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Mid-infrared attenuated total reflection spectroscopy of human *stratum corneum* using a silver halide fiber probe of square cross-section and adhesive tape stripping ☆

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Dedicated to Professor Bernhard Schrader

Abstract

Mid-infrared fiber probes allow an extended use of attenuated total reflection (ATR) measurements for topical in vivo skin analysis, which were otherwise not possible with conventional sample compartment accessories. Evanescent wave spectroscopy using a flexible fiber-optic probe from silver halide fibers of square cross-section was employed for *stratum corneum* characterization and keratinocyte quantification on adhesive tapes. Such a method of quantifying the amount of keratin, which can be repetitively removed from the skin surface by adhesive tape application, is essential for the study of substances topically applied and penetrating into the horny layer. For calibration, the weight of keratinocytes was determined using an ultramicro-balance. Best results were obtained with difference spectroscopy and the evaluation of the amide I absorption band intensity (correlation coefficient $r = 0.983$). Lowest amounts per cm^2 were reached for the range down to 5 $\mu g/cm^2$. The heterogeneity in the surface density of keratinocytes clinging to the tape was investigated by microscopy, and the thickness of some individual keratinocytes was tested by ATR-microspectroscopy and atomic force microscopy.

Keywords: Infrared spectroscopy of *stratum corneum*; Attenuated total reflection spectroscopy; Silver halide fiber-optic probe; Depth profiling in *stratum corneum* for dermal pharmaco-kinetics; Quantification of keratinocytes

1. Introduction

For many years infrared spectroscopy has now been used in skin analysis, and much research has been specialized in the characterization of the upper skin layer, which for most of the body parts consists of horny material, also called the *stratum corneum* [1,2]. In recent applications, the attenuated total reflection (ATR) technique was utilized for characterizing

☆ This contribution is in honor of Prof. Dr Ing. Bernhard Schrader, a friend and a pioneer in vibrational spectroscopy of biomaterials.

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the keratin-lipid composition of the human horny layer [3] or for the analysis of the percutaneous penetration of UV-filters [4]. In particular, the so-called horizontal ATR-accessory, still to be used within the standard spectrometer sample compartment, but with easy access to the crystal from above the instrument, facilitated skin surface measurements significantly. The ATR-crystal area, usually to be covered by skin, is a few square centimeters large. An example for the topical characterization of oral mucosa based on such an accessory is listed in our references [5]. Other recent applications, especially from the cosmetic industry, were concerned with the study of influencing the *stratum corneum* by cosmetic products [6]. On the other hand, the fairly large potential of ATR infrared spectroscopy for epidermal studies with the use of flexible silver halide fiber-optic probes has been shown by us, since such tools, based here on sensing fibers of square cross-section, can ease the epidermal surface analysis significantly [7–11]. It must be noted that the contact skin area in such investigations is much smaller when compared to the use of conventional ATR-accessories.

For chemical penetration and bioavailability studies, skin surface stripping with adhesive tapes has been used within dermatology, pharmacology, and cosmetics (skin care) on various occasions. With multiple strippings even a complete removal of the *stratum corneum* of the epidermis can be achieved. Such a technique, for example, was applied to examine the structurally heterogeneous biomembrane of the horny layer and its behavior with respect to water diffusion through this barrier [12,13]. Other applications were concerned with the investigation of the distribution profile of active components such as solvents, drugs, cosmetics and UV filters of sunscreen lotions within the horny layer [14,15]. As recent examples, different formulations with clobetasol propionate were tested to quantify its bioavailability within the *stratum corneum*; the substances found on the tape samples were determined by HPLC [16,17]. Another application of tape stripping in combination with a spectroscopic assay aimed at the determination of titanium dioxide microparticles that were part of sunscreen formulations [18]. Within the quantification of skin penetration processes, also different pathways have been discussed [19].

The amount of keratinocytes removed with a single adhesive tape application can vary considerably for different individuals, various skin regions and within the depth of the *stratum corneum* layer. Therefore, a fast routine method is desired to analyze the large number of tape samples, which usually accrue during work-extensive campaigns within dermal pharmacokinetic testing of the substances applied to the skin surface. The generally accepted quantitative method for determining the amount of keratinocytes on a tape strip is weighing, which can, however, give false results, when substances are topically applied [1]. For that reason, the first tape strip in drug penetration studies is often discarded. On the other hand, the latter usually contains also important information. Additionally to the restriction mentioned can such a method be rather time consuming and laborious.

