVA permeation in the skin. This is to be expected if we consider the unique ionic character of ILs. ILs can penetrate the SC by extracting the lipid from the SC,[4a] which is made possible by their surfactant character.[16] To check whether ILs can extract lipids, we investigated the solubility of phosphatidylcholine (PC), a class of phospholipids that is the major component in biological membrane, in IPM, IL and IPM +IL systems (Fig. S4 in ESI†). It was found that IL [C_{12}mim][Tf_2N] facilitated dissolution of PC in IPM. By comparing these results with other literature studies,[13a,15] we can conclude that the presence of IL in the IPM phase of S/O nanodispersions can promote disruption of the lipid bilayer in the SC and enable OVA-surfactant complexes to more easily penetrate into

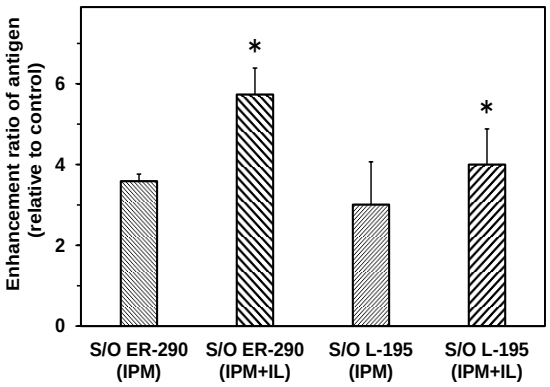

Fig. 2 Enhancement of OVA delivery into the skin relative to PBS (control) by inclusion of IL in a S/O nanodispersion. To account for variability between skin pieces the, PBS control was performed for each skin piece used. Error bars represent mean ± SD for n = 3. *P< 0.05 compared with control formulation by one way ANOVA, followed by Turkey's Post Hoc test using Graph Pad Prism 6. Error bars represent mean ± SD (n = 3).

the skin. However, further detailed study of the mechanism will be required to assess the contribution of IL to enhancing transcutaneous immunization. Compared with L-195, S/O nanodispersion with ER-290 provided better results, possibly due to its smaller droplet size.

OVA-release study from S/O in the presence of IL

We checked the *in vitro* OVA release efficiency of the S/O nanoparticles in a PBS solution at 37 °C (Fig. S5 in ESI†). In case of L-195 based S/O nanoemulsions, a fast release was found for the first 6 h, followed by a sustained release until 24 h. Interestingly, the percentage of OVA released from L-195 S/O nanoemulsions in the PBS solution decreased in the presence of IL in IPM (Fig. 3). It is known that the release of drugs from S/O nanoparticles depends on particle stability. As mentioned previously, IL can exist at the interface of S/O nanodispersions where it can enhance the stability of the system, leading to reduced OVA release. Compared with ER-290-OVA complexes, L-195-OVA showed better release efficiency, possibly due to the larger particle size, which is relatively unstable in a hydrophilic environment.[10f]

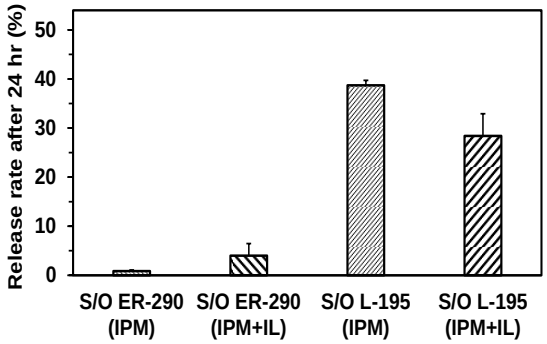

Fig. 3 Percentage release of OVA from S/O nanoemulsions to PBS buffer after 24 h. Error bars represent mean ± SD (n = 3).

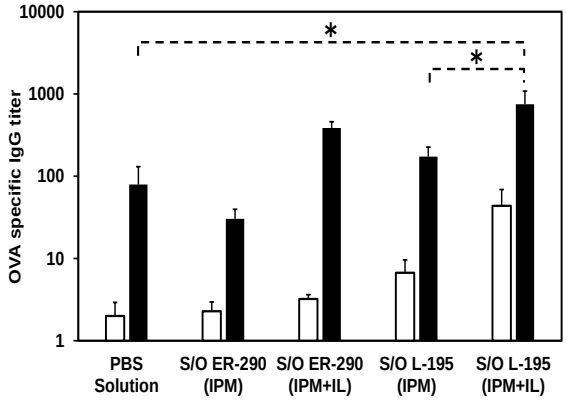

Fig. 4 OVA-specific IgG titers 2 weeks (white bar) and 3 weeks (black bar) after the first administration determined by ELIS Animals were vaccinated transcutaneously with PBS solution or S/O nanodispersion thrice at one-week intervals. Data a expressed as mean ±SD (n = 3–4, *P< 0.05).

