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ORIGINAL ARTICLE

Molecular weight dependence on bioavailability of FITC-dextran after administration of self-dissolving micropile to rat skin

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Abstract

Background: Self-dissolving micropiles (SDMPs) have been evaluated with macromolecular drugs like insulin and erythropoietin as a new percutaneous drug delivery system. To study the molecular weight dependence on the absorption of macromolecular drugs through the skin after administration of SDMPs, four kinds of SDMP were prepared using fluorescein isothiocyanate-labeled dextrans (FDs) having a molecular weight of 10, 20, 40, and 70 kDa. *Method*: In in vitro release experiments there were no significant differences on their release rates in the four SDMPs. The dependence of molecular weight of FD on the permeability coefficient was studied in the *in vitro* permeation experiment. Histological study on the skin after administration of FD SDMP (5.0 mg/kg) to rat was performed for 24 hours. In vivo experiment using rats resulted in slower absorption rate of FD-40 and FD-70 SDMP (5.0 mg/kg). *Results*: The permeability coefficient was 4.59, 4.69, 3.38, and 1.43×10^{-4} cm/h for FD-10, 20, 40, and 70, respectively. Histological examination showed that yellow color was still observed at 6 h after administration of FD-40, and FD-70 showed yellow color even at 24 h. Bioavailabilities of FDs from SDMP were 99.4 ± 6.93%, 88.3 ± 7.05%, 45.7 ± 4.77%, and 16.0 ± 1.15% for FD-10, 20, 40, and 70, and the dependency on molecular weight dependence was clearly observed. *Conclusion*: These observations supported that bioavailabilities of FD from SDMP decreased as the molecular weight of FD increased to more than 40 kDa.

Key words: *Enhancement; fluorescein isothiocyanate-labeled dextrans; micropiles; rats; self-dissolving; skin permeability*

Introduction

Recent advance in microfabrication technology has made it possible to prepare microneedles made of metal and silicon, and microneedles were found out to be useful for the percutaneous absorption of macromolecules that were not absorbed from the skin. The tested macromolecules were insulin[1], protein antigen, ovalbumin, bovine serum albumin[2], antisense oligonucleotides[3], and plasmid DNA[4], etc. Microneedle is classified into two categories: hollow microneedles and microneedle array. Hollow microneedles are made of metal and drug solution is injected into the skin through hollow microneedles. The microneedle array made of silastic and metals such as stainless steel[5] and titanium[6] are used in two ways. One method is the application of drug solution to the skin after physical microconduits are made by the insertion of metallic and/or silastic microarray[5]. The second method is to use microarrays whose surface is coated with drug[6]. After the insertion of microarrays into the skin, drug is dissolved and absorbed into the systemic circulation. However, these microneedles are not pharmaceuticals but belong to medical device.

On the other hand, we have been studying self-dissolving micropile (SDMP) as a new percutaneous drug delivery system[7]. SDMP is made of pharmaceutical additives as the base and drug, where drug is formulated as solid dispersion or suspension. Therefore, SDMPs are genuine pharmaceutical preparations. Skin is made of three layers, that is, stratum corneum, epidermis,

