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Research paper

Keratin film made of human hair as a nail plate model for studying drug permeation

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ABSTRACT

The limited source of human nail plate for studying drug permeation inspired us to develop a nail plate model made of human hair keratin. The manufacturing process consisted of keratin extraction, dialysis, molding, solvent evaporation, and curing, producing a water-resistant film. The permeability of the film was examined using three markers: sodium fluorescein, rhodamine B, and fluorescein isothiocyanate–dextran as water-soluble, lipid-soluble, and large molecule models, respectively. Bovine hoof was used for comparison. First investigation showed that keratin films (thickness 120 μm) resembled hooves (thickness 100 μm) except that these films were more permeable to rhodamine B compared with hooves (1.8-fold, $p < 0.01$). Subsequent investigations using ungual penetration enhancers (urea, thioglycolic acid, and papain) showed that keratin films were generally more susceptible than hooves. This study revealed that the produced keratin film offers a possibility as a human nail plate substitute. However, inclusion of the penetration enhancer must be carefully interpreted.

1. Introduction

Nail diseases, especially onychomycosis, which contributes to as much as 50% of nail disorders [1–3], should be efficiently treated to improve a patient's quality of life. Unlike skin diseases, for which medical treatments are continuously developed, treatments of nail diseases hitherto are not satisfying. The main obstacles are our limited knowledge of conquering the perfect barrier of the human nail and the limited source of human nails as the object of the study itself. Bovine hoof has been accepted until now as a substitute for human nail, but significant differences between them have been reported [4]. The limitation of animal hoof as a study object is its great water uptake during hydration, which could lead to the overestimation of drug permeability, when this is translated to human nail. Khengar et al. [4] reported that the water uptake by human nail was 27 ± 3%, whereas that of horse hoof was 40 ± 9%. Nail clippings have been used for permeation studies as well, but they are not the best model due to the limited nail bed [5] and the available contact surface with formulation.

Both human nails and animal hooves are composed of the same keratin type (i.e., α-keratin). Their main differences include the ratios of the amino acid components and their inner structures, with the latter due to their specific differentiation pathways *in vivo* [6]. However, both human nail and animal hoof possess α hair-type keratin which is well known for its high-sulfur protein content [7].

Keratin is not a single substance; in fact, it is composed of a complex mixture of proteins. Keratin is insoluble in many common solvents, such as dilute acids, alkalines, water, and organic solvents [8]. A common method to extract keratin involves the use of reducing agents because the native form is hard to obtain, due to its highly cross-linked state by disulfide bonds. The use of the Shindai method, which omits the use of detergent, provided a sufficient amount of extracted proteins for analysis and can avoid protein hydrolysis [9]. Reducing agents such as dithiothreitol and 2-mercaptoethanol work by cleaving keratin's disulfide bonds and thus increasing its solubility [8].

Mertin and Lippold [10] investigated the influence of drug lipophilicities and solubilities in water on their permeabilities across human nail and hooves. They found that drug permeability across both membranes was not dependent on its lipophilicity. Both materials were found to behave as a hydrophilic gel membrane rather than a lipophilic partition membrane, as in the case of human skin [10–12]. The mechanism of drug permeation across hoof resembled that in human nail plate, except that hoof retained more water. This was confirmed by a study with serial nicotinic acid esters that permeated across human nail and bovine hooves. Hoof permeabilities were found to be 10- to 30-fold greater than those across the human nail plate [10]. Nail hydration plays an important role in the ungual permeation by providing more spaces for the permeating substance. This has been observed by Walters et al. [11,13] as well, where the permeation of homologous alcohols was higher from aqueous solution than that from the neat liquids (undiluted).

Human hair is abundant in nature, and its keratin type is present in human nail [6,8,14–16]. Baden et al. [15] studied human

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keratinized tissues (stratum corneum, hair, and nail) and found that hair and nail showed many resemblances in their physical and chemical properties, despite their different morphological properties. Our question was whether the extraction of keratin from hair with the Shindai method and its re-assembly into a film would enable its use as a nail plate model. This was one purpose of this study. The properties of this nail model were examined by the permeation of several markers and the application of ungual penetration enhancers. Because hoof and nail showed many similarities, bovine hoof was chosen for comparison. Moreover, bovine hoof is available in the market and can be obtained for screening purposes in great quantities, whereas the availability of human nail is limited and costly. Since the physical properties of bovine hoof as an accepted model for human nail are well known, bovine hoof was utilized as a comparison for the keratin film in the numerous permeation experiments.

Three markers were chosen to represent drugs with different physicochemical properties. Sodium fluorescein (MW 376, log *P* −1.52), rhodamine B (MW 443, log *P* 2.38) [17], and fluorescein isothiocyanate–dextran (MW 4400, log *P* −2.0) [18] were chosen as water-soluble, lipid-soluble, and large molecule model drugs, respectively. The slight differences in size between sodium fluorescein and rhodamine B were not taken as considerable.

Three acknowledged nail penetration enhancers with different mechanisms were chosen, i.e., urea, thioglycolic acid, and papain from papaya latex to provide more information about the usefulness of keratin film made of human hair as a human nail plate substitute. Urea works by increasing nail hydration [19], thioglycolic acid by cleaving the disulfide bonds between keratin molecules [20,21], and papain, an endopeptidase enzyme that contains a highly reactive sulfhydryl group, by hydrolyzing these bonds, thus promoting the formation of pores between nail corneocytes [22,23].