As a result, several UV–Vis spectroscopic methods have been proposed to ease and accelerate the quantification. One option is to measure the keratin UV absorption around 275 nm (for spectral absorptivity data, see Ref. [20]), which was investigated by Marttin et al. for the quantitative analysis of keratinocytes attached to the tape, but a satisfactory assay based on UV-spectrometry could not be achieved [21]. Furthermore, topically applied substances often show significant absorption especially for this spectral range. Therefore, a discrimination from the faint and rather featureless spectral absorbances of keratin is hardly possible. The measurement of absorption and light scattering at 600 nm, also tested by the latter authors, yielded no significant improvement for a regression against area normalized *stratum corneum* weight. Weigmann et al. [22] studied the visible spectra of such tapes and correlated the absorbance at a single wavelength at 430 nm due to scattering and reflectance to the weight of the abrasive tapes after topical skin application. However, the selectivity and sensitivity of their spectroscopic method for a reliable quantification must be questioned.

In this paper, infrared ATR-spectroscopy is proposed for keratin quantification using a sensor head from silver halide fibers of square cross-section, which enables also reproducible measurements on adhesive tape surfaces. The infrared methodology is fast and—due to the high information content of its spectra—very selective and can be applied for small

skin and tape areas. The methodology allows to quantify the amount of keratinocytes taken off with improved confidence limits by their unique infrared spectral pattern. Exemplary results are presented here, which are combined with a microscopic inspection of the keratinocyte distribution on adhesive tape and an analysis of individual keratinocytes by IR-microspectroscopy using the ATR-technique and atomic force microscopy.

2. Experimental

For the experiments infrared spectra were recorded by an FTIR-spectrometer (model Vector 22; Bruker Optik GmbH, Ettlingen, Germany) equipped with a flexible silver halide optical fiber probe for remote ATR-spectroscopy (infrared fiber sensors, Aachen, Germany). The infrared beam exiting the FTIR-spectrometer is coupled into the silver halide optical fiber with a square cross-section of 750 μm × 750 μm and a numerical aperture of 0.5 by an off-axis parabolic mirror (see also Fig. 1). The bifurcated silver halide optical fiber probe had a shaft containing the ATR-measuring sensor head made from a u-shaped silver halide fiber piece with a bending radius of 4 mm. Opposite to fibers with a circular cross-section, fibers of square cross-section provide an exactly defined active measurement area (15 mm × 0.75 mm), which allows reproducible measurements with a defined contact pressure of about 5 kg/cm^2 for 10 s. One arm of the bifurcated fiber probe, equipped with a micro-lens at the fiber end, was directly coupled to a liquid nitrogen cooled mercury–cadmium–telluride-detector (MCT) from Infrared Associates, Stewart, FL (USA).

All spectra have been recorded with a spectral resolution of 4 cm^{-1}. The high transmission of the fiber probe renders low-noise spectra within a few seconds measurement time (e.g. for a scanning time of 5 s and a spectral resolution of 4 cm^{-1} a signal-to-noise ratio of larger than 8000 between 1100 and 1200 cm^{-1} was achieved).

Adhesive tapes (TESA® film No. 4204, BDF Beiersdorf AG, Hamburg, Germany) with a width of 19 mm were used for the removal of *stratum corneum* layers from the forearm of a test person. The weight of circular tape pieces of 15 mm diameter before and after skin application was measured using an ultra-micro-balance of type UMT5 Comparator from Mettler-Toledo GmbH (Greifensee, Switzerland).