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and dermis. Generally, there are three barriers for the absorption of drugs through percutaneous route. First one is the stratum corneum that has strong primary barrier function against exogenous compounds including drug. Second barrier is the viable epidermis which is 100–200 μm thick and contains a tissue such as living cells. However, there are no blood vessels in the epidermis. Deeper still, there are blood capillaries in the dermis that forms the bulk of skin volume and contains living cell nerves. For the absorption of percutaneously administered drug into the systemic circulation, there is the third barrier, that is, drugs must permeate through the microcapillary walls. Drug is added to dense solution, glue, of water-soluble thread-forming polymers such as chondroitin sulfate, hyaluronic acid, dextran, albumin, and gelatin, etc., and the resultant mixture is formed to be SDMP having sharp tops after drying in the room temperature. The polymers used to prepare SDMP are generally used as pharmaceutical additives. Therefore, their safeties have been proved. As these polymers have hydrophilic property, SDMP dissolves easily after inserted into the skin. In the case of water-soluble macromolecular drugs, they are easily formulated into SDMP base as solution. Therefore, we demonstrated that SDMP formulated with macromolecular-drug, such as insulin[8], erythropoietin[9], interferon-α (IFN)[10], human growth hormone[11], and low-molecular-weight heparin[12], were well absorbed from the skin into the systemic circulation in mice, rats, and dogs. However, when SDMP was applied to poly(ethylene glycol)-linked IFN (PEG-IFN), the obtained serum IFN levels were considerably lower than that obtained after administration of IFN SDMP[9]. The mean peak serum PEG-IFN level, C_{max}, obtained after administration of PEG-IFN SDMP to rat skin was 1.54 ± 0.43 IU/mL, though mean C_{max} of IFN from IFM SDMP was 8.2 ± 0.5 IU/mL. The molecular weight of PEG-IFN is 60 kDa, which is far larger than IFN (19.3 kDa). Therefore, a hypothesis arises, that is, the bioavailability (BA) of macromolecular drugs administered by SDMP decreases as the molecular weight increases. To clarify this hypothesis, several fluorescein isothiocyanate (FITC)-labeled dextrans (FDs) having a molecular weight of 10, 20, 40, and 70 kDa were used as a model drug of macromolecules and their absorption from SDMP have been studied in rats.

Materials and methods

Materials

FITC-labeled FDs, molecular weight = 10, 20, 40, and 70 kDa) were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Sodium chondroitin sulfate was obtained from Nacalai Tesque Co. Ltd. (Kyoto, Japan). Hiviswako 103 was obtained from Wako Pure Chemicals (Osaka, Japan). L-Lysine-L-glutamic acid was obtained from Ajinomoto Co. Ltd. (Tokyo, Japan). Male Wistar Hannover/Rcc rats were obtained from Nippon SLC Co. Ltd. (Hamamatu, Japan). All other materials used were of reagent grade and were used as received.

Preparations of FD SDMP, FD cream, and FD solution

After 150 mg of each FD was dissolved with 140 μL of distilled water, 115.4 mg of sodium chondroitin sulfate and 34.6 mg of L-lysine-L-glutamic acid were added and glue was obtained by mixing well, as previously reported[9]. The glue was centrifuged at 8060 × *g* for 5 minutes and was introduced into a 2.0 mL syringe having 22G needle whose length was shortened to be about 5.0 mm. The output was received on a Teflon plate and SDMPs were made in series by a cut at a right angle and a cut on the cross alternately. The plate was kept in an incubator at 50°C for 1 day and test SDMP preparations were obtained as shown in Figure 1. The mean diameter, mean length, and weight were 0.51 ± 0.02 mm, 6.10 ± 0.03 mm, and 3.53 ± 0.68 mg, respectively. One micropile was administered to one rat. FD cream was prepared by adding 1.0 mg of each FD and 0.5 mL of phosphate-buffered saline (PBS), pH 6.0, to Hiviswako 103 solution, which was dissolved with 50.0 mg of Hiviswako 10.3 and 0.5 mL of distilled water. After centrifuging at 12,000 rpm for 5 minutes, resultant cream was used for skin permeation experiment. FD solution for i.v. injection study was prepared by dissolving 3.0 mg of each FD with 1.0 mL of PBS (pH 6.0)[13].

In vitro *release study*

In vitro release studies were carried out with FD SDMP loaded in 1.0 mg of FD. FD SDMP was kept in 15 mL of

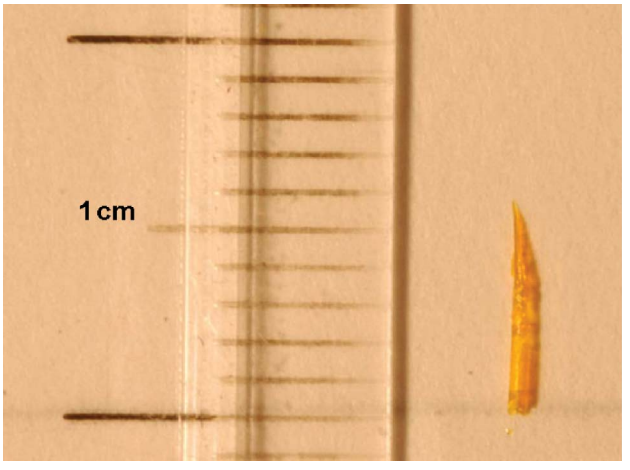

Figure 1. Microscopic photograph of test SDMP preparation containing FD.