2. Experiments

2.1. Materials

Blond hairs were obtained from a local hairdresser, bovine hooves were purchased from an online pet shop (Edingershops, Germany), and healthy nail clippings were donated from volunteers in the university surroundings. Shindai solution was prepared from urea, thiourea (Carl Roth GmbH, Karlsruhe, Germany), 2-mercaptoethanol, and Tris base (Sigma, USA) according to a previous method [9]. A Spectra/Por membrane (MWCO: 6–8000 Da Spectrum Laboratories, Inc. Rancho Dominiguez, Canada) was used as dialysis tubing. Sodium fluorescein (SF) was purchased from Fluka (Steinheim, Germany), rhodamine B (RB) from Fluka (Sweden), thioglycolic acid (TA) from Merck (Hohenbrunn, Germany), fluorescein isothiocyanate–dextran MW 4000 (FD4) and papain (from papaya latex) from Sigma–Aldrich (Steinheim, Germany). Phosphate-buffered saline pH 7.4 (PBS) was prepared according to Eur. Ph. 6.0. Disodium hydrogen phosphate anhydrous and sodium chloride were obtained from Merck (Darmstadt, Germany) and potassium dihydrogen phosphate was from Carl Roth GmbH (Karlsruhe, Germany). Sodium hydroxide (NaOH) was supplied from BASF (Darmstadt, Germany). Water for the permeation study was used in double-distilled quality. Acrylamide (30% solution), N,N,N′,N′-tetramethylethylenediamine, and ammonium persulfate were obtained from Sigma–Aldrich, sodium dodecyl sulfate from Acros Organics (Geel, Belgium), glycine from ICN Biomedicals, Inc. (Aurora, Ohio, USA), and Serva Blue G (dye based on Coomassie® brilliant blue G-250) from Serva Electrophoresis GmbH (Heidelberg, Germany). Spectra™ multicolor broad range protein ladder #SM 1841 (10–260 kDa) from Fermentas GmbH (St. Leon-Rot, Germany) was used as the size marker for electrophoresis.

2.2. Keratin film (KF) manufacture

Keratin from hair was extracted under reducing conditions with the so-called Shindai method [9,24]. The hair was pulverized using a ball mill MM301 Retsch (Haan, Germany) at 29 Hz for 10 min. An amount of 25 g of hair powder was mixed with 500 ml Shindai solution containing 25 mM Tris pH 8.5, 2.6 M thiourea, 5 M urea, and 5% (v/v) 2-mercaptoethanol and was extracted at 50 °C for 72 h. The mixture was filtered with medical gauze and further with filter paper with a 2.5-μm pore size to remove the insoluble hair. The extract was centrifuged at 4500*g* for 15 min; the supernatant could be stored at −20 °C and thawed, if required. Dialysis against demineralized water was conducted to remove the rest of the Shindai components using Spectrapore® tubing, MWCO 6000–8000 Da, for 48 h at ambient temperature. The dialysate was further centrifuged at 10,000*g* for 30 min to remove coarse aggregates and immediately used for KF manufacture. The dialysate's protein content was determined using the Bradford colorimetric method [25] using bovine serum albumin as the standard. Prior to film manufacturing, 1% (w/w) glycerol was added to the dialysate as film plasticizer. Rings as molding were prepared in our studio; they were made of polytetrafluoroethylene (PTFE/Teflon) with 2 cm inner diameter. A siliconized polyethylene terepthalate (PET) foil from LTS Lohmann (Andernach, Germany) was used as base. A volume of 2 ml from this mixture was optimum to produce about 100-μm-thick KFs. This volume was added stepwise (2 × 1 ml within 4 h) and dried at 40 °C for 24 h. The produced soft films were punched out (diameter 1.5 cm) before curing. Curing at 110 °C for 3 h was essential to oxidize the disulfide bridges between keratin molecules, producing stable, water-insoluble films. The thicknesses of the prepared KF and hoof membrane were measured with a micrometer screw gauge after a complete hydration in PBS for 1 h.

2.3. Bovine hooves preparation

Only the sole part of the bovine hooves was used for this study. Soles were first cut in squares (2 cm × 2 cm) and submerged overnight in water before being sliced with a rotational microtome MICROM HM 355S (Walldorf, Germany) to a thickness of 100 μm. For the permeation study, these membranes were then punched out (15 mm diameter).

2.4. Water absorption profile from keratinous materials under study

All studied keratinous materials, i.e., hoof membranes, KFs, and human nail clippings, were submerged in double distilled water and repeatedly weighed until a constant weight. Prior to weighing, the water on the surface was wiped off with tissue paper. The amount of water absorbed was compared to the dry weight.

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli [26] to separate the protein components of the keratinous materials under study according to their molecular weights. The aqueous dialysate and the hair extract in the Shindai solution were previously diluted 5-fold with water. The other samples were previously extracted with the Shindai solution. Nail clippings (2.5% (w/w)) were extracted and agitated overnight, whereas KF and hoof (5% and 2.5% (w/w), respectively) were extracted for 72 h at 50 °C. All samples were finally diluted 2-fold with Laemmli buffer (Sigma, Deisenhofen, Germany) and boiled for 5 min before running. The

sample volume for the separation was 5 μl, except for hoof extract and marker, which was 7.5 and 10 μl, respectively. A vertical slab gel electrophoretic system (EC 120, 80 × 100 mm, EC Apparatus Corp., Holbrook, US) with a 4% stacking gel and 12% separation gel was used. The system was run at 180 V, 80 mA, and 25 W. After running, the gel was stained with 0.1% (w/v) Coomassie brilliant blue in a mixture of methanol: acetic acid: water (4:1:10) and further destained with this acidic methanol solution for 30 min.

2.6. Permeation studies

2.6.1. Finding the thickness analogue of KF

The thickness analogue of KF, in terms of permeability and marker retaining ability, was compared with 100-μm-thick bovine hoof. KFs with thicknesses of 110, 120, and 130 μm were examined. Permeation experiments were carried out using modified Franz diffusion cells [27] at 32 °C. The cell opening diameters were between 0.418 and 0.534 cm^2, and the volumes of the receiver compartments varied between 5.18 and 6.37 ml. The diffusion cells were calibrated in terms of volumes of the receiver compartments and the measurement of the cell opening diameters.

