INTRODUCTION

Imaging modalities help in the development of topical drugs and cosmetics and also contribute to the early diagnosis, monitoring and grading of various pathological or physiological conditions in the skin. Different techniques are available in the toolbox of dermatologists and cosmeto-scientists including dermoscopy, confocal microscopy, optical coherence tomography, high-frequency ultrasound, Raman spectroscopy, fluorescence imaging and multispectral opto-acoustic tomography. All these methods have advantages and limitation which are summarized in details by Schneider et al, 2019a and 2019b.¹² This review article aimed to focus on Raman spectroscopy in skin research. This technology uses near-infrared lasers which generate photons from the test preparation (e.g. skin tissue) that scatter at the same and at different energies at each chemical bond within the structure. The changes are detected by the device and...
translated into highly specific and characteristic graphs (spectra). It can be applied both on in vivo and ex vivo/in vitro samples. The main strength of Raman spectroscopy is that it is a non-invasive, non-destructive method, which makes possible the in vivo sample analysis and further investigation of ex vivo samples after Raman analysis. Also, the coupling of Raman spectroscopy with confocal microscope enables the possibility of making axial screening. These screening steps can be combined with automatic focus which enables the measurements to be made automatically. No sample preparation is required which simplifies the method and shortens the time collecting the experimental results.3

In the current paper after some introductory words about the history and principle of Raman techniques, the main types of the Raman spectroscopy in dermatological research, the most important application possibilities in skin analysis, and the use of machine learning in data analysis are summarized. Finally, a short chapter about the regulatory aspects is presented.

2 | HISTORY OF RAMAN SPECTROSCOPY

The principle of Raman spectroscopy is based on the Raman effect which has been discovered more than 90 years ago. The phenomenon of inelastic scattering of light (Raman scattering) was discovered by Dr. C.V. Raman (1888–1970) in 1928. Sir Chandrasekhara Venkata Raman was an Indian physicist known about his work in the field of light scattering.4 He developed a spectrograph, and together with his student, K. S. Krishnan discovered that when light traverses a transparent material, a shift is happening in the deflected light in its wavelength and frequency.5 This phenomenon is called “modified scattering” which was subsequently termed as Raman effect or Raman scattering. Raman received the Nobel Prize in 1930 in Physics for this discovery, and he was the first Asian to receive a Nobel Prize in any branch of sciences.

Originally, extraordinary measures were required to obtain Raman spectra due to the low sensitivity of the technique. Typically, the sample was held in a long tube and illuminated along its length with a beam of filtered monochromatic light generated by a gas discharge lamp. The photons scattered by the sample were collected through an optical flat at the end of the tube. To maximize the sensitivity, the sample was highly concentrated and relatively large volumes (5 ml or more) were used. The use of Raman spectroscopy diminished when commercial infrared (IR) spectrophotometers became available in the 1940s. However, the discovery of the laser in the 1960s resulted in simplified Raman spectroscopy instruments and also boosted the sensitivity of the technique. This has renewed the use of Raman spectroscopy as a common analytical technique.4

In the late 1970s, Raman spectroscopy combined with an optical microscope was introduced and was used for microanalysis in several fields. Micro-Raman spectroscopy became an important tool in biology, especially for single-cell studies. Now, many researchers in various fields use micro-Raman spectroscopy. The application of this technology is widespread in analytical fields for industrial, biological, medical and environmental samples and also for art and archeology.8 Non-destructive Raman analysis has been used for cultural heritage diagnostics9 and to evaluate the ageing and degradation of plenty of precious artefacts, paints and statues.10 During the last almost one century, the market for Raman spectroscopy has had plenty of time to mature, and users are continuously working on to find new application areas.

The continuous development of the technology resulted in (1) increased light transmission due to the more advanced optical components, such as the lens, mirror and, especially, optical filters for the removal of scattered light; (2) improved stability due to developments in the spectrometer; and (3) enhanced light detection sensitivity with the use of detectors such as charge-coupled devices (CCDs).

Developments in coherent Raman scattering microscopy applications such as stimulated Raman scattering and coherent anti-Stokes Raman scattering microscopy offer exciting potential in biological imaging applications (see later in the article). Moreover, they offer huge potential for sub-diffraction limited microscopic approaches such as tip-enhanced Raman scattering (TERS), which are now crossing boundaries of spatial resolution that have previously not been accessible with conventional Raman microscopy. Another advancement is surface-enhanced Raman spectroscopy (SERS). It uses nanostructured metal surfaces to generate Raman spectra that are several orders of magnitude higher than regular ones.

3 | THE PRINCIPLE OF RAMAN SPECTROSCOPY

Among the methods of material science, non-destructive vibration spectroscopy (including Raman spectrometry) is known, which is a set of structural analysis methods based on the excitation of vibrational energy transitions and suitable for the production of chemical maps also. Raman scattering is an indirect way to excite vibrational transitions. Photons emitted by irradiated (monochromatic) high-intensity laser light undergo a change in frequency when they collide inelastically with a molecule. The magnitude of the frequency shift is the same as the vibration frequencies characteristic of the molecules of the test substance, so that the vibrations of the test substance can be identified from the spectra. The condition is a molecular structure, or a system of atoms connected by covalent bonds, as these result in a spectrum of sufficiently narrow bands to facilitate the identification of a given substance. Raman spectrometry usually detects molecular vibrations in the range of 3200–200 cm⁻¹.