PBS, pH 6.0, and was incubated at 37°C in a shaking water bath with a shaking speed of 36 rpm. The dissolution medium was degassed by sonication at room temperature and maintained at 37°C throughout the test period. To determine the amount of drug released from the test preparations, 0.3 mL of the dissolution medium was removed for analysis at the predetermined time and replaced with fresh dissolution medium. The FD content in the withdrawn medium was measured spectrofluorometrically, where excitation and emission wave lengths were 495 and 515 nm, respectively. The cumulative amount of FD released from SDMP is defined by the following equation:

$$\text{Cumulative amount released} = \left(\sum_{t=0}^{t} M_t / M_{\text{actual}} \right) \times 100\%,$$

where M_t is the amount of FD released at time t and M_{actual} is the actual amount of FD loaded in the micropiles as described above. $T_{50\%}$, the time when half of the total amount of FD was released from the SDMP, was determined by an interpolation method.

In vitro *skin permeation experiment*

Male Wistar Hannover/Rcc rats, 300–340 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 mg/kg, and were fixed in the supine body position on an operation plate. After the hair of the abdominal skin was removed and shaved, the stratum corneum was removed by stripping 10 times with a tape[14]. Thereafter, stripped skin specimens (3.0×3.0 cm^2 each) were freshly excised from the abdominal region of the rats and mounted on the Franz-type diffusion cell (diameter of the cell was 1.3 cm.) kept at 37°C[15]. For the skin permeation experiments of FD, 100 μL of FD cream that includes 1.0 mg of FD was applied onto the skin surface. The receptor compartment of diffusion cell was filled with 17 mL of isotonic phosphate buffer, pH 6.0[15].

Permeation experiment was performed at 37°C. After the start of the experiment, samples, 0.3 mL, were taken from the receptor compartment at 0.5, 1, 2, 3, 4, 5, and 6 hours were immediately replaced by the same volume of the buffer. The obtained samples were immediately frozen in a deep freezer at −80°C until assay.

Histological examination

Male Wistar Hannover/Rcc rats, 300–340 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 mg/kg. After the hair of the abdominal skin was removed, the photograph of the skin was recorded by a digital camera, Nikon D-100. One-test FD SDMP was inserted to the rat skin. Thereafter, photographs were recorded at 0.5, 1, 2, 4, 6, and 24 hours.

In vivo *absorption experiments*

Male Wistar Hannover/Rcc rats, 300–390 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital, 50 mg/kg, and the hair of the abdominal skin was removed. One group was composed of three to five rats. At 5 minutes before administration, blank blood sample, 250 μL, was obtained from the left jugular vein. One-test FD SDMP was percutaneously inserted into the skin (5.0 mg/kg), that is, the FD SDMP was administered into epidermis. When the FD SDMP was administered, the site of administration was not the dermis but the epidermis after confirming that there was no hemorrhage. Because, though the dermal vasculature is in the dermis, it is not contained in the epidermis. At 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, and 24 hours after the administration, blood samples, 250 μL, were obtained from the left jugular vein into a heparinized syringe. After centrifuging at 12,000 rpm for 15 minutes, plasma samples were obtained. For i.v. injection study (1.0 mg/kg), FD solution was prepared by dissolving FD solution, 1.0 mg/mL, in PBS (pH 7.4) and was injected into the right jugular vein of another group of rats after blank blood sample, 250 μL, was obtained from the left jugular vein. Thereafter, blood samples, 250 μL, were also obtained at 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, and 240 minutes. By centrifuging the blood sample at 12,000 rpm for 15 minutes, 100 μL of the plasma samples was obtained. All the plasma samples were immediately frozen in a deep freezer at −80°C until analysis. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation, Kyoto Pharmaceutical University.