Infrared spectra from 3000 to 600 cm^{-1} were collected from the epidermal keratinocytes attached to the tape and from different skin surfaces after repeated stripping. The measurements on the tape surface were performed with the shaft of the silver halide optical fiber probe fixed in a mechanical holder, while the adhesive tape was attached to the plane outer surface of the u-shaped ATR-fiber probe. The infrared rays which enter the u-shaped fiber piece under a broad range of large grazing reflection angles, lead to a rather small sampling depth, which is lower than the typical thickness of the flat keratinocytes (a comparison of the sensitivity of an u-shaped fiber ATR-probe compared to a fiber-coupled micro-prism diamond with two reflections at 45° has recently been given by

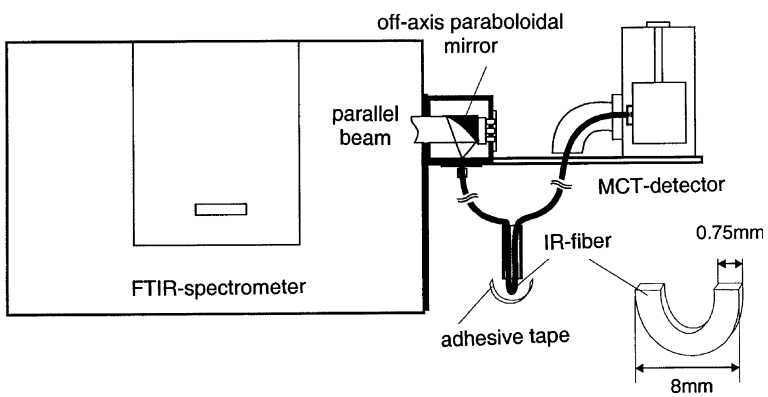

Fig. 1. Experimental set-up with a FTIR-spectrometer and the silver halide fiber-optic probe.

us [23]). Therefore, it was possible to separate the spectral features of the adhesive tape from those of the flat keratinocytes by difference spectroscopy. Cleaning of the fiber surface was done using pure ethanol.

Transmission spectra from adhesive tape samples were recorded using the beam focus in the Vector 22 spectrometer sample chamber. Routinely, a thermal DTGS-detector was used for such spectral measurements. In order to estimate the thickness of different keratinocytes, micro-spectroscopic measurements were carried out employing an FTIR-spectrometer (model PE 2000, Perkin Elmer, Überlingen, Germany), which was equipped with an Autoimage-microscope including the micro-ATR option with a Germanium crystal. A CCD-camera allows for the recording of micro-images. In addition, atomic force microscopy (AFM) of single corneocytes on adhesive tape was carried out by a NanoWizard™ AFM-instrument from jpk Instruments (Berlin, Germany) using the non-contact mode. The cantilever probe (ultrasharp, non-contact type nsg10) was from the company NT-MDT Co. (Moscow, Russia).

3. Results and discussion

The flexible fiber probe enabled us to carry out measurements on various skin locations. Due to the square cross-section of the fibers also reproducible spectral recordings under a defined contact pressure can be done. A spectrum of a test person's forearm skin is shown in Fig. 2. Most intensive bands can be assigned to the so-called protein amide I vibration at 1640 cm^{-1} and amide II vibration at 1540 cm^{-1}, respectively. A more complete assignment of different bands to vibrations of molecular sub-structures was given recently by Lucassen and coworkers [2]. Skin spectra from different patients, using the remote sensing capability of our fiber-probes, can exhibit a wide range of variance, especially within the fingerprint region; for recent investigations, see Refs. [10,11].

The possibilities of quantifying the amount of material sticking to the tape surface were first evaluated by conventional transmission infrared spectroscopy, similarly to previously proposed UV–Vis spectroscopic methods. In contrast to those measurements, where absorption bands of the tape

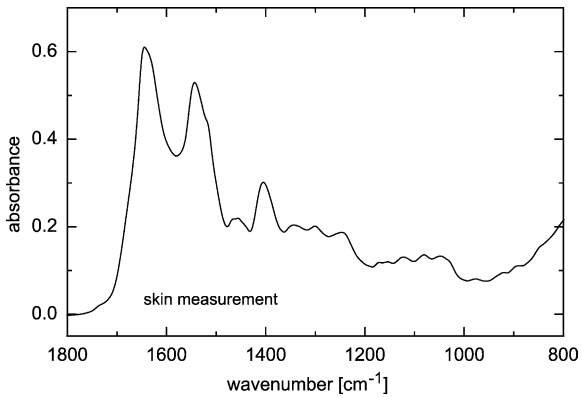

Fig. 2. ATR-infrared spectrum of *stratum corneum* from the forearm of a test person using the flexible fiber probe in contact with the skin surface.