Bovine hoof membranes and KF were equilibrated for 1 h with PBS and were then mounted between donor and receiver compartments. The receiver compartments were filled with PBS, whereas the donor compartments were filled with marker solutions in PBS with the concentration of 500 μg/ml for SF, 250 μg/ml for RB, and 1000 μg/ml for FD4 (all w/v). Samples of 100 μl were taken from the receiver for 40 h and replaced by the same amount of fresh buffer. The first sample was taken at 10 h; afterwards, 3- to 4-h intervals were employed over the remaining time. The samples were placed in a 96-well plate from Corning (Roskilde, Denmark), and the fluorescence intensities were directly measured after terminating the permeation using a fluorescence plate reader, Tecan Genios (Switzerland), with λ excitation at 485 nm, λ emission at 535 nm (filters) for SF and FD4 and λ excitation at 535 nm, λ emission at 590 nm for RB. Calibrations for the markers were made in PBS within the range of 0.1–5 μg/ml for SF and RB and 1–10 μg/ml for FD4 with r^2 more than 0.999. The permeability coefficient P_{app} (cm/s) of each marker was calculated according to:

$$P_{app} = dQ/(dt\ C_0\ A) \quad (1)$$

where dQ/dt is the flux of the marker, C_0 is the initial donor concentration (g/cm^3), and A is the cell area (cm^2). Flux values were obtained from the slope of a plot of the permeated amounts (g/cm^2) versus time (s).

2.6.2. The application of ungual penetration enhancer (PE)

PEs were not mixed into the marker solutions to avoid any interaction between the PE and the marker. After assembling the diffusion cells, the receivers and the donors were filled with PBS and PE solution, respectively. All PEs were dissolved in water, except for papain which was dissolved in PBS (pH 7.4 is the optimum papain pH), with the concentration of 40% for urea, 5% for TA, and 2% for papain (all w/w). The pH values of the PE solutions were 9.23, 1.63, and 2.93 for urea, TA, and TA/urea combination, respectively. The treatment lengths were 3 days for urea and 15 h for TA and papain. Combinations (15 h) and serial treatments (3 days urea + 15 h TA) were examined as well. The treated KF or hoof was kept at ambient temperature during treatment, except for papain, which was equilibrated in a water bath at 32 °C. Both compartments were rinsed twice with double-distilled water and PBS after the treatment. The receivers were ultimately filled with fresh PBS and the donor with the marker solution. Samples of 100 μl were taken from the receiver for 7–40 h (depending on the treatment) and replaced by the same volume of fresh buffer. Sampling time was adjusted for every PE. The use of TA, its mixture, and serial application allowed for a sampling time of only up to 7 h before the KF was damaged. Therefore, the sampling was performed hourly for all markers in the presence of TA. This was also the case for papain and urea, except for FD4 the sampling interval was employed as in Section 2.6.1. The fluorescence intensities of the samples were measured using a fluorescence plate reader with the same parameters as in Section 2.6.1.

2.7. Marker extraction from membranes

Hooves and KFs (without PEs) were isolated directly after terminating the permeation. The membranes were rinsed with double-distilled water twice to remove the rest of the donor solution. The residual humidity was absorbed with tissue paper, and the membranes were finally placed in reaction vessels of 1.5 ml volume (Roth, Karlsruhe, Germany). These vessels were stored without lids in a desiccator for 48 h. The dried membranes were extracted using 1 ml aqueous solution of 2% (w/v) NaOH. These membranes were agitated overnight (3 days for RB) with an orbital shaker IKA Vibrax-VXR (Staufen, Germany) at 150 rpm. The fluorescence intensities were measured afterward, as in Section 2.6.1. Calibration curves were obtained in 2% NaOH aqueous solution within the same concentration ranges as in the permeation study with r^2 values greater than 0.999.

2.8. Determination of the extraction recovery in hooves and KFs

Hoof of 100 μm thickness and KF of 120 μm thickness (each $n = 6$) were given about 10-μl drop of a defined concentration of marker solution ($c_{standard}$) in PBS and then weighed. The marker concentration was similar as in Section 2.6.1. Blanks for both membranes were dropped with buffer only (PBS). Afterward, drying and extraction process were carried out as in Section 2.7. The recovery of the extraction process was calculated as follows:

$$\%\ \text{Recovery} = \frac{\text{Detected concentration } (\mu g/ml)}{\text{Actual concentration } (\mu g/ml)} \times\ 100\% \quad (2)$$

The detected concentration was obtained through the fluorescence measurement; the actual concentration was calculated as follows:

$$\text{Actual conc. } (\mu g/ml) = \frac{\text{Weight of the solution } (g) \times c_{standard}\ (g/ml) \times 10^6}{\text{Density of the solution } (g/ml) \times 1\ (ml)} \quad (3)$$

The amount of accumulated marker in the membrane from Section 2.7 was adjusted according to the recovery result of the individual marker.

2.9. Statistical data analysis

Statistical analysis was performed with the software program SPSS® v. 17.0. The Shapiro–Wilk test was applied to assess the normality of the data. The test showed that the resulting permeation data were normally distributed. On this basis, *F*-test was performed to examine the variances between the groups, continued with the Student's *t*-test (two tails, $\alpha = 0.05$) for testing the significance of the difference between the means. The *p*-values of less than 0.05 and 0.01 were considered as significant and highly significant differences, respectively.

3. Results

3.1. Keratin film manufacture

The lyophilization of the dialysate yielded ~60% of the dry hair weight used in the keratin extraction procedure (Section 2.2). This was in accordance with data reported by Nakamura et al. [9], who found more than 65% with the same method. However, this method was time-consuming in terms of determining the extraction efficiency. Therefore, the Bradford method, a quicker method, was further performed to determine the protein content of the keratin dialysate using bovine serum albumin as standard. The protein content of the keratin dialysate was found to be 13 ± 3.7 mg/ml. This refers to approximately 26% from initial hair weight being distinctly lower than what had been detected with the lyophilization method (~60%). However, the Bradford method is easier to perform and sufficiently reliable to monitor the recovery of the extraction process.

With 2 mL of keratin dialysate mixture, different KF thicknesses of 90–120 μm were obtained. This range remained the same for different batches. However, as expected, this range would decrease when the protein content in the keratin dialysate was lower and also vice versa. Variation in KF with similar thicknesses can be seen from the standard deviation of the marker P_{app} in the permeation study (without PE) in Table 1.

After evaporating the solvent from the keratin solution, a clear, translucent, and brownish film was achieved. The subsequent curing process gave this film a water-resistant property as well as mechanical stability through the oxidation of disulfide bonds between keratin molecules. The intermediate product, finished keratin films, and bovine hooves membranes for the permeation study are displayed in Fig. 1.