Analytical procedures

For the in vitro release study and in vitro skin permeation experiment, each sample was diluted with 0.9 mL of 0.1 N NaOH. In the case of the FD content in the plasma sample was diluted with 1.0 mL of 0.1 N NaOH solution. The FD content in the sample was measured spectrofluorometrically, where excitation and emission wave lengths were 495 and 515 nm, respectively. The calibration was linear over 0–50.0 μg/mL.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the plasma FD concentration–time data using WinHARMONY software developed by us[16]. The time when plasma FD concentration reaches its maximum concentration, $T_{\max}$,

and the peak plasma FD concentration, C_{max}, was determined from the plasma FD concentration versus time data. The area under the plasma FD concentration versus time curve (AUC) was calculated using the linear trapezoidal rule up to the last measured plasma FD concentration. The absolute BA was calculated from the AUC_{SDMP} and $AUC_{i.v.}$ by the following equation:

$$\%BA = (AUC_{SDMP} / AUC_{i.v.}) \times (Dose_{i.v.} / Dose_{SDMP}) \times 100.$$

Statistics

All values are expressed as their mean ± SE. Statistical differences were assumed to be significant when $P < 0.05$ (one-sided *t*-test).

Results and discussions

The results of the in vitro release study of FDs from the test FD SDMP are shown in Figure 2. The FDs from SDMP were immediately released and the mean $T_{50\%}$ values were 1.5 ± 0.1 minute for FD-10, 1.5 ± 0.3 minutes for FD-20, 2.4 ± 0.6 minutes for FD-40, and 2.4 ± 0.2 minutes for FD-70, respectively. In all cases, FDs were completely released from FD SDMP within 5 minutes. Although a threshold appears in this figure on the release rate of FDs from SDMPs between FD-20 and FD-40, there were no significant differences between the release rates of FD from four SDMPs.

To study the absorption characteristics of FDs from the four test SDMPs, test preparations were inserted into the rat skin and plasma FD concentration versus time profiles were obtained. Figure 3 shows the results.

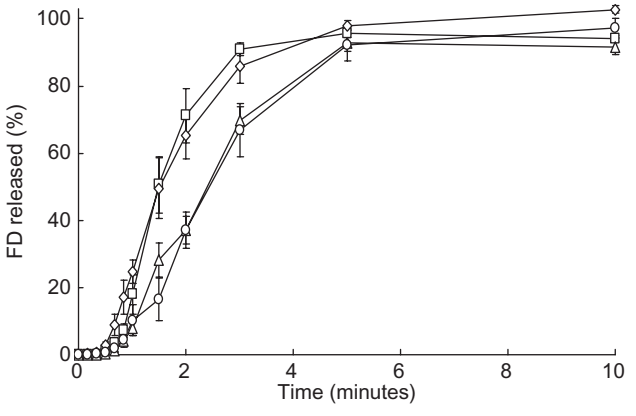

Figure 2. In vitro release profiles of FD from SDMP. □, FD-10; ◇, FD-20; △, FD-40; ○, FD-70. Each point shows the mean ± SE of three or four experiments.

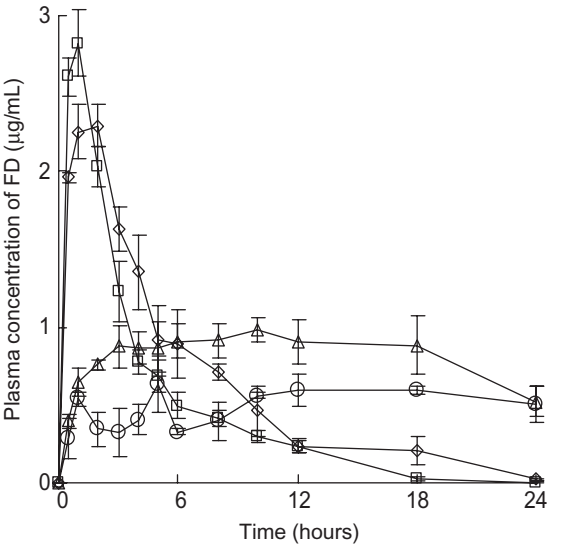

Figure 3. Effect of molecular weight of FD on the plasma FD level versus time profiles after percutaneous administration of SDMP to rats, 5.0 mg/kg □, FD-10; ◇, FD-20; △, FD-40; ○, FD-70. Each point shows the mean ± SE of three or four rats.

