European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 432–440

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

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

Lusiana, Stephan Reichl, Christel C. Müller-Goymann *

*Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Braunschweig, Germany*

ARTICLE INFO

*Article history:*
Received 11 October 2010
Accepted in revised form 31 January 2011
Available online 15 February 2011

*Keywords:*
Keratin
Bovine hoof
Hair
Nail
Permeation study
Marker

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

* Corresponding author. Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstraße 1, 38106 Braunschweig, Germany. Tel.: +49 531 3915650; fax: +49 531 3918108.
*E-mail address:* c.mueller-goymann@tu-bs.de (C.C. Müller-Goymann).

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doi:10.1016/j.ejpb.2011.01.022

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