material were found within the 250–300 nm UV-region [24], some IR-bands from the polymer are extremely strong, so that absorption with complete opaqueness takes place, see absorbance spectrum in Fig. 3(A). Additionally, sinusoidal interference fringes in the spectral baseline that cause disturbing effects for keratin quantification can be observed due to multiple beam interference within the free standing polymer film. For the transmission mode, signal saturation for those bands could possibly be avoided by using a reduced tape thickness, which would also enlarge the fringe period, but such a material is not commercially available. Subtraction of the spectral features of a clean adhesive tape applied to a spectrum of a tape with attached keratinocytes from the *stratum corneum* leads to the difference spectrum shown in Fig. 3(B). The spectral quality is poor, providing also evidence of the problem from uncompensated interference fringes, which complicates the quantification of the cellular material stripped off by the adhesive tape.

In Fig. 3(A), an adhesive tape spectrum, as recorded by our ATR fiber technique with the gluey side in contact with the evanescent field sensing fiber, is also presented. The band intensities realized with the fiber probe are adequate for compensation work, which can be demonstrated with the spectrum shown in Fig. 3(B). The difference spectrum, which renders the spectral contribution from the epidermal keratinocytes attached to the tape, shows impressively

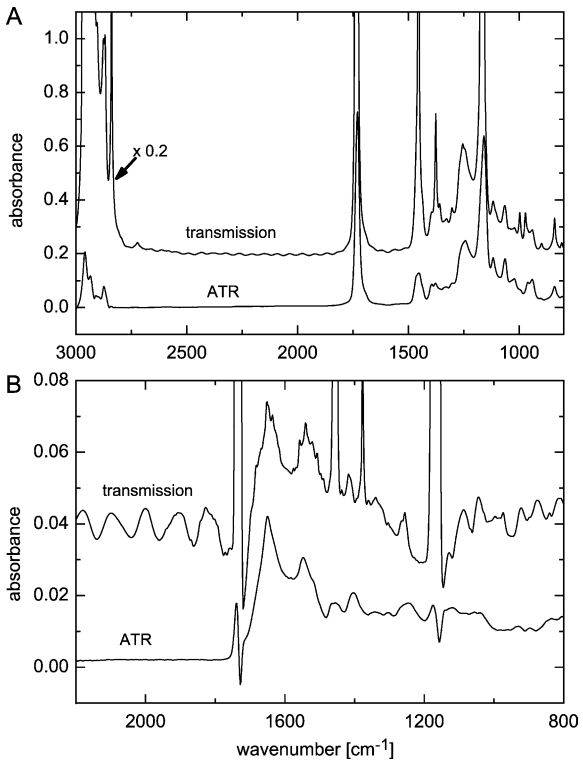

Fig. 3. Comparison of mid-infrared spectra of a sample of untreated adhesive tape and tape used in keratinocyte removal, using transmission and ATR measurement techniques, respectively. (A) Measurement of whole untreated tape by transmission and surface ATR-measurement with attachment of the gluey tape side to the ATR-crystal. (B) Difference spectra obtained for adhesive tape containing sticking keratinocytes using scaled absorbance subtraction with the corresponding spectra shown above.