3.2. Water absorption profile from keratinous materials

The water absorption profiles of all materials under study can be seen in Fig. 2. The water uptake of hoof was the greatest, by about 45%, followed by nail and KF, around 30% and 5%, respectively (referred to the initial dry weight).

3.3. SDS–PAGE

The protein content of KF, its raw, and intermediate materials were separated using SDS–PAGE, and the respective protein bands were compared to those of human nail and bovine hoof extracts. The gel matrix after the separation is depicted in Fig. 3. All human keratin materials from hair and nail showed similar band profiles. Major fragments at approximately 42 and 52 kDa were clearly observed in both hair and nail. Further bands from nail extract (b) were situated at approximately 11, 16 and 24, 95, and 135 kDa. The protein from hair extract (c) and its aqueous suspension after dialysis (a) showed similar major fragments, although the other bands were slightly diffuse. The finished KF (d) resembled the characteristic hair bands from hair extract in the same position. Curing altered the intensities of these protein bands (e) and

Table 1
P_{app} of the markers [$\times 10^{-7}$ cm/s].

Marker	Hoof 100 μm	KF 120 μm	KF/hoof ratio
SF	5.00 ± 1.35	5.85 ± 1.12	1.2
RB	17.68 ± 2.22	32.29 ± 3.94	1.8**
FD4	0.56 ± 0.15	0.41 ± 0.11	0.7

n = 4–12
** $p < 0.01$.

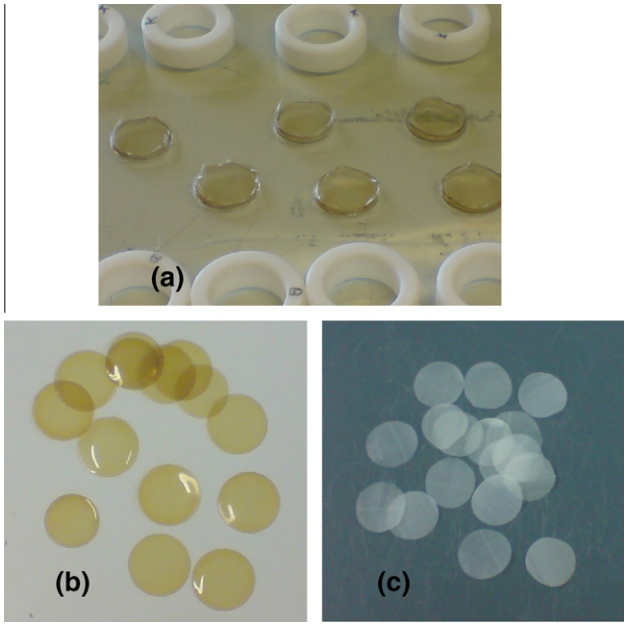

Fig. 1. (a) The intermediate products of keratin films before punching and curing; (b) Keratin films after curing; (c) Bovine hooves for the permeation study; membrane diameter = 15 mm; Teflon rings are shown in (a). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

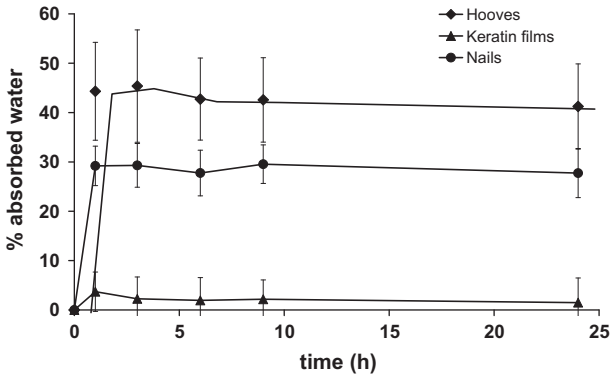

Fig. 2. Water absorption profile from hooves, keratin films, and human nail clippings – a comparison (n = 5).

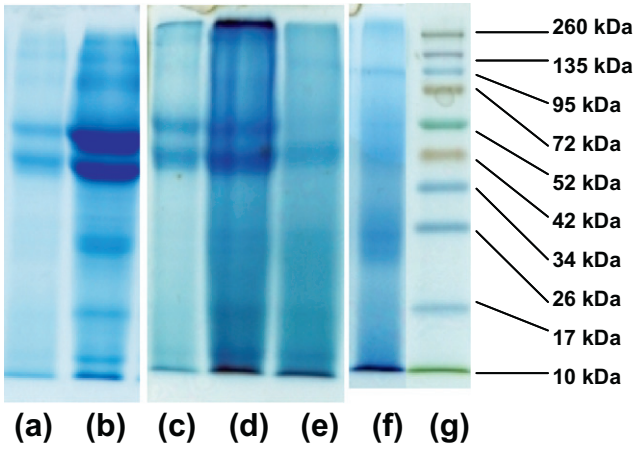

Fig. 3. SDS–PAGE of (a) aqueous keratin suspension after dialysis; (b) human nail extract; (c) hair extract; (d) and (e) keratin film before and after curing, respectively; (f) hoof extract; (g) molecular weight marker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

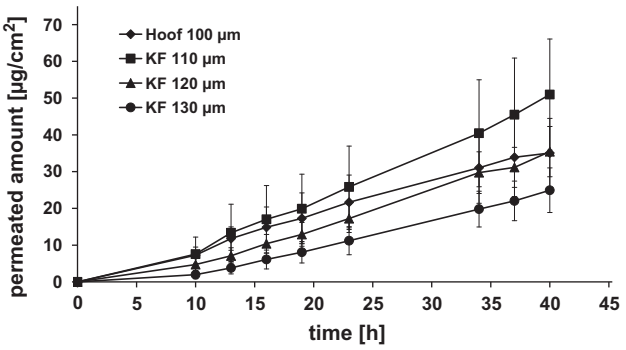

Fig. 4. Permeation of SF across different thicknesses of KFs (110–130 µm) and 100-µm-thick hooves (n = 4–6).

increased the intensity of diffuse bands in the lower molecular weight area (10–26 kDa), which hinted at fragment cleaving to smaller sizes. Hoof extract (f) showed a different profile to that of human hair and nail. Two diffuse major fragments were obvious at 20–26 and around 42 kDa. Another thin band appeared at 95 kDa.