Raman spectrometry complements infrared spectroscopy well, as both analyse the molecular vibrations of matter. The relative sensitivity of the two methods is different, so it is possible to study different molecular bonds and bond groups. There are several vibrational transitions that are inactive in infrared spectroscopy but active in Raman spectrometry, and vice versa. Raman spectrometry is mainly concerned with the most common chromophore groups, that is C-O, C-N, C=S, S=S, C-Cl bonds and C=C,
C=O, C=O, or C≡C bonds and bond groups, while IR spectroscopy is more sensitive to polar bonds and functional groups. Water with an intense IR absorption spectrum shows only a weak Raman scattering, so its presence during the measurement is not disturbing.

Chemical mapping is currently one of the most dynamically evolving areas of spectroscopy. The term “chemical mapping” refers primarily to methods based on vibrational (infrared and Raman) spectrometric techniques. Chemical mapping techniques combine the advantageous properties of a spectrometer and a microscope. The information carried by the vibration spectra, which is very characteristic of each molecule, can be combined with the possibility of spatial mapping, so the concentration and location of the components can be determined in heterogeneous systems. This method is becoming increasingly important in many fields of science and industry, from the semiconductor industry, medicine, the paper industry, the food industry, and the plastics industry to pharmaceutical technology.

A practical advantage of Raman spectrometry is that in most cases no sample preparation is required. Another major advantage is that due to the high power of the irradiated light, it is also possible to measure the samples through glass and plastic. The disadvantage of this method is that it can cause degradation in more sensitive samples and some substances show fluorescence, which significantly impairs the signal-to-noise ratio.

4 | RAMAN SPECTROSCOPIC TECHNIQUES

Raman spectroscopic investigation of the skin, changes in skin composition due to age or diseases, or analysis of drug delivery to the skin are mostly done by either acquiring spectra along a line perpendicular to the skin surface or by two-dimensional imaging. The former gives penetration depth profiles similar to those obtained by conventional methods such as tape stripping. The latter enables the visualization of drug (or other substances) distribution in the skin.

4.1 | Types of data acquisition

In general, spectra may be acquired in a point-by-point mapping (using a spot focus), line scanning (using a line focus) or global imaging approach (using wide-field illumination). Point-by-point mapping is the most frequently used approach. Here, spectra are acquired in each point in an x-y-coordinate system. In each point, only a small dimension (typically below 1 μm) is illuminated with the laser and a full spectrum from each point is recorded. Subsequently, a data cube is generated comprising the spectra and x-y-coordinates. From this datacube, parts of the information are extracted to form, for example a 2D colour-coded image of a drug substance inside a skin sample. This part of information may, in the easiest case, contain the intensities of the signal in one single wavenumber at each point in the x-y-coordinate system. In line scanning, the sample is illuminated along a line and the detector simultaneously detects the signals along this line. This is achieved either by conversion of the circular beam to a line focus using a cylindrical lens or by using a point focus to scan along a line. In global imaging, a two-dimensional area of the sample is illuminated. To this end, the laser is defocused which in turn leads to lower intensity of the incident light at the sample. The light is guided towards a detector comprising many thousand elements that acquires all signals simultaneously. Here, each wavenumber is detected one after the other. For imaging purposes, an area of interest may thus be mapped in a point-by-point approach or imaged by global imaging (depending on the type of instrument used). In point-by-point mapping, a full spectrum is acquired in each point. While the acquisition of one spectrum is very fast, the need for acquiring many spectra slows down the process. In global imaging, all points are illuminated at the same time but only one wavenumber is detected. In general, less light is guided to the detector than in point-by-point mapping, thus requiring longer acquisition times. This means that in the case that only one signal at one wavenumber is of interest, this approach is faster. But, if a full spectrum needs to be acquired, the process is slowed down substantially and may then be even slower than point-by-point mapping.

4.2 | Transmittance Raman spectroscopy

In transmittance Raman spectroscopy, the whole sample or a large part of it (typically several mm) is illuminated and the transmitted light is detected. The detected spectrum contains an average spectrum of the sample that it diffused through. It is thus not useful for generating spatially resolved information like it is aimed, for example in skin penetration research. If alterations in the overall spectrum are high enough, it may still be used, for example for quick detection of cancerous tissue. The main area of application remains non-destructive quantitative analysis of dosage forms like ointments, tablets or capsules.