The profiles showed two patterns, that is, fast absorption rate and slow absorption rate. FD-10 and FD-20 showed high peak plasma levels and rapid disappearance from the systemic circulation. On the other hand, FD-40 and FD-70 were slowly absorbed into the systemic circulation and gradually disappeared from the plasma. Therefore, low plasma FD levels were maintained for long period, about 15 hours. Pharmacokinetic analysis was performed with those data and the calculated pharmacokinetic parameter values are shown in Table 1. The C_{max} of FD-10 and FD-20 were 2.82 ± 0.21 and 2.29 ± 0.14 μg/mL, respectively. FD-40 and FD-70 had C_{max} of 0.98 ± 0.08 and 0.64 ± 0.18 μg/mL, respectively. AUC values were 11.42 ± 0.80 for FD-10, 14.81 ± 1.18 for FD-20, 19.67 ± 2.06 for FD-40, and 12.12 ± 0.87 μg·h/mL for FD-70, respectively. To determine the absolute BA of FDs, FDs were i.v. injected into another group of rats, 1.0 mg/kg. Figure 4 shows the plasma FD levels versus time profiles. FD-10 disappeared rapidly from the systemic circulation. By comparing the AUC values, mean BAs were calculated to be 99.4 ± 6.9 for FD-10, 88.3 ± 7.1 for FD-20, 45.7 ± 4.8 for FD-40, and 16.0 ± 1.2% for FD-70, as shown in Table 1. Figure 5 shows the relation between BA and the molecular weight of FDs. Good reverse linear relation was obtained ($r^2 = 0.9719$). Thus, as the molecular weight of FD increased, the BA of FD from SDMP decreased. This result suggested that the barrier function of the epidermis was an important factor affected on BA. As FD-70 has large molecular weight, it was not easy to permeate the epidermis. Therefore, the low BA value was obtained from FD-70 SDMP.

Table 1. Pharmacokinetic parameters of FD after percutaneous administrations of SDMN to rats.

	i. v. solution		SDMP		
Test preparation	AUC_{0-24} (μg•h/mL)	C_{max} (μg/mL)	T_{max} (h)	AUC_{0-24} (μg•h/mL)	BA (%)
FD-10	2.30 ± 0.25	2.82 ± 0.21	0.67 ± 0.16	11.42 ± 0.80	99.4 ± 6.93
FD-20	3.36 ± 0.08	2.29 ± 0.14	1.67 ± 0.33	14.81 ± 1.18	88.3 ± 7.05
FD-40	8.61 ± 0.86	0.98 ± 0.08	10.25 ± 2.78	19.67 ± 2.06	45.7 ± 4.77 [a, b]
FD-70	15.15 ± 1.45	0.64 ± 0.18	7.33 ± 2.33	12.12 ± 0.87	16.0 ± 1.32 [a–c]

Each point shows the mean ± SE (n = 3–4).
[a] $P < 0.05$ compared to FD-10.
[b] $P < 0.05$ compared to FD-20.
[c] $P < 0.05$ compared to FD-40.

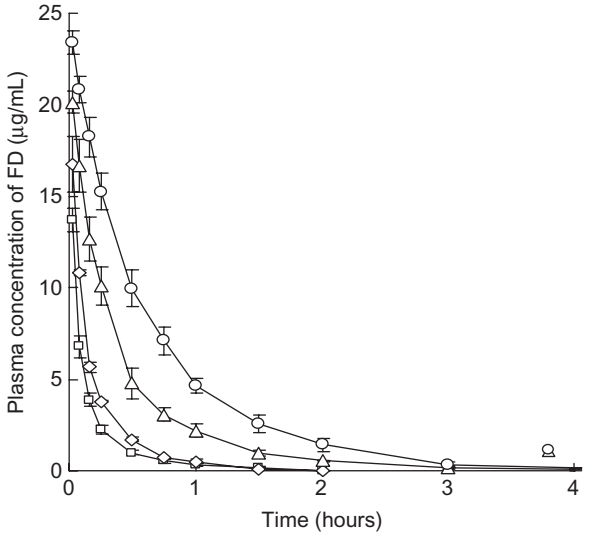

Figure 4. Plasma FD level versus time profiles following intravenous injection of FD solutions to rats, 1.0 mg/kg. □, FD-10; ◇, FD-20; △, FD-40; ○, FD-70. Each point shows the mean ± SE of three or four rats.