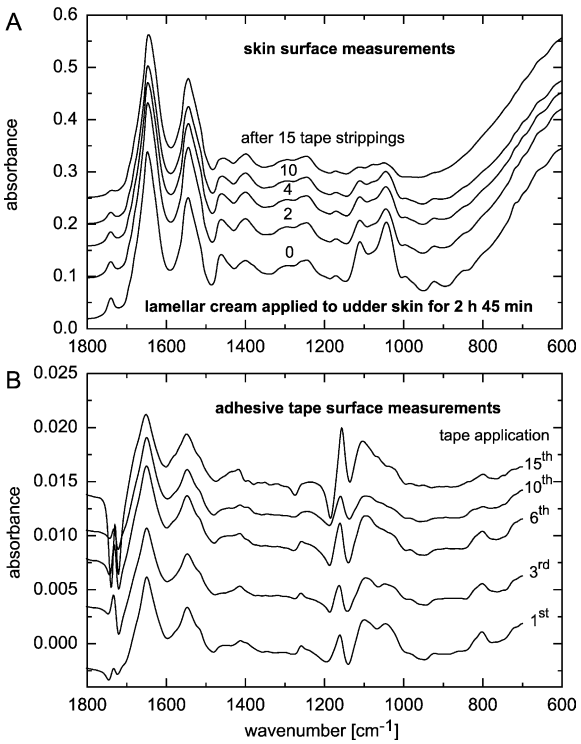

Fig. 4. Skin surface ATR-measurements using a silver halide fiber probe after topical cream application and repeat tape stripping using the bovine udder skin (BUS) model (A) and complementary measurements on the tape surfaces after topical application (B).

the advantages of the ATR technique. Still minor artifacts from the spectral subtraction can be observed for the two most intensive bands within the fingerprint region, but their perturbations are rather limited with respect to small spectral intervals. The spectral quality is fine when compared to a skin surface measurement as shown in Fig. 2. The spectral measurements are also free from atmospheric water and carbon dioxide absorptions, which are usually a problem with sample compartment based accessories. A further advantage is certainly that skin surface and tape measurements can be carried out successively using the same ATR-probe. The skin area that can be investigated is of only several square millimeters, so that inhomogeneities can easily be accessed by the fiber probe.

Another example of measurements on the skin surface after repeated tape stripping and on the individual adhesive tapes used for removal of keratinocyte layers is shown in Fig. 4. Here, the experiment was carried out using the in vitro model of the isolated perfused bovine udder skin (BUS-model), which has been proposed as a substitute for human in vivo tests [25]. Due to the continuous perfusion, the horny layer demonstrates active barrier and reservoir functions. The in vitro model is widely used in dermatological and cosmetic research as well and exhibits hair follicles and sebaceous glands, providing the corneal compartment with sebum similar to the human in vivo situation. The stripping was done after topical application of a lamellar cream (for further details on composition, see Ref. [24]) and subsequent exposure to the skin surface for nearly 3 h. The complementary nature of the tape surface measurements with regard to the cream constituents,

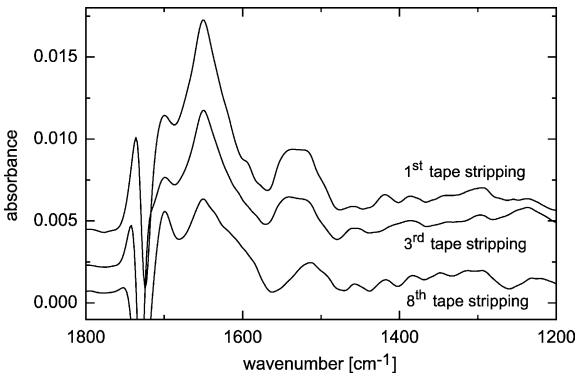

Fig. 5. Difference spectra obtained for small amounts of keratinocytes, clinging to the adhesive tape surface, after tape spectrum compensation.

when compared to the skin spectra recorded from the surface layer after the respective treatment, is illustrated.

An example of experiments carried out with *stratum corneum* from the flexor forearm is shown in Fig. 5. The intensity of the amide I band in the difference spectrum did not change with clear evidence within a range of different scaling factors that were employed for the scaled subtraction using a virgin adhesive tape spectrum. In this case after successive strippings, the bandshape of the amide II band is changed slightly. We observed area normalized weights for the stripped keratinocytes between 150 and 10 μg/cm^2. In Fig. 6 results from a series of tape strippings are shown (see part A), and the regression of the amide I band maximum versus area normalized weight is given with a linear line fitted through the graph origin (part B). The standard deviation of the fit was $\sigma = 2.2$ μg/cm^2 and the correlation coefficient $r = 0.983$, which is significantly improved, compared to the results from previous UV–Vis spectral evaluations, as discussed in the introduction. These preliminary results for quantifying the keratin amount that has been individually removed by adhesive tape may underline the potential of fiber-based ATR-measurements for a fast and quantitative method for depth profiling of topically applied substances within the *stratum corneum*. However, also intrinsic skin components such as ceramides can be quantified as needed for sebumetry; for its potential, see Ref. [11].

