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HR-MAS NMR Spectroscopy of Reconstructed Human Epidermis: Potential for the *in Situ* Investigation of the Chemical Interactions between Skin Allergens and Nucleophilic Amino Acids

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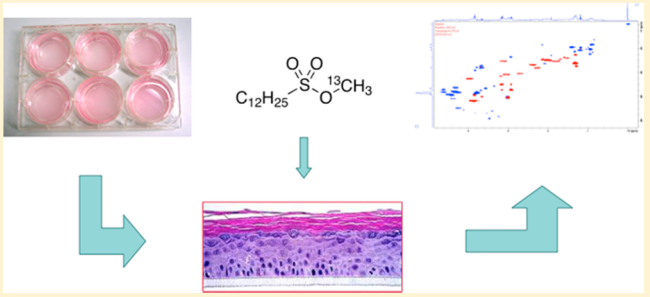
Supporting Information

ABSTRACT: High-resolution magic angle spinning (HR-MAS) is a nuclear magnetic resonance (NMR) technique that enables the characterization of metabolic phenotypes/metabolite profiles of cells, tissues, and organs, under both normal and pathological conditions, without resorting to time-consuming extraction techniques. In this article, we explore a new domain of application of HR-MAS, namely, reconstructed human epidermis (RHE) and the *in situ* observation of chemical interactions between skin sensitizers and nucleophilic amino acids. First, the preparation, storage, and analysis of RHE were optimized, and this work demonstrated that HR-MAS NMR was well adapted for investigating RHE with spectra of good quality allowing qualitative as well as quantitative studies of metabolites. Second, in order to study the response of RHE to chemical sensitizers, the (^{13}C)methyldodecanesulfonate was chosen as an NMR probe, and we compared adducts formed on human serum albumin (HSA) in solution and adducts formed in RHE. Thus, while the modification of proteins or peptides in solution takes several days to lead to a significant amount of modification, in RHE the modifications of nucleophilic amino acids were observable already at 24 h. The chemioselectivity also appeared to be different with major modifications taking place on histidine, methionine, and cysteine residues in RHE, while on HSA, significant modifications were observed on lysine residues with the formation of methylated and dimethylated amino groups. We thus demonstrated that RHE could be used to investigate *in situ* chemical interactions taking place between skin sensitizers and nucleophilic amino acids. This opens perspectives for the molecular understanding of the skin immune system activation by sensitizing chemicals.



INTRODUCTION

High-resolution magic angle spinning (HR-MAS) is a nuclear magnetic resonance (NMR) technique[1−3] that enables the characterization of metabolic phenotypes/metabolite profiles of cells, tissues, and organs, under both normal and pathological conditions.[4,5] This technique affords a detailed and accurate analysis of the metabolic composition of intact tissue specimens without resorting to time-consuming extraction techniques. Several human gynecological, neurological, and urological malignancies have thus been studied by HR-MAS with promising results, and the technique has been applied to a variety of pathologies[6] in the brain,[5,7−10] breast,[11,12] prostate,[13−15] kidney,[16−18] cervix,[19,20] esophagus,[21] ovary,[22] and colon.[23,24]

More recently, our studies have demonstrated HR-MAS to be a promising tool in new domains such as organ transplantation by allowing the assessment of the quality of the graft[25] and surgical operations as an additional diagnostic tool providing real-time information.[26]

In this article, we explore a new domain of application of HR-MAS, namely, reconstructed human epidermis (RHE) and the *in situ* observation of chemical interactions between carbon 13 substituted skin sensitizers and nucleophilic amino acids. Indeed, allergic contact dermatitis (ACD) or skin allergy is a very common inflammatory disease resulting from a cutaneous contact with chemical allergens. In the physiopathology of this disease, the key step for induction and/or elicitation of a skin allergic reaction is the interaction between a reactive chemical or hapten and nucleophilic residues on amino acid side chains of skin proteins.[27,28] Such modified proteins, recognized as