4.3 | Coherent Raman scattering microscopies

Coherent Raman spectroscopy comprises two techniques: coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS). Both are stimulated and thus need two lasers: “pump” and “Stokes.” The frequency difference between the two lasers is tuned to match the molecular vibration of interest, that is a specific vibration of a drug substance or excipient. By creating a coherent, stimulated condition, signals many orders higher than in conventional Raman spectroscopy are created. In CARS, the molecular vibration, the lasers have been tuned to, creates a photon in the anti-Stokes part of the spectrum which is then detected. In SRS, the energy transfer from the Stokes...
to the pump laser is detected via a modulation transfer scheme. The CARS spectrum may be contaminated by non-resonant contribution which makes quantification challenging. Further, signal-concentration dependence is not linear but quadratic which needs to be compensated for using appropriate algorithms. The SRS spectrum does not contain non-resonant contamination and the signal-concentration dependence is linear. Both methods are most powerful when a specific molecular vibration of the substance under investigation either falls within the range of 2000–2700 cm\(^{-1}\), where there are no vibrations of the skin itself, or if they are deuterium labelled which makes vibrations fall into this range, too. In this range, the methods show limits of detection around 100 mM but this may increase drastically when signals overlapping with those of the skin are to be used. Tuning the lasers to multiple frequencies, that is acquiring hyperspectral data would improve this situation as more than one wavenumber could be used for evaluation but this drastically slows down acquisition speed. Further, many setups have limited tuning range.

### 4.4 Surface-enhanced Raman scattering (SERS)

Raman spectroscopy of the skin is often hampered by fluorescent background in the Raman spectrum and superposition of the signals of interest with the spectrum of the skin itself, making it difficult to generate high signal-to-noise ratios. Surface-enhanced Raman spectroscopy (SERS) uses nanostructured metal surfaces to generate Raman spectra that are several orders of magnitude higher than regular ones. To this end, a very thin sample specimen is adsorbed to a nanostructured metal surface like gold or silver. When illuminating the sample with an excitation wavelength that matches the plasmon resonance of the atoms at the surface of the specimen, a strong electromagnetic field is induced. The generated electromagnetic wave propagates along the surface of the specimen. As the Raman signal intensity relates to the amplitude of the electromagnetic field, the Raman modes of the molecules close to the metal surface are strongly enhanced. As a result, the incident and the scattered lights intensities are increased. Raman signal intensity enhancement can be explained by the high intensity of the electromagnetic field near the plasmonic substrate and thus by classical electromagnetic theory. On the contrary, there are also chemical enhancement effects. They are derived from the interactions of the samples’ molecules with the SERS substrate and can be explained by quantum mechanical electronic structure theory. SERS requires the sample to be prepared as a very thin sheet, carefully fixed to the metal surface which makes sample preparation rather elaborate. SERS substrates themselves require multiple factors to be carefully balanced which makes them elaborate to prepare as well. Furthermore, depth profiling is not feasible unless a large number of such very thin samples are prepared. The technique is therefore only rarely used in skin penetration analysis. Technical progress such as tip-enhanced SERS or the fabrication of microneedle probes will probably lead to more versatile instrumentation in the future making the method more convenient and increase its use in skin research.

### 4.5 Confocal Raman spectroscopy/microscopy

CRS is by far the most frequently used Raman technique to investigate penetration of exogenous substances into the skin or to analyse the skin in healthy or diseased state. Combined with a movable scan stage that enable precise positioning of the sample in three dimensions, imaging in two or three dimensions can be performed, also referred to as confocal Raman microscopy. A confocal Raman microscope consists of a laser light source, objective(s), beam splitter, pinhole, motorized scan stage, detector and optical fibres to guide the light from the laser source to the objective and from the objective to the detector. Furthermore, the fibres may also be used as pinhole to achieve confocality. The principle of confocality and the factors affecting it as well as resolution will be explained briefly hereafter. As in every optical method, the resolution is defined by the Rayleigh criterion (Equation 1).  

\[
\Delta x = 0.61 \times \frac{\lambda}{NA}
\]

where \(\Delta x\) is the distance or resolution or laser focal volume [nm], \(\lambda\) is the wavelength of the light [nm] and NA is the numerical aperture of the objective.

**Figure 1** shows an illustration of the Rayleigh criterion. On the left, the distance \(x\) between two point-like light sources is larger than \(\Delta x\) so that the two points can be differentiated. The situation in the middle shows two point-like structures at a distance \(x\) which is smaller than \(\Delta x\). The two points cannot be distinguished and appear as one spot. The distance \(\Delta x\) beyond which two point-like light sources cannot be detected as two points depend on the wavelength of the light and the numerical aperture of the objective. The longer the wavelength of the light and the smaller the numerical aperture of the objective the larger \(\Delta x\) will become. As a result, resolution decreases. The used wavelength is determined by the laser used and can only be altered if different lasers are at hand. The numerical
aperture is defined by the objective used and can more easily be altered by simply attaching different objectives to the microscope. In general, water or oil immersion objectives show higher numerical apertures compared to metallurgical objectives. Typically, green (532 nm) or red (785 nm) lasers are used in confocal Raman spectroscopy. With these lasers and an oil immersion objective with the numerical aperture of 1.25, the maximum theoretical resolution which can be achieved is 260 nm or 383 nm, respectively. In practice, the resolution also depends on the quality of the objective as well as on the sample and the interaction of the light with the sample. In depth profiling, a laser is focused to different depths inside the skin and spectra are acquired from these depths. Inside the sample, the light is scattered and absorbed which leads to depth-dependent signal attenuation. Furthermore, the laser focal volume broadens and the resolution therefore decreases with increasing depth inside the sample (Figure 1, right). As a result, spectra can only be obtained from up to 50 μm inside the skin. To counteract the effect of depth attenuation, the spectra acquired from deeper depths inside the skin need to be normalized to the signal at the skin surface. Otherwise, the decrease in drug concentration with increasing depth in the skin depth profiling in order to reject all rays from out of focus regions. Otherwise, spectra of substances, for example the drug on the skin surface may be erroneously detected inside a certain skin depth and lead to false penetration profiles.