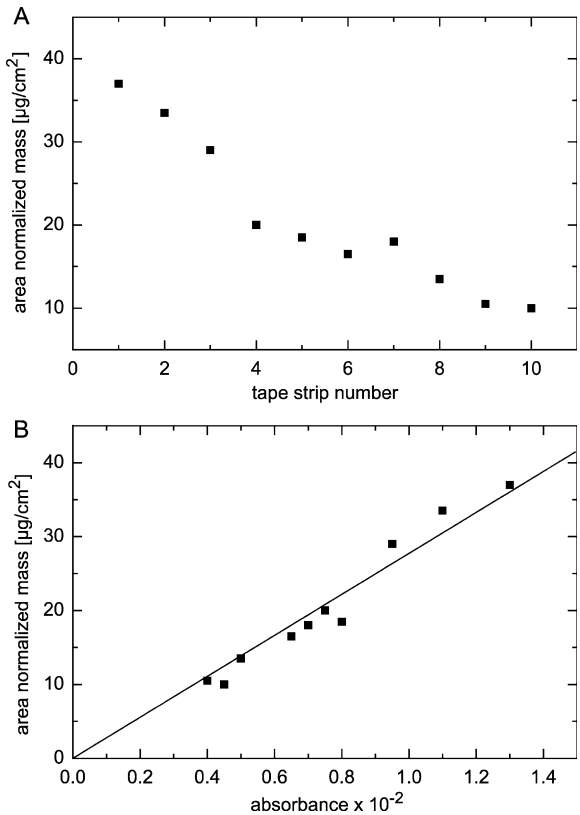

Fig. 6. Experiments using the weight of successively stripped tapes and employing infrared spectrometry for determining area normalized weight. (A) Diagram showing the decrease of removed keratinocyte material after successive tape strippings. (B) Regression of area normalized weight of stripped keratinoytes versus amide I absorption band maximum as measured against the baseline level at 1800 cm^{-1} (for regression results, see also text).

Further investigations were made into the heterogeneity of keratinocyte distributions on the adhesive tape. Microscopic images were scanned from small areas and one example is shown in Fig. 7(A). Usually, the density of corneocytes attached to the tape is reduced after repeated stripping, which was also documented by us, but is not shown here. The photographic image already gives an impression of the surface structure of the corneocytes. For further investigation, also AFM topographic measurements were carried out, and an exemplary result is shown in Fig. 7(B). The width of the square area studied is 30 μm and the whole surface roughness scanned for this individual corneocyte is contained within a height variation of 2 μm.