3.4. Finding the thickness analogue of KF and marker accumulated amount in the membranes

In order to find the thickness analogue of KF versus bovine hoof, permeation studies were carried out with SF first. The permeation data showed that the KF of 120 µm thickness resembled hooves of 100 µm thickness, as can be seen in Fig. 4. The 120-µm-thick KF was further tested for the permeation of FD4 and RB and those permeability coefficients were then compared to the 100-µm-thick bovine hoof again. A similar profile was shown by FD4, but a significant discrepancy ($p < 0.01$) was obvious with RB (Table 1). Both bovine hooves and KFs revealed the same rank order of marker permeability coefficients (i.e., RB > SF > FD4).

The recoveries of the marker extraction process were between 63% and 100%. The actual accumulated amount for every marker can be seen in Table 1. A great affinity of keratin to the lipophilic molecules could also be seen from this table. Both membranes retained more RB than other hydrophilic markers. KF bound more RB than hoof (42% versus 26%, $p < 0.01$) as well as SF ($p < 0.05$). The accumulations of FD4 in hoof and KF were comparable and amounted to only 0.8% from the initial donor concentration.

The KF/hoof ratios, in terms of P_{app} (without PE treatment) and accumulated amount, for every individual marker were about in the same magnitude. The KF/hoof ratios were about 1.2 for SF, 1.6–1.8 for RB, and 0.7–0.98 for FD4 (see Tables 1 and 2).

3.5. The Application of ungual penetration enhancer (PE)

3.5.1. Influence of PEs on permeability coefficients of the markers

All permeability coefficients after treatment with PEs can be seen in Table 3. The permeability coefficients of the three markers were generally higher in KFs compared with hooves after treatment with PEs. Exceptions could, however, be seen in the case of SF after treatment with urea and RB after treatment with TA, where the P_{app} in hooves were higher than those in KFs.

The P_{app} of SF across hooves and KFs increased after being treated with all PEs. KF was more susceptible to PEs, especially after treatment with TA, as well as after PE combination and serial

Table 2
Percentage of the accumulated marker in the membranes after 20-h permeation study (40 h for RB).

Marker	Accumulated amount in hoof 100 µm (%)	Accumulated amount in KF 120 µm (%)	KF/hoof ratio
SF	1.52 ± 0.12	1.93 ± 0.16	1.27*
RB	25.97 ± 5.73	42.13 ± 5.14	1.62**
FD4	0.83 ± 0.06	0.82 ± 0.11	0.98

n = 3–6
* $p < 0.05$.
** $p < 0.01$.

Table 3
P_{app} of the markers after treatments with PEs [$\times 10^{-7}$ cm/s] and its ratios.

Marker	PE	P_{app} Hoof	P_{app} KF	Enhancement factor (versus no PE)		KF/ hoof ratio
				Hoof	KF	
SF	No PE	5.00 ± 1.35	5.85 ± 1.12	–	–	1.2
	Urea 3d	22.49 ± 8.50	9.67 ± 2.80	4.5*	1.7*	0.4
	TA 15 h	55.63 ± 8.26	88.41 ± 14.15	11.1**	15.1**	1.6**
	Combination urea/TA	53.62 ± 5.25	95.20 ± 7.51	10.7**	16.3**	1.8**
	Serial urea/TA	45.82 ± 5.77	83.09 ± 11.16	9.2**	14.2**	1.8**
	Papain 15 h	9.78 ± 4.09	20.80 ± 3.62	2.0	3.6**	2.1*
FD4	No PE	0.56 ± 0.15	0.41 ± 0.11	-	-	0.7
	Urea 3d	0.19 ± 0.08	0.51 ± 0.24	0.3**	1.2	2.7*
	TA 15 h	0.31 ± 0.04	5.20 ± 2.48	0.6**	12.7**	16.8**
	Combination urea/TA	2.19 ± 1.53	6.87 ± 2.22	3.9**	16.8**	3.1*
	Serial urea/TA	8.13 ± 5.19	11.60 ± 0.13	14.5**	28.3**	1.4
	Papain 15 h	1.62 ± 1.29	2.79 ± 1.19	2.9**	6.8*	1.7
RB	No PE	17.68 ± 2.22	32.29 ± 3.94	-	-	1.8**
	Urea 3d	9.62 ± 6.10	43.07 ± 11.26	0.5*	1.3*	4.5*
	TA 15 h	86.48 ± 28.92	68.32 ± 19.21	4.9**	2.1**	0.8
	Combination urea/TA	17.02 ± 11.95	93.55 ± 15.26	1.0	2.9**	5.5**
	Serial urea/TA	5.97 ± 2.11	119.10 ± 35.20	0.3**	3.7**	19.9**
	Papain 15 h	10.40 ± 5.83	59.90 ± 50.29	0.6*	1.9	5.8

n = 3–12.
* $p < 0.05$.
** $p < 0.01$.

treatment. The ratios of KF versus hoof permeation after the application of PEs to SF were at maximum 2.1-fold.

All PEs increased FD4 permeability across KF, although the urea impact was not significant. Interestingly, the single treatment of both TA and urea decreased FD4 permeability across hooves, but its combination and serial treatment enhanced it. Papain was able to increase FD4 permeation across hooves as well. The highest ratio of KF versus hoof permeability was observed by TA treatment being 16.8-fold.

RB permeability across hooves increased after being treated with TA, while the urea treatment, the serial treatment, and papain reduced it. KF was susceptible to all treatments, although for papain was not statistically significant. Remarkable differences between KF and hoof were observed with urea when its combination and serial treatment were applied (i.e., KF versus hoof ratios 4–19.9-fold).

From the preliminary experimental setup, papain was expected to be a potential PE because concentrations of 3% and 5% damaged both membranes within one hour (data not shown). Therefore, a papain concentration of 2% was chosen for the further permeation study. However, a penetration enhancement could not be shown for RB across hooves.