In principle to types of microscope setups exist, upright and inverted microscopes. For skin Raman analysis, upright microscopes are used for in vitro or ex vivo studies whereas inverted microscopes are used for in vitro cell culture and in vivo measurements. In the upright setup, the skin sample is placed onto a movable scan stage and beneath the objective. In the upright setup, the skin sample is placed onto a movable scan stage and beneath the objective. A small pinhole and high degree of confocality are of pivotal importance in skin depth profiling. The distance between objective and sample can be adjusted by the scan stage. Thereby, the laser will be focused into different depths of the skin. In most cases, the skin is incubated beforehand in Franz diffusion cells or transwell plates. The samples are then withdrawn from the cells or plates and mounted onto special sample holders which guarantee skin hydration throughout the Raman analysis. A more recent development is a modified diffusion cell which can be mounted directly beneath the objectives of the microscope. The setup enables the incubation of the skin with the respective solution of formulation directly underneath the Raman microscopes objective. Thereby, Raman spectra can be acquired at any timepoint, enabling in situ analysis of penetration kinetics.

For in vivo analysis, an inverted microscope setup is used. It comprises an oil immersion objective which is located beneath a measuring window onto which the respective body part may be placed for analysis. The depth into which the laser is focused is adjusted through the distance between objective and measuring window. The use of the inverted setup shows the advantage that the skin is always in full contact with the measuring window and will thus not be moved out of focus due to involuntary movements of subjects. A disadvantage is that (at least to date) this system does not allow for measurements in

![Figure 2](image)

**Figure 2** Illustration of reflection—sample surface, and microscope setup of confocal microscopy. Left: metallurgical objective, middle: oil immersion objective and right: the propagation of light through a confocal microscope.
FIGURE 3  The schematic profiles of the off-line device (A) of Franz diffusion cell for skin incubation and in-line device (B) of skin incubation cells used (reproduced from Pharmaceutics 2021, 13, 67. https://doi.org/10.3390/pharmaceutics13010067; Creative Commons Attribution (CC BY) licence (https://creativecommons.org/licenses/by/4.0/)).

FIGURE 4  Number of publications in PubMed database with the search term (A) "Raman spectroscopy" and (B) "Raman spectroscopy skin." Changes over time (C) in the proportion of skin research in all Raman spectroscopy articles and (D) in the number of publications on Raman spectroscopy in skin research regarding in vivo/ in vitro/ ex vivo applications (The PubMed search was finalized by 25 February 2022).
two or three dimensions thereby prohibiting imaging/mapping. Solving this issue should only be a matter of software capabilities, and it can be expected that this drawback will be resolved in the future.

5 | RAMAN SPECTROSCOPY IN SKIN RESEARCH FROM THE BEGINNINGS BASED ON PUBMED DATABASE

Though the Raman effect underlying the Raman spectroscopy was described in 1928, it could become widespread only after the invention of lasers in the 1960s. The PubMed database contains 15 publications between 1947 and 1960 with the keywords “Raman spectroscopy.” In the next decade, an average of less than 5 publications per year was published, followed by a few dozen articles per year in the 1970s, when the microscopic Raman with an optical microscope equipped with a Raman spectrometer has also been invented. Since the introduction of the near-infrared region Fourier transform Raman spectroscopy enabled the application of the technique in biomedical studies, the number of relevant papers has steadily increased, reaching more than 1000 articles in 2005 and over 4400 in 2021, as it is shown in Figure 4A,B.

There are a few articles between 1975 and 1990 which are related to skin studies, but in these cases, they are mostly about the study of different molecules isolated from the skin (e.g. peptides isolated from the skin of Xenopus laevis, proteodermatan sulphate from pig skin). The first studies on the skin composition were published in the late 1990s (e.g. comparing the structure of water, proteins and lipids in intact human skin, or analysing the molecular structure of 5200-year-old skin). The annual number of papers on the use of Raman spectroscopy in skin research has already reached 100 in recent years. Which means that, although this is only a few per cent (2%-3%) of all Raman spectroscopy papers, its proportion is slowly increasing, presented in Figure 4C,D.

Nearly one third of hits on Raman spectroscopy in skin research are not labelled either in vitro, in vivo or ex vivo, including, for example, structural studies of molecules isolated from skin. A further one third of the articles deals with in vivo applications, such as diagnosis of skin cancer, or monitoring the changes due to ageing. The remaining part of the relevant papers used ex vivo analysis for instance for cancer discrimination, or for classification of burn severities, and in vitro applications, such as using in vitro skin substituents.

