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Research paper

Towards drug quantification in human skin with confocal Raman microscopy

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ABSTRACT

Understanding the penetration behaviour of drugs into human skin is a prerequisite for the rational development and evaluation of effective dermal drug delivery.

The general procedure for the acquisition of quantitative drug penetration profiles in human skin is performed by sequential segmentation and extraction. Unfortunately, this technique is destructive, laborious and lacks spatial resolution. Confocal Raman microscopy bares the potential of a chemically selective, label free and nondestructive analysis. However, the acquisition of quantitative drug depth profiles within skin by Raman microscopy is impeded by imponderable signal attenuation inside the tissue.

In this study, we present a chemical semi-solid matrix system simulating the optical properties of human skin. This system serves as a skin surrogate for investigation of Raman signal attenuation under controlled conditions. Caffeine was homogeneously incorporated within the skin surrogate, and Raman intensity depth profiles were acquired. A mathematical algorithm describing the Raman signal attenuation within the surrogate was derived from these profiles. Human skin samples were incubated with caffeine, and Raman intensity depth profiles were similarly acquired. The surrogate algorithm was successfully applied to correct the drug profiles in human skin for signal attenuation. For the first time, a mathematical algorithm was established, which allows correction of Raman signal attenuation in human skin, thus facilitating reliable drug quantification in human skin by confocal Raman spectroscopy.

1. Introduction

The analysis of dermal drug absorption is essential for rational development of novel drug delivery options via the skin. As composition and barrier function of animal skin differ from human skin, the transferability of absorption data from animal testing to human skin is exacerbated [1]. Thus, for appropriate *in vitro* testing of dermal drug delivery, excised human skin is the gold standard specimen.

Human skin comprises a complex assembly of different layers with varying composition. The stratum corneum as the outermost skin layer represents the main barrier. Therefore, most *in vitro* penetration and permeation studies focus on this particular layer. According to the brick–mortar model, the stratum corneum consists of hydrophilic corneocytes surrounded by an extracellular lipid matrix [2]. Most substances enter the skin via an extracellular pathway, diffusing along this lipid matrix. The analysis of penetration processes in the different skin layers is a complex and laborious procedure which requires destructive segmentation as well as extraction [3]. Furthermore, the analytical determination lacks spatial resolution.

Recently, biophysical techniques like confocal laser scanning microscopy [4] or two photon microscopy [5,6] have been proven to provide spatially resolved information about kinetics and depth of dermal penetration and molecular interaction with the skin. However, quantification of drug penetration by two photon microscopy is limited to autofluorescent drug compounds in the absence of dyes or labels. Linking drugs with dyes or labels potentially introduces changes in substance physicochemical properties like lipophilicity or molecular weight which impair reliable transferability of the data. Unlike the former mentioned techniques, vibrational spectroscopy provides direct molecular information of the sample without labelling. For instance, infrared (IR) spectroscopy was successfully used to track lipids within the skin [7]. Unfortunately, the use of this technique is limited by its sensitivity to water.

In this context, Raman spectroscopy as a complementary analytical technique to IR spectroscopy bears a lot of potential for the analysis of skin. In contrast to IR, Raman spectroscopy is not

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constraint by the presence of water. By detecting the frequency shift of scattered laser light after irradiating a sample, chemically selective information of the sample composition is acquired. Furthermore, combining Raman spectroscopy with a confocal microscope provides spatially resolved analysis of the sample. Thus, confocal Raman microscopy is a promising analytical approach for label free and nondestructive follow-up of substances within human skin.

Confocal Raman spectroscopy has already been applied for analysis of skin hydration status [8], the effect of penetration enhancers [9] and the epidermal antioxidative potential [10,11] of skin. *In vitro* follow-up studies of metronidazole [12] and phospholipids [13] indicated the potential of confocal Raman microscopy for penetration experiments.

Recently, several authors described mathematical models to correct depth determination uncertainties of confocal Raman microscopy in polymer films [14–16]. Previous studies used these models to improve accuracy of confocal depth determination in skin [17]. Determination of the exact depth of the focal plane inside human skin allows the accurate acquisition of qualitative drug penetration follow-up profiles.