3.5.2. Comparison between markers

The application of PEs astonishingly did not always increase the permeability coefficients of tested markers. While the application of urea increased the P_{app} of SF across hooves, this was not the case for FD4 and RB. Again the P_{app} of SF and RB across hooves increased after treating hooves with TA, but this was not the case for FD4.

3.5.2.1. Permeation across hooves. PEs could increase the amount of permeated SF (up to 11.1-fold) across hooves. In the case of FD4, only papain, the combination, and serial applications of urea/TA were effective. The permeability coefficient of RB increased after treating hooves with TA only.

3.5.2.2. Permeation across KFs. All PEs increased marker permeability coefficients, although for both urea and papain, the impact on FD4 and RB permeation was not significant, respectively.

3.5.2.3. KF versus hoof. In the case of SF permeation, hooves and KF permeability differed only up to 2.1-fold when PE treatment was performed. Greater discrepancies of 16.8- and 19.9-fold were found for FD4 and RB permeation when TA and serial application were used, respectively. KF versus hoof ratios of 2- to 3-fold were observed when urea and its combination with TA were applied to FD4. Urea and its combination with TA resulted in about 5-fold ratios for RB.

Fig. 5 shows the relation between permeability (P_{app}) and octanol/water partition coefficient of the markers (log *P*) under study. As a note, Fig. 5 is actually not intended to explain the dependency of the markers' partition coefficients (lipophilicities) with their permeation coefficients, as it was clear that both parameters were independent in terms of permeation across nail and hoof. Rather, this plot is an effort to explore the behavior of the tested membranes, especially their response to markers with different physicochemical properties.

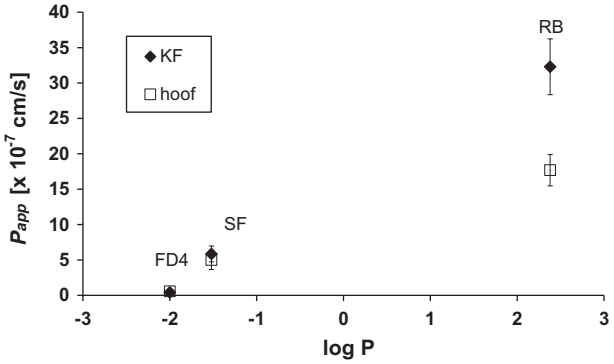

Fig. 5. Plot of permeability coefficients (P_{app}) and log octanol water partition coefficients (log *P*) of the markers.

4. Discussion

4.1. KF manufacture

Keratin belongs to the family of water-insoluble proteins [28]. Furthermore, it is insoluble in many solvents and even robust against digestion of enzymes, such as pepsin and trypsin. The keratin extraction involves reducing agents that turn keratin into a more soluble form [8] via cleavage of the keratin disulfide bonds. The Shindai solution was found to be sufficient to extract the keratin from human hair to produce the KF under study. Blond or gray hair was the best raw material for KF production because colored hair may influence or disturb the extraction process (according to our preliminary study), due to the rich content in melanin. Bleaching could, however, be applied before extraction to diminish this problem.

During dialysis, the keratin molecules aggregated together, changing their appearance from a clear to an opaque solution, hinting at the growing size of keratin aggregates. Further solvent evaporation led to film formation. Curing was essential to oxidize the disulfide bonds between keratin molecules and thus produced water-resistant films.

The drying process and the plasticizer amount played an important role. A mild evaporation at 40 °C for 24 h was the optimum condition. A higher drying temperature led to a rapid evaporation and evoked trapped air bubbles, producing uneven films. Insufficient plasticizer (i.e., glycerol) resulted in brittle KF, whereas an excessive use led to a moist film. The optimum concentration for this purpose was found to be 1% glycerol.

4.2. SDS–PAGE

The major protein bands from KF at around 42 and 52 kDa were similar to those from human nail. This finding is in accordance with a previous study from Baden et al. who also found similar SDS–PAGE patterns for human keratinized tissues [15]. In addition, they also found that human hair and nail showed nearly equivalent water absorption profiles at various relative humidities. Keratin is composed of proteins with molecular mass between 40–70 kDa and variations in their isoelectric points [8,29]. To resolve all keratin proteins, two-dimensional electrophoresis is needed, i.e., resolving them according to their molecular weights (MW) and thus isoelectric points. Human keratin proteins can be grouped as acidic/type I with pI 4.9–5.4 and basic/type II with pI 6.5–8.5. In bovines, pI values are slightly different being <5.6 for type I and >6.0 for type II. In addition, to enable the characterization of keratin proteins according to their MW, immunoblotting assay is needed since some proteins in certain tissues possess similar MW, others do not. K13 is a good example; its size is 51 kDa in humans and 43 kDa in bovines [8].

There are two major protein groups of hair and nail keratin, i.e., low-sulfur and high-sulfur proteins. The former has higher apparent MW in the range of 55–76 kDa, whereas the latter is in the range of 26.5–43 kDa [16,30]. Human hair and nail share similar proteins in both groups, although their amino acid and half-cystine

contents are slightly different [15]. At least seven proteins were common to hair and nail in the high-sulfur group with additional proteins for nail at MW 38.5 and 32 kDa. On the other hand, 6 proteins in the low-sulfur group have been identified in both materials including additional proteins at MW 61 kDa in hair and 72 or 76 kDa in nail [16]. Both groups were evident in our study from the hair and nail samples. However, to identify each single keratin protein, further application of two-dimensional electrophoresis in different pH is needed. The thick bands at around 52 kDa presented the low-sulfur proteins group; meanwhile those at around 42 kDa belong to the high-sulfur proteins group. Additional bands from each keratin group were shown distinctly by the nail extract compared to hair or KF at e.g. 76 kDa.

Cooper and Sun [31] listed some bovine proteins from previous reports. Hoof keratin comprises proteins with MW of 48, 50, 51, 52, 54, 56, 58, 60, 63 and 68 kDa. In addition to this, Kvedar et al. [32] found specific keratin proteins in bovine hoof, labeled as a_1–a_4 and b_2 with MW of 44–58 kDa and pI 5.2–6. These descriptions can however not explain the thick band with MW 20–26 kDa appeared from the hoof extract lane (f). On the other hand, the other band at around 42 kDa is the protein group, which is about similar to human keratin as in hair and nail. A more detailed discussion to bovine hoof keratin contents and its immunoblotting assay is given in Ref. [31,32].