6 | UTILIZATION OF RAMAN SPECTROSCOPY IN SKIN RESEARCH

6.1 | Skin composition studies

Confocal Raman Spectroscopy (CRS) is being extensively used to analyse skin composition. The basic analysis of skin compounds under physiological conditions comprises a large body of data. Among other vibrational spectroscopic techniques available for this task such as Attenuated Total Reflection Fourier Transform Infrared Spectroscopy, CRS has been shown to provide significantly richer spectroscopic detail and to differentiate between SC and underlying epithelial layers in skin tissue sections of human skin and artificial skin models. Data on skin biochemistry by CRS has stepwise been established for intact and diseased skin in the last 20 years. The steps along the way are discussed in the following section and recent examples are given.

CRS has been used to study the molecular composition of human skin in vivo, tissue sections ex vivo, and skin substitutes in vitro. The Raman spectrum of human SC is dominated by the vibrational bands of its structural proteins, amino acids and lipids. Both human skin and animal skin can be analysed by CRS (Figure 5).

Spectral bands were first assigned by Barry et al. and are summarized in recent literature. The most prominent bands are given in Table 1.

Changes in barrier function of a reconstructed human epidermis model caused by frozen storage have been studied. Both storage time and temperature were relevant factors. Molecular properties of human skin in vivo have been analysed for different age groups; differences in skin biochemistry have been shown for elderly and infant skin.

Apart from human skin, animal skin models have been assessed. Porcine skin is perhaps most frequently used due to its relative similarity to human skin in morphology and barrier function. Differences of skin models to their natural counterpart based on CRS analysis of their molecular composition provide insights into the underlying cause of lower barrier function as known for reconstructed skin models and porcine skin ex vivo. Choe et al. conducted an in-depth analysis of SC molecular profiles of human skin in vivo and porcine ear skin. The SC depth profiles of porcine skin exhibited lower natural moisturizing factor (NMF) content throughout the entire SC and depth-dependent differences in hydrogen bonding states of water. A higher content of keratin in β-sheet form and higher hexagonal lateral packing order of intracellular lipids was observed at 10%-50% SC depth, which permits penetration of both hydrophilic and lipophilic entities more easily than in human skin.

Of particular interest when investigating the effect of dermal preparations is skin lipid composition. The effect of surfactants on the SC lipid matrix can be observed by CRS through changes in lipid content via lipid/protein ratio, changes in conformation and in lateral packing order. Non-ionic polysorbate emulsifiers and ethoxylated ethers with a small number of oxyethylene groups were found to cause smaller lipid perturbations than, for example PEG-20 ethers. Further, the NMF as a collection of hygroscopic molecules derived from the protein filaggrin in the SC can be analysed by CRS; the contribution of free amino acids such as serine, glycine, pyrrolidone-5-carboxylic acid, arginine, ornithine, citrulline, alanine, histidine and urocanic acid is usually taken into account to obtain information on the state of skin hydration and barrier function. The analysis of NMF in atopic skin has been validated against tape stripping/HPLC analysis; comparable results were obtained with both methods.
The water content within the skin, in particular the SC, is important to keep the skin barrier functional and is therefore routinely investigated in dermatological studies. CRS can be used to calculate the water mass concentration profile as a function of depth based on the ratio between the intensity of the OH vibration and keratin vibration.\textsuperscript{52} Skin hydration, that is the water molecules present in the SC that are involved in maintaining elasticity and physiological molecular structures, has been intensively studied by CRS.\textsuperscript{77,86,87} For the mentioned aspects, the bound water content is of particular importance since it is associated with intracellular lipids and proteins via hydrogen bonds.\textsuperscript{86} In this context, the hydrogen bonding state of water as determined by CRS was found to reach a maximum at a SC depth of 30\% of its entire thickness, correlating well with the maximum lateral packing order of the intercellular lipid matrix and the NMF content.\textsuperscript{86} Atmospheric relative humidity was reported to affect the partially bound states of water within the SC.\textsuperscript{88} At intermediate relative humidity around 60\% both lipid organization and protein deployment were optimal, representing the maximal SC water binding capacity. An increased content of unbound water within the SC, however, was associated with disordered lipid and protein states. Recently, the application of heavy water was investigated by CRS\textsuperscript{89}; different ethoxylated emulsifiers were dissolved in D\textsubscript{2}O to probe their effect on skin biochemistry while avoiding additional effects on the OH bond of the skin spectrum through externally applied water. The D\textsubscript{2}O distribution within the skin was well discernible due to its OD stretching band and was clearly affected by the co-applied selected ethoxylated emulsifiers in dependence of their chemical structure and the positive control sodium laureth sulphate (SLES).