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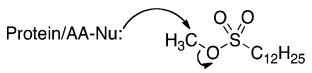
foreign by the immune system, will initiate a cascade of immunological events leading to the selection and activation of T-cell subpopulations with an appropriate T-cell receptor able to recognize the chemical modification induced on proteins.[29] Thus, chemical allergens (or their metabolites) are low molecular weight molecules, lipophilic enough to cross the horny layer and penetrate the epidermis, and with an electrophilic chemical reactivity allowing the formation of covalent links with nucleophilic residues on amino acid side chains. On the basis of this mechanistic understanding, a number of studies have been conducted in order to detect, characterize, and quantify the process of protein haptenation. So far, investigations have been performed using either isolated model nucleophiles such as *n*-butylamine and isolated *N*-acetyl amino acids[30−32] or more complex targets such as GSH, model peptides, and proteins.[33−35] Such studies have provided a set of information on the reactivity between specific chemicals and nucleophilic amino acids but also evidenced a mechanistic complexity not initially predicted.[36] Indeed, these studies carried out in solution (buffer or in semiorganic) are very far from modeling interactions that may happen between a xenobiotic and nucleophiles in a complex heterogeneous tissue. There is therefore a very strong need to develop more complex models in order to study and better understand the mechanism of skin sensitization.

Since the 1980s, various reconstructed human epidermis (RHE) models have been developed as alternative methods to animal experiments for the development of cosmetic, chemical, and pharmaceutical compounds. As keratinocytes represent the major cell type in the epidermis and play a key role in skin inflammatory reactions, keratinocyte cultures were chosen for the development of these models. RHE models show high similarity with the *in vivo* human epidermis in terms of morphology and metabolic activity[37,38] and have therefore been used for metabolic studies,[39] for assessment of skin penetration,[40] for the evaluation of the skin irritancy and corrosion potential of chemicals,[41,42] and for predicting the epidermal responses to irritants and sensitizers.[43,44]

In order to study the response of RHE to chemical sensitizers, (^{13}C)methyldodecanesulfonate was chosen as an NMR probe (Scheme 1). Indeed, this easily accessible methylating agent was previously reported as a good model to identify the nature of reactive amino acids in proteins.[45] Moreover, methyl alkanesulfonates were demonstrated to cause severe ACD in guinea pigs[46] and mice.[47]

Scheme 1. Structure and Reactivity of Methyldodecanesulfonate toward Nucleophilic Residues of Amino Acids/Proteins



We now report the results of our investigations on RHE and chemical sensitizer treated RHE by using HR-MAS. On the basis of high−resolution spectra, we were able to determine the individual metabolites and amino acids as well as fatty acids and macromolecules present in this RHE model. Our results also demonstrate for the first time, the potential of the HR-MAS NMR spectroscopy applied to RHE in the investigation of chemical sensitizers/epidermal tissue that may result in improved accuracy and comprehension of skin allergies.

It must be emphasized that reliable results on RHE samples required adjustments of protocols at various stages of this work and the use of a carbon 13 substituted allergen. The methodology described in the following text was embedded in the context of a medical environment where tissue samples are collected, stored, and analyzed in a reliable matter to prevent sample cross-contamination and sample alteration.[48]

EXPERIMENTAL PROCEDURES

Caution: *Skin contact with methyl dodecanesulfonate must be avoided. As a potential sensitizing substance, this compound must be handled with care*

Chemicals and Reagents. Methyl dodecanesulfonate and (^{13}C)-methyl dodecanesulfonate were synthetized as previously described.[45] (^{13}C)methyl iodide and deuterated solvents were purchased from Euriso-Top (Saint Aubin, France). All other chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) unless otherwise noted and used without further purification. Aqueous solutions were prepared with deionized water. Human serum albumin (HSA) fraction V was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Reconstructed Human Epidermis. For this study, the SkinEthic RHE model (SkinEthic, Lyon, France) was chosen as it could be obtained in a large 4 cm^2 format. SkinEthic RHE are normal human keratinocytes cultured for 17 days on an inert polycarbonate filter at the air−liquid interface. The 3D reconstructed epidermis were received on day 18, aseptically removed from the transport medium, and preincubated for 2 h in a growth culture medium (SkinEthic, Lyon, France) at 37 °C, 5% CO_2, and humidified atmosphere, according to SkinEthic's procedures.