The discrepancy between human and bovine keratin suggests actually that KF made of human hair is a strong candidate for a nail plate substitute. KF still retained its SDS–PAGE pattern after film formation while a reduction in intensity occurred after curing. This could hint at the denaturation of KF protein into smaller sizes, which appeared as diffuse bands throughout the lane. Reichl [24] investigated human hair keratin as cell culture/tissue engineering substrate and showed that the arrangement of the keratin filament in KF produced with this method did not resemble that in the native hair or nail. The arrangement was observed as a tightly packed and well-connected nanostructure of the keratin dialysate, as has been verified with cryo-TEM micrographs [24]. This artificial arrangement explains the susceptibility of KF to all PEs.

4.3. Permeation study: finding the analogue of KF

The search for KF analogue revealed an optimal thickness of about 120 μm, compared with the 100-μm-thick hoof. This was confirmed by the permeability coefficients of three markers (SF, FD4, and RB) as well as by their accumulated amounts in the membranes. A significant discrepancy was shown for RB, where its permeability coefficient across hoof was lower than that across KF ($p < 0.01$). Again this discrepancy was shown for the RB accumulated amount as well. A strong binding of lipophilic RB to KF hinted at some specific binding of hair-type keratin compared with hoof keratin. This could be attributed to the different amino acid compositions from both materials, as reported by Baden et al. [15,33]. Furthermore, the graph shown in Fig. 5 emphasizes that a large discrepancy between hoof and KF permeabilities could be expected when the solute is a lipophilic substance, although this explanation is in contrast to some literature reports [10,12,21,34].

It has been acknowledged that human nail, hooves, and accordingly KFs behave as hydrophilic membranes [10,11,34], where the permeation should not be controlled by the lipophilicity of the substance, but rather by its molecular weight [10,12,21,34]. Both SF and RB are not very different in their sizes (376 and 443, respectively), but SF is an acidic dye and RB is a basic one. Keratin, with a pI around 5 [16], is negatively charged in a neutral environment and supports the diffusion of a positively charged molecule, e.g., RB at pH 7.4. This explains thus the greater P_{app} of RB compared with SF across hoof and KF in this milieu as shown in Table 1. This phenomenon has been described by Mertin and Lippold before [10] and was attributed to the Donnan equilibrium phenomenon [35,36]. The electrostatic repulsion between the SF anion and the negative co-ion of keratin in this milieu at the membrane interface is responsible for its smaller P_{app} compared with RB.

The amounts of SF and FD4 bound in both membranes were inconsiderable (maximum 1.9%), whereas the amount of bound RB was much higher. This was in agreement with the work of DeLauder and Kidwell [37], who found no binding between fluorescein and hair samples after exposure of 10 mg/ml dye in 10 mM phosphate buffer, pH 5.6 at 37 °C for 2 h. They found that anionic substances such as SF could not be absorbed into hair due to the electrostatic repulsion at the hair interface, which was negatively charged in this milieu (pH > 5). Rhodamine 6G, a cationic dye, was found to be almost completely absorbed in the hair samples after the same defined exposure. This finding supported once more the phenomenon of Donnan equilibrium and confirmed the great influence of environmental pH to the diffusion of charged or dissociated molecules across nail or hoof.

Interestingly, the ratio of KF/hoof from each marker was almost similar in terms of P_{app} and accumulated amount. As example, despite the discrepancy shown by RB between KF and hoof, the ratios remained constant for the permeated (P_{app}) and accumulated amount (ratio KF/hoof 1.6–1.8). This implies that KF and hoof were comparable by a factor, which was determined by the marker physicochemical property.

4.4. The application of ungual PE

The chosen PEs have various mechanisms in enhancing the permeation of markers. TA disrupts keratin disulfide bonds which is then followed by nail swelling [20,21]. Urea works by increasing nail hydration only [19] in contrast to urea H_2O_2 which is able to oxidize disulfide bonds and to increase nail hydration as well [21,38]. PE increased the diffusion of all markers across KF, yet for several treatments not in a statistically significant extent. Hooves, on the other hand, exhibited rather selective barrier properties after the application of PEs. The single PE (urea or TA) was not effective for FD4, and only the single TA could increase RB permeation.

The permeation of SF across both membranes was similar after PE application, with differences up to 2-fold only. The application of TA alone or in combination resulted in a remarkable increase of up to 16-fold, indicating its effectiveness versus the PE-free SF permeation. The benefit that SF received from the acidic TA (pH of TA solution 1.63) was in agreement with the Donnan effect.

In contrast to SF, the use of single PE (urea and TA) was not sufficient to increase FD4 permeation across hooves, whereas all PEs increased FD4 permeation across KF. Hoof exhibited a selective barrier for FD4, and interestingly, only the serial and the combination of PE treatment could increase FD4 permeation. This hinted at a synergistic effect of PEs, probably due to different mechanisms in creating diffusion passages for FD4. The serial application resulted in a remarkable increase. Obviously, the cleaving action of TA was better in a fully hydrated hoof and/or in a basic pH after urea application. This synergistic PE effect has been previously reported by Brown et al. [21], where the permeation of terbinafine HCl increased remarkably after the sequential application of 5% TA and 17.5% urea H_2O_2. The resulting flux was 2.2 and 23.8-fold greater than the application of single TA and urea H_2O_2, respectively. They also found that reversing the PEs sequence did not always increase the drug diffusion across nails, as was shown by their tested solutes, i.e., caffeine, methylparaben, and terbinafine HCl. A similar phenomenon was investigated even earlier by Nakamura et al. [9], in conjunction with the synergistic work of PEs, showing that the combination of several reducing agents to extract keratin from hair was able to increase the yield of the extraction process. For

example, the use of 3 M thiourea, 5 M urea, and 5% 2-mercaptoethanol yielded 60% keratin, while the use of 8 M urea, and 5% 2-mercaptoethanol yielded only 27% (all were determined with the dry weight method).