The effect of dermally applied preparations on hydration status and NMF levels has been explored by CRS for washing procedures with different cleaning products\textsuperscript{90,91} and cosmetic emollients,\textsuperscript{92} but also for drug delivery systems in vivo.\textsuperscript{93,94} Normal skin, but also the lip region have been investigated.\textsuperscript{95} Titanium dioxide nanoparticles, as frequently used in cosmetics for sun protection, were found to promote structural rearrangement of the SC lipid bilayers under controlled indoor illumination,\textsuperscript{96} which underlines the advantages of CRS in toxicological investigations.
Apart from the effect of chemical stressors, the impact of physical procedures such as tape stripping, microneedling or hair removal, is possible, but less promising since not all physical stressors evoke significant changes in the chemical composition of the skin. In addition, potential changes in SC thickness due to physical removal of corneocytes have to be taken into account in data evaluation. In a recent study, the effect of iontophoresis and sonophoresis on barrier function alone and in combination with azone as enhancer was shown for a model permeant using mouse skin and surface-enhanced Raman spectroscopy; the best option for oxaprozin delivery was found to be sonophoresis method, it is non-invasive, faster and requires less sample processing. Thus, CRS has become an important asset in cosmetic and pharmaceutical formulation development. Likewise, it is used to evaluate potentially hazardous permeants in toxicological studies. For these tasks, different membranes are used: human skin in vivo, human skin ex vivo, in vitro skin substitutes and porcine skin. Rodent skin is rarely due to its inherent differences to human skin.

6.2 | Skin penetration studies

In analogy to its role in skin composition analysis, CRS has found a solid place in skin penetration studies. In comparison with other vibrational spectroscopic techniques used to analyse drug penetration, for example the ATR-FTIR-spectroscopy/tape stripping method, it is non-invasive, faster and requires less sample processing. Thus, CRS has become an important asset in cosmetic and pharmaceutical formulation development. Likewise, it is used to evaluate potentially hazardous permeants in toxicological studies. For these tasks, different membranes are used: human skin in vivo, human skin ex vivo, in vitro skin substitutes and porcine skin. Rodent skin is rarely due to its inherent differences to human skin.

Caffeine, lidocaine, procaine, tetracaine, salicylic acid, ibuprofen, flufenamic acid, imiquimod, trans-retinol, resorcinol, niacinamide, sulfathiazole sodium, trimacinolone acetone and oxaprozin are among the most investigated permeants in the CRS penetration studies. Next to these drugs, the penetration of enhancers such as DMSO, propylene glycol, polyoxyethylene-23-lauryl ether and other ethoxylated emulsifier systems has been evaluated.
The skin penetration of classic anionic surfactants such as sodium dodecyl sulphate (SDS) or sSLES has likewise been investigated in numerous studies, often as positive control.\textsuperscript{54,123,124} Regarding skin penetration of additives used in cosmetic or pharmaceutical preparations, data on the penetration behaviour of oils or waxes,\textsuperscript{49} preservatives\textsuperscript{120} or sunscreen agents\textsuperscript{125} have been presented.

In dependence of the experimental setup used, both two- and three-dimensional information on the location of applied permeants is obtained. To facilitate visualization of drugs next to solvents or enhancers such as octanol or propylene glycol and to obtain lateral spatial distribution of the applied materials, the use of deuterated compounds is a valid approach.\textsuperscript{105} When using complex vehicles, overlapping of formulation compounds with numerous skin bands has to be avoided.\textsuperscript{104} However, monitoring the spatial distribution of chemically similar species in CRS penetration studies, for example prodrug and drug, is possible without labelling for compounds with a suitable spectroscopic profile.\textsuperscript{125}

The experimental conditions for CRS penetration studies should be kept constant during the analysis. Artefacts can arise from changes in skin biochemistry during analysis caused by prolonged measurement time (occlusion, changes in temperature, etc.). Skin temperature and hydration of porcine skin samples were found to affect results,\textsuperscript{109} potentially due to crystallization of the applied drug. This should be kept in mind when comparing results obtained with different experimental setups.

An important question in CRS penetration studies is how to obtain quantitative drug penetration profiles. So far, most studies have delivered information on permeant distribution within the skin on a relative scale. Approaches for quantitative analysis of permeants have been proposed.\textsuperscript{128} In recent years, Caspers et al.\textsuperscript{119} have moved forward in this respect; the first fully quantitative approach analysing the skin penetration of niacinamid from various formulations into human skin was studied both in vitro using classic Franz-type diffusion cells and in vivo by quantitative CRS. The correlation after linear regression between cumulative drug amounts in vitro and penetrated drug at 2 μm SC depth in vivo was reported with $R^2 = 0.98$. Further research will provide more insight into quantitative CRS analysis for different applications to determine its role in future bioequivalence studies.