However, for rational development and *in vitro* testing of novel dermal drug delivery systems, quantitative analysis of drug penetration processes is mandatory. Unfortunately, so far drug quantification within skin based on Raman microscopy is exacerbated by Raman signal attenuation, as with increasing depth a decrease in Raman signal intensity distorts reliable drug quantification. One approach to overcome the influence of Raman signal attenuation in skin was already performed by relating the intensity of a drug Raman peak to the intensity of a skin derived Raman peak [12,18]. Unfortunately, potential inhomogenities due to the complex skin structure cannot be taken into consideration with this relative method. For reliable quantification of substances inside human skin by Raman microscopy, the exact extent of Raman signal attenuation has to be determined. As Raman microscopy offers unique possibilities for nondestructive and chemically selective analysis of substances in human skin, there is a strong need to overcome the analytical pitfalls exacerbating a reliable quantification of drugs in skin samples.

In this study, we present a novel approach to quantify Raman signal attenuation in human skin. We successfully developed a simplified and reproducible surrogate system simulating the optical properties of human stratum corneum. A comprehensive physical characterisation comprising spectroscopic as well as thermoanalytical methods was performed on the skin surrogate as well as on its single components followed by a comparison to excised human skin samples. Caffeine was homogeneously incorporated within the skin surrogate, and Raman depth profiles of caffeine were acquired. To address the issue of Raman signal attenuation, these profiles were mathematically fitted, and a correction algorithm was derived. Furthermore, excised human skin samples were incubated with caffeine, and Raman intensity depth profiles were acquired. Using the surrogate algorithm, these human skin intensity depth profiles were finally corrected for Raman signal attenuation.

2. Materials and methods

2.1. Skin surrogate fabrication

The main chemical components of human stratum corneum were used to create a suitable skin surrogate, namely keratin as the main protein component, purified water and a specific lipid mixture (Table 1). The lipid mixture was based on the work of Jaeckle et al. [19] and contains fatty acids, cholesterol and triglycerides in predefined ratios.

Table 1
Chemical composition of the skin surrogate.

Ingredients	Skin surrogate composition (w/w) [%]
Keratin	42
Water	28
Lipids	30
Cholesterol	9.3
Triglycerides	9.3
Fatty acids	11.4
Myristic acid	0.27
Palmitic acid	4.35
Stearic acid	1.23
Oleic acid	4.14
Linoleic acid	1.41

Keratin, extracted from rabbit fur (Chemos GmbH, Regenstaufen, Germany), and purified water were mixed in a melamin bowl by dropwise adding water to the keratin powder. The fatty acids myristic acid (Edenor C 14®), palmitic acid (Edenor C 16®), stearic acid (Edenor C 18®), oleic acid (Edenor Ti05®) and linolic acid (Edenor SB05®), provided by Cognis Oleochemicals GmbH (Duesseldorf, Germany) were mixed and molten in a metal bowl at 75 °C forming a clear liquid and stirred during cooling down to room temperature. This mixture is referred to as 'fatty acids' in the text. Triglycerides (Witepsol H5®, Sasol, Hamburg, Germany) and cholesterol (Sigma–Aldrich, Steinheim, Germany) were added, and the procedure was repeated as described. The cold lipid mixture was added to the keratin in water and finally homogenised. First approaches to form a homogeneous matrix involved gentle physical mixing of the components in a melamin bowl leading to inhomogeneous distribution of hydrophilic and lipophilic compounds and the incorporation of air bubbles. Further processing by means of a porphyrisator improved homogeneity but multiplied the air contribution, resulting in a foamy consistence.

The optimised fabrication process involves premixing in a melamin bowl and final homogenisation using a three-roll-mill. The appearance of the final product was a creamy matrix of bright brown colour. The refractive index of the skin surrogate was determined using a Abbe-refractometer. The final skin surrogate was stored at room temperature in a scintillation vial sealed with parafilm.

2.2. Incorporation of caffeine

Micronized caffeine (Sigma–Aldrich, Steinheim, Germany) as a model drug was incorporated in the surrogate by adding the powdered substance to the keratin water mixture. The subsequent fabrication of the skin surrogate follows the protocol as described above. A caffeine concentration of 30 mg caffeine per 1 g surrogate was used as reported for penetration experiments in human skin [20]. This concentration could be detected by Raman microscopy without any constraints. To verify the homogenous distribution of caffeine, the surrogate is spread on a glass slide, and at least six single spectra are recorded at random positions on the surrogate surface.

2.3. Human skin preparation

Human skin was obtained from plastic surgery of female Caucasians (Department of Plastic and Hand Surgery, Caritaskrankenhaus, Lebach, Germany). Only abdominal skin was used. After excision, the stratum corneum was cleaned with purified water, and the fatty tissue was removed with a scalpel. The skin was stored in impermeable polyethylene bags at −26 °C. For stratum corneum isolation, the protocol of Kligman et al. [21] was followed. First, punches of 25 mm in diameter were taken from the frozen