Treatment of RHE with Methyl Dodecanesulfonate and Controls. SkinEthic RHE were topically treated with methyl dodecanesulfonate or (^{13}C)methyl dodecanesulfonate in acetone (0.6M, 100 μL) and postincubated for 24 and 48 h, respectively. This concentration is about 10% of the usual doses used for skin irritation tests routinely performed on RHE.[44] RHE negative controls were either nontreated or treated with acetone (100 μL) and postincubated for 24 h. After postincubation, the RHE were washed with fresh medium, separated from the polycarbonate support using a treatment with Dispase II (neutral protease, grade II, Roche, Mannheim) in HEPES buffer *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), washed with water, and stored at −80 °C before NMR sample preparation.

Rotor Preparation. Each sample was prepared at −20 °C by introducing 15 to 20 mg of frozen RHE into a disposable 30 μL KelF insert. To provide a lock frequency for the NMR spectrometer, 10 μL of D_2O containing 1% w/w TSP was also added to the insert. The insert was then sealed tightly with a conical plug and stored at −80 °C until the HR-MAS analysis. The insert insures that the entire RHE sample is detected by the radio frequency coil of the probe and that no leaks occur during the HR-MAS analysis. Shortly before the HR-MAS analysis, the insert was placed into a standard 4 mm ZrO_2 rotor and closed with a cap. The ensemble was then inserted into a HR-MAS probe precooled at 3 °C. All HR-MAS experiments were performed at 3 °C and were started immediately after the temperature inside the probe had reached equilibrium conditions (5 min). Upon completion of the HR-MAS analysis, the insert was taken out of the rotor and stored back at −80 °C. Therefore, complementary NMR analysis can be performed at a later stage on any of the stored samples inserts.

HR-MAS Analysis: Data Acquisition. HR-MAS spectra were recorded on a Bruker Avance III 500 spectrometer (Hautepierre University Hospital, Strasbourg) operating at a proton frequency of 500.13 MHz. The spectrometer was equipped with a 4 mm double resonance (^{1}H, ^{13}C) gradient HR-MAS probe. A Bruker Cooling Unit (BCU) was used to regulate the temperature at 3 °C by cooling down the bearing air flowing into the probe. All NMR experiments were conducted on samples spinning at 3502 Hz in order to keep the rotation sidebands out of the spectral region of interest. Shimming procedure of the HR-MAS probe is described elsewhere.[49] For each sample, a one-dimensional proton spectrum using standard one-pulse and Carr−Purcell−Meiboom−Gill (CPMG)[50] pulse sequences was acquired (Bruker *cpmgpr1d* pulse