Among the most important conclusions derived from the summarized penetration data is the importance of selecting suitable experimental parameters for CRS instrumentation such as choice of objective and pinholes,\textsuperscript{43,44} and skin samples. Recently, penetration studies performed in parallel with a common multi-purpose confocal Raman spectroscopy by WiTec (alpha 500) and the River Diagnostics device (gen2 SCA) were found to deliver comparable outcomes.\textsuperscript{127} Binder et al. obtained similar enhancement ratios for procaine HCl when using polyoxyethylene23-lauryl ether to promote skin penetration. This is promising considering that instruments of two different suppliers were used in different laboratories, working with different objectives, pinholes and laser wavelengths. Aspects to be kept in mind are the specific mode of data evaluation and substance-specific aspects such as resonant effects.\textsuperscript{127}

### 6.3 | Skin diseases

As mentioned before, articles on the applicability and utility of Raman spectroscopy for dermatological purposes first appeared in the 1990s.\textsuperscript{1,2} Shortly afterwards, increasing number of results were published on Raman spectroscopic investigation of cancerous skin tissues.

Next to healthy skin, analysis of pathological conditions has been the target of numerous CRS investigations, for example on atopic dermatitis,\textsuperscript{128,129} and psoriatic lesions.\textsuperscript{130} A very specific application in context with unphysiological skin conditions is the identification of multicoloured pigments in tattooed human skin preceding laser removal.\textsuperscript{131} The effect of applied substances on skin biochemistry, in particular on the SC lipid matrix and barrier function, has been investigated for numerous common additives in dermal preparations: oils,\textsuperscript{95,132} emulsifiers,\textsuperscript{54,83,84,133,134} solvents or other penetration enhancers.\textsuperscript{52} Strategies have been proposed on how to optimize biochemical analysis of the skin by CRS considering spectral variability,\textsuperscript{97} impact of applied chemicals,\textsuperscript{87} or keratin distribution throughout the SC.\textsuperscript{135}

Skin cancer is one of the common cancers. Several types of melanomal and non-melanomatous (basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)) cancers\textsuperscript{136} can be distinguished. The first step in the medical diagnosis of the disease is a visual examination,\textsuperscript{137} followed by skin biopsy and histopathology.\textsuperscript{136,137} Thanks to increasingly sophisticated data processing methods, it became possible to fully differentiate between cancerous and non-tumorous tissues\textsuperscript{3-5} and between different types of skin cancers, and to determine a demarcation line of the diseased tissue.\textsuperscript{6-9} The technical advances have made it possible to apply the technique clinically for examination and diagnosis of various, primary skin cancers.\textsuperscript{10-15} On application of Raman spectroscopy for diagnostic purposes, some sample papers are shown in Table 2.

### 6.4 | Applications in cosmetoscience

In addition to the dermatological use of Raman spectroscopy, its use for cosmetology purposes is gaining ground, as it is becoming important to know the mechanism, exact extent and depth of penetration of different active ingredients and drugs furthermore provides information on the distribution of these molecules in different layers of the skin. In vivo Raman spectroscopy is a non-invasive, sensitive method that offers an effective, easy-to-use solution for all these requirements.

The penetration of several popular molecules in the cosmetic industry has been investigated by Raman spectroscopy. The tested active ingredients provide solutions to various skin problems (e.g. acne, skin ageing, hydration, anti-inflammatory problems and sun protection). Essendoubi et al. studied three different hyaluronic acid derivatives (Cristalhyal (1000–1400 kDa), Bashyal (100–300 kDa) and Renovhyal (20–50 kDa)) on plastic abdominal skin samples removed by plastic surgery.\textsuperscript{138} Tfaili et al.\textsuperscript{139} performed penetration
The large amount of data from Raman spectroscopy allows, and at the same time may require the use of artificial intelligence in data analysis.
to extract meaningful information and make predictions based on the data collected. In the literature, a myriad of different application fields is available, where the measured Raman spectra were the input data of machine learning, mostly by classification algorithms. A large portion of the studies focuses on non-medical applications, with an emphasis on adulteration detection in the food industry. Other part of the articles describe Raman spectroscopic techniques combined with machine learning algorithms to improve diagnostic measures for identifying diseases such as infections, cancer, neurodegenerative disorder or different skin conditions. A summary on application of artificial intelligence in Raman spectroscopic data analysis can be found in Table 3.

8 | REGULATORY ASPECTS OF RAMAN SPECTROSCOPY IN SKIN TESTING

Modelling drug permeation through the skin is a complex challenge. Although there are many quantitative and qualitative methods for following-up skin penetration, the different techniques are not fully equivalent but complement each other. Therefore, the regulation of dermal and transdermal formulations has received increasing attention nowadays. There are more and more guidelines to provide harmonization for dermal and transdermal testing. The Organization for Economic Cooperation and Development (OECD) published several documents about this topic, including: Guidance Notes on Dermal