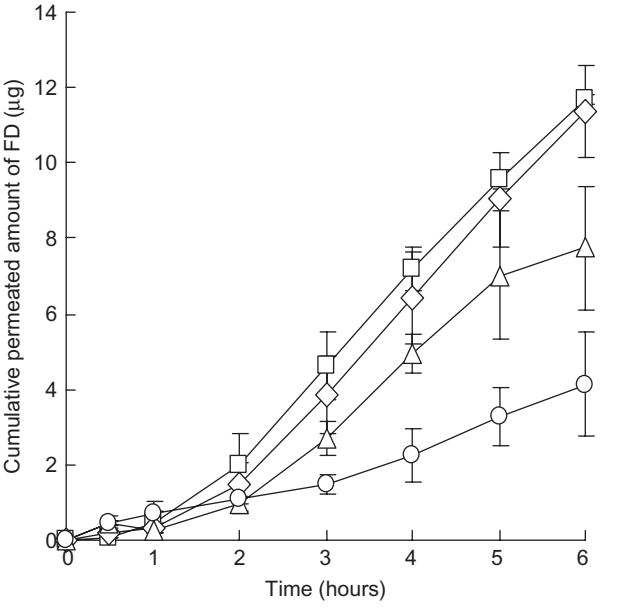

Figure 6. Permeation profiles of FDs from SDMP. □, FD-10; ◇, FD-20; △, FD-40; ○, FD-70. Each point represents the mean ± SE of three or four experiments.

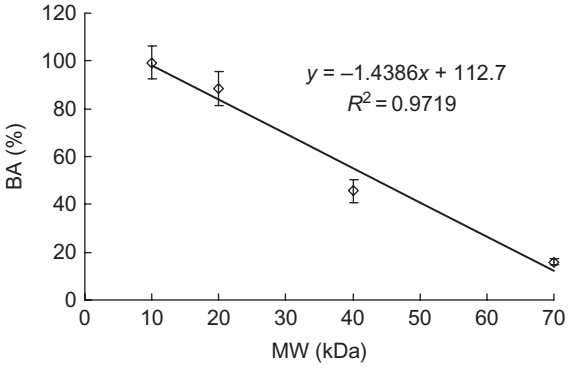

Figure 5. Relationship between molecular weight of FDs and bioavailability.

In the case of SDMP that is inserted into the epidermal junction, the first barrier, stratum corneum, is out of consideration. Therefore, to study the contribution of the second and third barriers, permeation experiment was performed after the stratum corneum was removed with tape stripping method and the results are shown in Figure 6. Although the results are not shown in the figure, all the FDs did not permeate through the intact skin. As shown in this figure, the permeated amount of FD through the stripped skin decreased as the molecular weight of FD increased. As the molecular weight of FD increased, both the diffusion rate through the epidermis and the permeation rate through the microcapillary wall were thought to decrease. As a result, BAs of FD-40 and FD-70 were thought to decrease.

To study the diffusion of FDs through the epidermis, histological experiment was performed in the in vivo experiment and the results are shown in Figure 7. When FD-10 and FD-20 SDMP were inserted into the rat skin, diffusion started at 0.5 hour and almost completed within 6 hours. However, in the case of FD-40, yellow

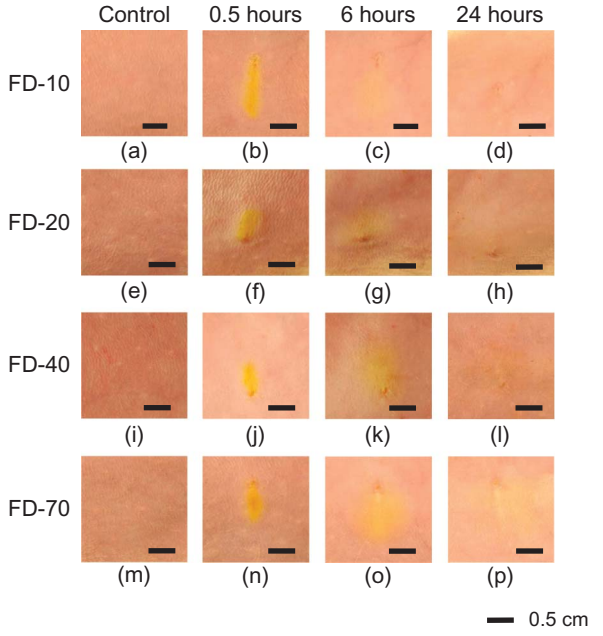

Figure 7. Photographs of rat skin after administration of FD SDMPs. (a–d) FD-10, (e–h) FD-20, (i–l) FD-40, (m–p) FD-70.

color because of FD-40 was still observed at the administered rat skin at 6 hours after administration. In addition, in the case of FD-70, yellow color was observed at 24 hours after administration. Thus, the BA of FD from SDMP was dependent on the molecular weight of FD administered to the rat skin.