All PEs were able to increase RB permeation across KF; however, only the single TA application was effective for hooves. It is presently, however, not fully understood why TA, despite its acidic pH, still increases RB permeation across hooves, because in this acidic milieu, keratin is positively charged. This contradicts the Donnan effect, where the acidic pH of TA should hinder the permeation of RB.

PE was previously found to be penetrant specific [21], and this is confirmed by our study. A careful conclusion must be made if the tested substance is a large or a lipophilic molecule. The different water uptakes from hoof and KF and their different inner structures (natural versus artificial) were possibly responsible for all discrepancies found after PE application. Hoof with the water uptake up to 45%, in contrast to KF, is accordingly able to retain the PE solution within the matrix longer; this would retain the matrix acidity/alkalinity after PE application as well. This environment will affect the solute permeation across the membrane. Human nail absorbs water up to around 30%, and this is between the water uptake values observed for hoof and KF. Just considering the water uptake only, one would underestimate the permeability of KF in comparison with nail. This was actually not the case, as indicated by an even higher KF permeability compared with hoof, despite its low water uptake.

KF is supposed to be less susceptible to urea, which works via an increase in hydration. This was indeed seen for all markers across KF, where the increases after urea application were the lowest among other PEs. However, the different water uptake between hoof and KF did not contribute to any distinct difference when the hydrophilic marker was applied. Finally, KF was very susceptible to TA, a common permanent waving agent [39].

To explain the synergistic works of TA and urea, TA as waving agent is usually employed in alkaline pH of about 9–10 to maximize disulfide bonds cleavage by the thiolate ion [38–40]. At acidic pH, heat is required to carry out the waving process. The presence of thiolate ion $^{-}S–CH_2–COO^{-}$ in alkaline pH plays the main role for the reduction in the keratin disulfide bonds [38]. In low pH, the dominant species in the system is the un-ionized molecule HS–CH_2–COOH. This explains thus the P_{app} increases across KF upon application of serial urea/TA or in combination with urea where the environments of these systems were alkaline. Meanwhile, the pH of 5% TA solution was 1.63, and in combination with urea the pH increased to 2.93. The pH increases of TA solutions were followed by P_{app} increases across KF except those for SF which were comparable. Single, serial, or combination of TA did not give any differences to SF's P_{app} across hooves; meanwhile, urea incorporation or serial application reduced RB's P_{app} across hooves.

To cleave disulfide bonds in hair, TA must reach the cortex through the hair cuticle [40]. Following this, Cannell [39] explained the reaction mechanism as follows:

$$\text{kSSk} + \text{RSH} \leftrightarrow \text{kSH} + \text{kSSR}$$

In this step, hair keratin disulfide bond (kSSk) cleaves upon addition of thiol compound (RSH) and produced a cysteine residue (kSH) and the mixed disulfide of the thiol compound with the hair keratin (kSSR). Upon contact with the second thiol molecule, a second cysteine residue and the symmetrical disulfide of the thiol RSSR are formed:

$$\text{kSSR} + \text{RSH} \leftrightarrow \text{kSH} + \text{RSSR}$$

In the final step, the application of an oxidizing agent restores hair disulfide cross-links (kSSk) through this reaction:

$$\text{kSH} + \text{HSk} \xrightarrow{\text{oxidizing agent}} \text{kSSk} + \text{H}_2\text{O} \quad (4)$$

Urea in aqueous solution acts as electron donor and keratin is negatively charged in this milieu due to the alkaline pH. Theoretically, this alkaline environment should actually support RB permeation as in the case of its permeation without PE at pH 7.4. However, this was not proven. Thereafter, the electrostatic repulsion with urea molecule at the diffusion interface as RB entering hooves could be responsible for this phenomenon. This phenomenon was not seen in KF where urea application led to permeation enhancements for all markers.

Interestingly, although the water uptake of hooves was high, the application of urea did not always increase the permeation of markers, which can be seen from FD4 and RB. The permeated amounts of FD4 and RB decreased, respectively, suggesting the unsuitability of urea as PE in these cases.

Papain was proven to be a potential PE, with a concentration of 2% still showing permeation enhancements across both membranes. The mechanism of papain in increasing diffusion was not clear until now. Quintanar-Guerrero et al. [23] suggested a passage formation by papain and salicylic acid, whereas Mohorčič et al. [22] proposed a hydrolysis of the keratin network by a keratinolytic enzyme, as was shown by keratinase in their study. KF was found to be more susceptible to papain compared with hooves. KF/hoof ratio was around 2-fold for SF and FD4 and around 6-fold for RB. This indicated that keratin bonds in KF were more fragile than the ones in hoof.

There is unfortunately no universal ungual PE for all penetrants. In fact, the permeation of the penetrant is strongly depending on the PE concentration, treatment duration, and the physicochemical character of the penetrant itself. Brown et al. [21] reported that all tested PEs (TA, urea H_2O_2, sequential and reversal TA/urea) showed no universal enhancements to all their tested substances (caffeine, methylparaben, and terbinafine HCl). Caffeine as a model of a small hydrophilic drug did not show any enhanced penetration after the sequential application of 15% urea H_2O_2 followed by 5% TA, but in the present study SF did. Indeed, one should consider the influence of environmental pH on the dissociation of penetrant as well. Mertin and Lippold [10] found that benzoic acid permeated to a higher extent across nail at pH 2.0 than at pH 7.4. Vice versa, this was also the case for pyridine, a basic substance, which permeated more at pH 7.4 than at pH 2.0. This phenomenon indicated that the undissociated form facilitated the permeation across nail.

5. Conclusions

A film made of human hair keratin was tested as a substitute for nail plate. The produced film was appropriate for permeation experiments with regard to its mechanical stability and water-resistant property. The similarity of KF to hooves in terms of permeability and accumulated amount of model compounds showed that KF offers a possibility as human nail plate substitute, especially for hydrophilic substances. The study involving lipophilic drug and any application of ungual penetration enhancer must be carefully interpreted because the use of KF could overestimate the permeated amount. Further improvements are needed for testing lipophilic substances and increasing film strength upon the application of ungual penetration enhancer.

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