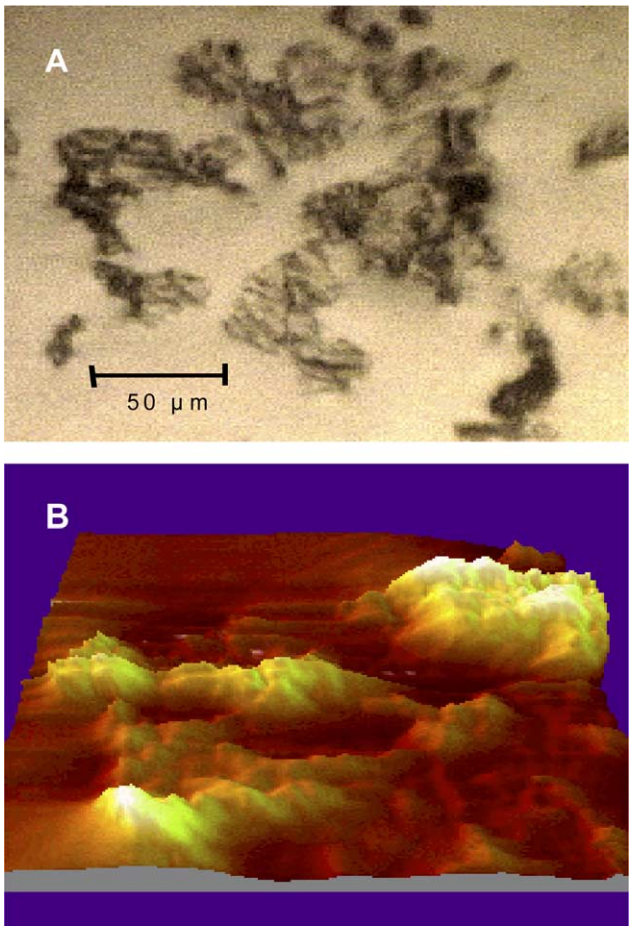

Fig. 7. (A) Microscopic image of keratinocytes on adhesive tape after a first stripping showing their area distribution, and (B) atomic force microscopy of a single corneocyte on adhesive tape (area $30 \times 30\ \mu m^2$, total roughness variation shown is within 2 µm).

Using the ATR-micro mode of our PE IR-microscope, several measurements were made on individual flattened keratinocytes for probing the thickness of the flat cell layers. In a few cases, the spectral features from the underlying adhesive tape are clearly noticeable, whereas others are optically thicker than the sensing radiation penetration field (see also Fig. 8). However, it must be noted that Germanium was used as crystal material having a refractive index of 4.0 around $1000\ cm^{-1}$, whereas the refractive index of the silver halide material is significantly lower ($n = 2.2$) leading to a larger sampling depth when based on the same reflection angle; see, for example [26]. For the fiber probe, however, the angles of incidence for most of the probing infrared radiation are much larger-leading to a smaller penetration depth into the sample medium-than for the ATR-microscope, for which the angles under the experimental conditions chosen are closer to those from which onwards total reflection is observed. A further difference is certainly the wider range of reflection angles at the interface of crystal and sample material, which exists for the fiber probe, in contrast to the conditions found for the ATR microscope.

Recently, we ran parallel transmission measurements on tapes loaded with keratinocytes using a UV–VIS-NIR spectrophotometer, model CARY 5G from Varian (Darmstadt, Germany). However, for

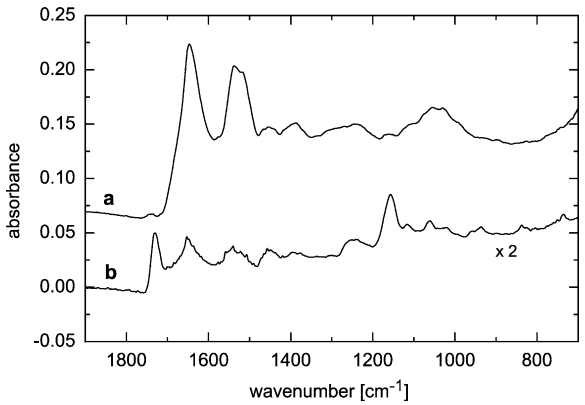

Fig. 8. ATR-infrared micro-spectroscopic analysis of flattened keratinocytes on adhesive tape: (a) an 'optically thick' sample and (b) a thin sample with spectral tape features seen through the flat corneocyte material.

keratin quantification the UV–Vis-spectra we recorded, as discussed above, were not appropriate, although components such as vitamin E or compounds acting as UV-filters, topically applied through a cream or lotion, can be conveniently quantified due to their strong absorbing chromophores [24].

4. Conclusions

Using the ATR fiber-optic probe, a fast and reliable quantification of the keratinocyte material stripped off by adhesive tape is possible. Applications were investigated for area normalized keratinocyte concentrations up to 150 $\mu g/cm^2$ and lowest values possibly down to 5 $\mu g/cm^2$, using simple difference spectroscopy as an intermediate step for spectrum processing. Compared to skin surface measurements, signals smaller by a factor of 300 can be observed at least for the strongest amide I and II vibration bands of keratinocytes found on adhesive tape samples. Due to the larger number of absorption bands within the mid-infrared spectrum compared to UV–Vis spectra, much better discrimination between, for example, topically applied drugs or sun-filter compounds and keratin material can be achieved. In this context, it is helpful to apply the versatile tool of difference spectroscopy also for isolating and quantifying individual components found within the skin surface by using their spectral absorption bands.

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