Transcutaneous immunization by using S/O nanoemulsions with and without IL

Finally, transcutaneous administration using IL-based S/O nanodispersion in BALB/c mice was evaluated. As shown in Fig. 4, transcutaneous vaccination using the S/O nanodispersion with IL obtained high levels of OVA-specific serum IgG compared with both S/O nanodispersions without IL and PBS control. This finding is consistent with the *in vitro* results that demonstrated the favorable effect of IL as a skin penetration enhancer. In addition, the S/O nanodispersion prepared with L-195 (with or without IL) produced a higher titer of antibodies compared with S/O with ER-290. This positive effect of L-195 is related to the favorable chemical properties of L-195 as a surfactant over ER-290.[10c] In fact, L-195 provides a better drug release profile from nanoparticles as shown in Fig. 3. To check the toxicological effect of IL on mice, we monitored the weight change of mice for 3 weeks and found no significant weight changes (Fig. S6 in ESI†). Direct toxicological assessments require further studies.

Conclusions

In conclusion, IL-mediated transcutaneous administration with S/O nanodispersions was effective for high molecular weight protein /drug molecules. IL-based S/O nanodispersions can enhance antigen-specific antibody creation without any adjuvant – derived immune stimulation. Although the toxicological issues of ILs is a matter for debate, ILs can be designed to be nontoxic by using pharmaceutically compatible cations and anions in their synthesis.[13a] We believe that this study could play an important role in the development of IL as skin penetration enhancers in drug delivery systems that take advantage of the many attractive properties of ILs. Future efforts should focus on optimizing systems for maximal transcutaneous administration delivery efficiency and determine their efficacy in attenuating cytotoxicity towards the targeted cells.

Acknowledgements

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Ionic liquid mediated transcutaneous protein delivery with solid-in-oil nanodispersion

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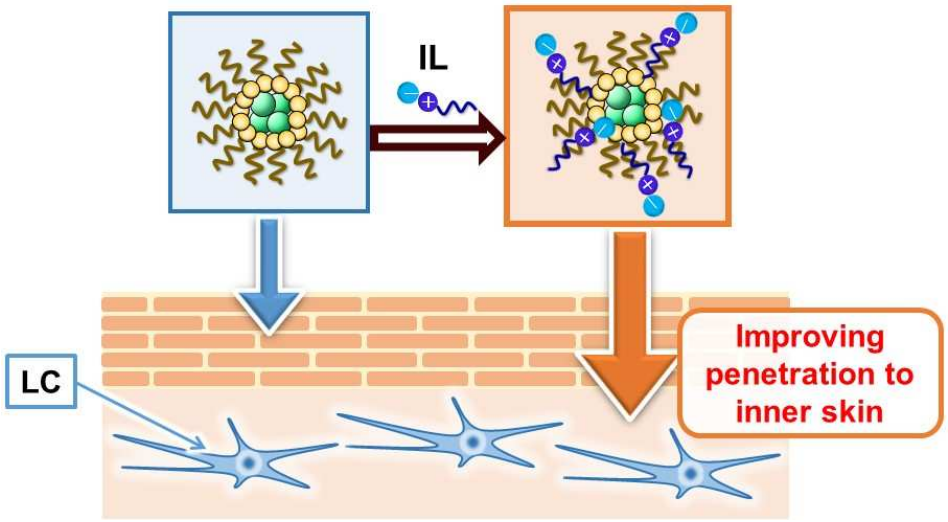
Graphical Contents



We report a novel ionic liquid (IL)-mediated transcutaneous vaccine formulation consisting of a solid-in-oil nanodispersion of antigen coated with pharmaceutically accepted surfactants dispersed in IL-containing oil. The introduction of IL in the formulation significantly enhanced the skin permeability of ovalbumin, a model antigen.