We previously reported the efficiency of SDMP for the percutaneous delivery of IFN that had a molecular weight of 20 kDa at the IFN dose of 5000 IU/kg, where the mean C_{max} was 8.2 ± 0.5 IU/mL, mean T_{max} was 0.27 ± 0.12 hours, and AUC was 19.42 ± 4.6 IU • h/mL, and high BA, 378.32 %, was obtained[10]. On the other hand, when-PEG-IFN having a molecular weight of 60 kDa was formulated into SDMP and was administered to the rat skin at PEG-IFN dose of 8400 IU/kg, the mean C_{max} was 1.54 ± 0.43 IU/mL, T_{max} was 2.33 ± 0.88 hours, and AUC was 2.97 ± 1.01 IU • h/mL. The dependence of molecular weight on BA from SDMP also explains the reason why low plasma IFN levels were observed after the administration of PEG-IFN SDMP to rat skin. Wu et al.[17,18] reported the permeability of FD-4, 10, 40, and 70 after microneedle puncture depended on the molecular weight and could be predicted by a model function of the number of pores in the skin barrier. And the permeation was fairly dependent on the pre-treated area of stratum corneum-stripped skin. Unfortunately, a small diffusion area can be produced in the stratum corneum barrier by needles. Although they evaluated the permeability only in the in vitro experiment, these were inconsistent with our results, that is, the permeability of FD by microneedle correlated with the molecular weight. Moreover, Verbaan et al.[19] performed a feasibility study to use microneedle arrays manufactured from commercially available 30G hypodermal needles, and absorption enhancement of macromolecules was shown up to the molecular weight of 72 kDa FD. The BA of FD-40 decreased as compared to FD-10 and FD-20 though the BA value was still high, 45.7%. Therefore, we may state that absorption enhancing effect of macromolecules can be obtained up to 40 kDa of FD. The discrepancy on the cutoff of the molecular weight between Verbann et al. and us is ascribed to the difference of the experimental method. Verbann et al. used in vitro permeation experiment and we used in vivo rat absorption experiment. However, both experimental results were in agreement with the decrease of permeated amount of 70 kDa. The barrier function would be a large factor in the permeation through the epidermis. To increase the permeated amount of macromolecular drugs, another factor for depression of the barrier function would be needed. FDs belong to polysaccharide. As polysaccharide drug, few drugs including low-molecular-weight heparin and heparin are clinically used. Most of the macromolecular drugs used clinically are protein drugs. Moreover, in the case of proteins, their three-dimensional structures are important factors for their permeation through the biological membrane. Therefore, further studies are needed using protein drugs to precisely show the effect of molecular weight for the percutaneous absorption enhancement of SDMP.

Conclusion

To clarify whether there is a threshold on the absorption of macromolecules through the skin after administration by SDMP, four kinds of SDMPs were prepared where FDs having different molecular weights, 10, 20, 40, and 70 kDa, were formulated. After administration of FD SDMP to rat skin, plasma FD levels were measured. As the molecular weight of FD increased from 10 to 70 kDa, BA decreased from 99.4% to 16.0%. The dependence of molecular weight on BA from FD SDMP was clearly demonstrated. On the other hand, the FDs were released from SDMP immediately and did not show significant differences. In vivo diffusion experiment suggested the difference of the release rate between FD SDMPs. In the in vitro permeation experiment using stripped skin, the permeated amounts of FDs showed the dependence on the molecular weight. The difference of the BA between FDs is ascribed both to the diffusion rate in the epidermis and to the permeation rate through the microcapillary wall existing in the dermis. These observations supported our hypothesis, that is, the BA of macromolecular drugs administered by SDMP decreases as the molecular weight increases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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