| Sample | Details | Machine Learning methods | Ref. |
|--------|---------|--------------------------|------|
| Non-medical applications | | | |
| Butter | Detection of adulteration of butter with margarine | PCA, PCR, PLS, ANN | 173 |
| Caviar | Discrimination between three different caviar types | PCA, LDA, ANN | 174 |
| Edible oils | Edible oil authentication (sesame, hemp, walnut, linseed, pumpkin, sea buckthorn) | Ensemble classifier-subspace KNN when the PCA was disabled | 175 |
| Fruit distillates | Trademark fingerprint differentiation, geographical discrimination | DT, DA, SVM, KNN other ensemble classifiers | 176 |
| Honey | Detection of low-concentration adulterated Suichang native honey | CNN, PNN, SVM | 177 |
| Milk | Differentiation between milk from different species (cow, buffalo, goat and human) | PCA, RF | 169 |
| Minerals | Recognition of minerals and estimation their elemental composition | CNN, KNN, SVM, extremely randomized trees, weighted-neighbours | 178 |
| Poplar | Prediction of the lignin content in poplar wood samples | DT, SVM ensemble classifiers (LightGBM, CatBoost, XGBoost) | 179 |
| Medical applications | | | |
| Alzheimer’s disease | Alzheimer’s disease diagnosis based on the analysis of cerebrospinal fluid | ANN, SVM-DA | 180 |
| Alzheimer’s disease | Alzheimer’s disease diagnosis based on the analysis of saliva | ANN, GA | 181 |
| COVID-19 infection | Diagnosis of COVID-19 infection based on saliva samples | MIL | 182 |
| Tuberculosis infection | Distinction between tuberculosis positive (diseased), negative (cured) and control (healthy) serum samples | PCA, HCA | 183 |
| Breast cancer | Classification of breast cancer subtypes | PCA-DFA, PCA-SVM | 184 |
| Colorectal cancer | Prediction the effect of immunotherapy | SVM, RF | 185 |
| Lung cancer | Cytopathological diagnosis of lung cancer | KNN, SVM | 186 |
| Skin cancer (basal/ squamous cell carcinoma) | Distinction between basal cell carcinoma, squamous cell carcinoma and healthy skin tissues and cells | CNN, LR, SVM | 187 |
| Skin cancer (melanoma) | Distinction between benign versus malignant melanoma tissues | LightGBM, KNN, XGBoost | 188 |
| Atopic dermatitis | Stratification of severity in atopic dermatitis | SVM | 189 |
| Burn injury | Classification of burn injury | LR, SVM, RF | 190 |
Absorption (No. 156), and Test Guideline 427 (in vivo methods) and Guidance Document for the Conduct of Skin Absorption Studies. There are some other documents: World Health Organization International Programme on Chemical Safety (WHO/IPCS) Environmental Health Criteria 235, European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Monograph 20, United States Environmental Protection Agency (USEPA) report on dermal exposure assessment, European Food Safety Agency (EFSA) Guidance on dermal absorption for plant protection products, and the European Medicines Agency (EMA) document, Draft Guideline on Quality and Equivalence of Topical Absorption (No. 156), Test Guideline 427 (in vivo methods) and Guidance Document for the Conduct of Skin Absorption Studies. There are some other documents: World Health Organization International Programme on Chemical Safety (WHO/IPCS) Environmental Health Criteria 235, European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Monograph 20, United States Environmental Protection Agency (USEPA) report on dermal exposure assessment, European Food Safety Agency (EFSA) Guidance on dermal absorption for plant protection products, and the European Medicines Agency (EMA) document, Draft Guideline on Quality and Equivalence of Topical Absorption (No. 156), Test Guideline 427 (in vivo methods) and Guidance Document for the Conduct of Skin Absorption Studies.

9 | SUMMARY

Simplicity in using and collecting data, as well as conducting analyses without the need for prior labelling and complicated sample preparation, has resulted in increased interest and a significant increase in the use of Raman spectroscopy in the field of life sciences including skin research and dermatologic diagnosis. Raman spectroscopy techniques used for research on biological material, that is skin tissues, provide insight into the structure and organization of the dermal barrier. Raman microscopy is a precise tool for the study of the structural polymers, metabolites, lipids, proteins and water content in the tissues. Owing to its exceptional sensitivity, this method can distinguish even subtle differences among regions with different chemical composition and structure. It allows the researcher to study the processes taking place in the skin and the effectivity of different topical formulations. The introduction of new and improved Raman techniques by researchers, as well as continued technological development of the apparatus, has resulted in an increased significance of Raman spectroscopy in the discovery and definition of tissues and the processes taking place inside them. The Raman technique is a promising direction for science resulting in the discovery of skin and artificial skin tissues as well as their identification and characterization.

This method offers extensive potential for use in further scientific work on dermatological disease diagnosis, drug and cosmetic penetration studies and evaluation of enhancer technologies (both chemical and physical methods). In the future, special attention should be paid to the use of Raman techniques for carrying out the research on the impact of various internal and external factors, such as ageing, disease conditions, environmental changes and environmental stress on biological processes in the skin barrier.

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AUTHOR CONTRIBUTIONS

DL: Raman spectroscopic techniques, VK: Utilization of Raman spectroscopy in skin research, DK: Analysis of PubMed database for Raman spectroscopy; artificial intelligence for evaluation of Raman results, FE, ZV-M: Applications of Raman spectroscopy in skin diseases and cosmeceuticals; ZV-M: list of references SB: Principle of Raman spectroscopy and regulatory aspects, FE: History of Raman spectroscopy, introduction and summary.

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CONFLICT OF INTEREST

No conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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