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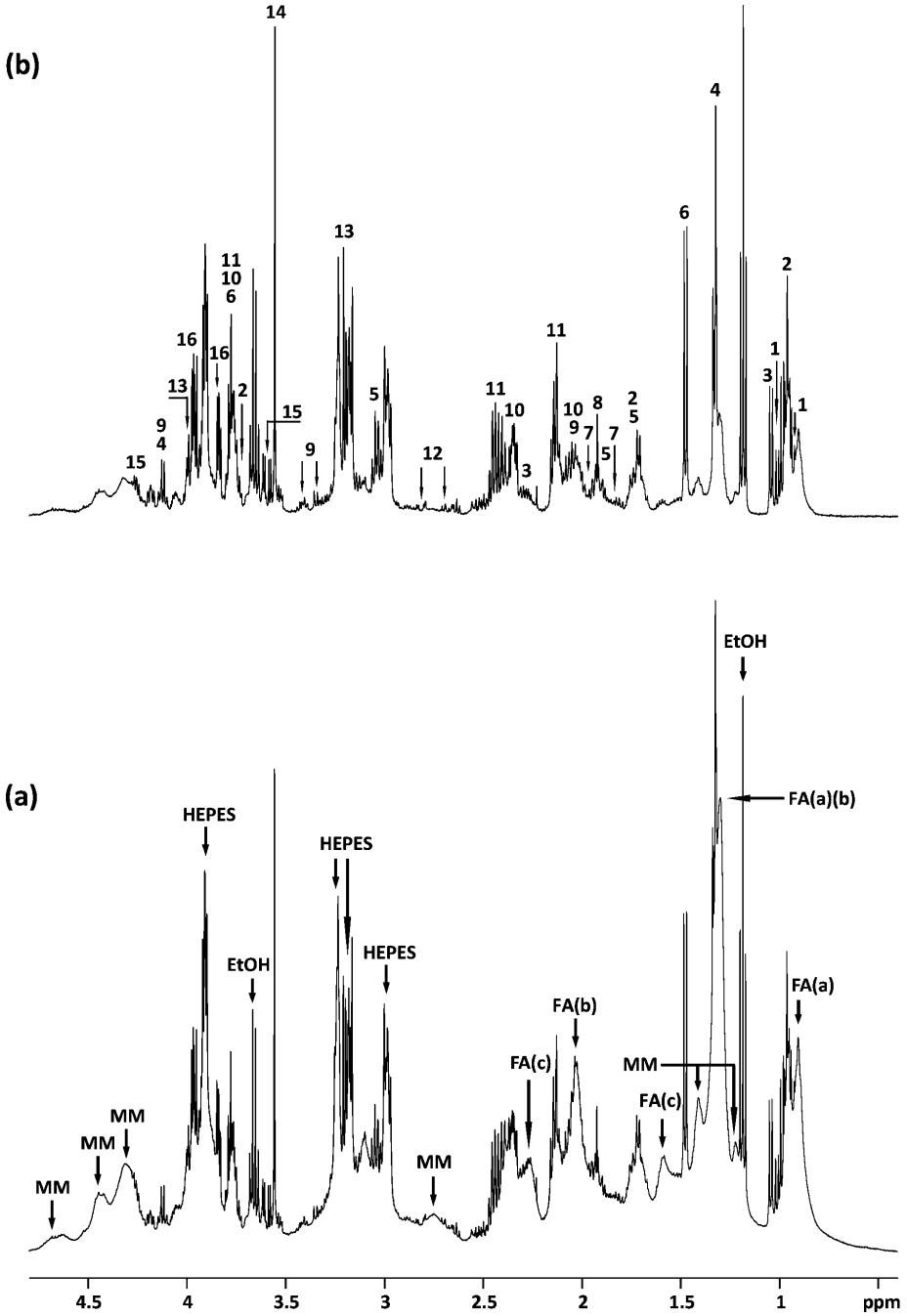

Figure 1. Representative 1D ^{1}H HR-MAS (a) One-Pulse and (b) CPMG spectra of reconstructed human epidermis sample at 500 MHz. Characteristic metabolites, fatty acids, and macromolecules are annotated. The numbers refer to the metabolites listed in Table 1.

sequence). Both sequences were coupled with water suppression/presaturation. The CPMG sequence allows a better observation of signals by reducing the intensity of those of macromolecules and lipids in the spectrum. The interpulse delay between the 180° pulses of the CPMG pulse train was synchronized with the sample rotation and set to 285 μs ($1/\omega_r = 1/3502 = 285\ \mu s$) in order to eliminate signal losses to B_1 field inhomogeneities.[50−52] The number of loops was set to 328 giving the CPMG pulse train a total length of 93 ms.

In order to quantify the amount of molecules present in each sample, a calibrated digital Eretic signal[48] was included in all the 1D experiments. It must be emphasized that some metabolites remain hidden and do not give sharp resonances because of the restricted motion due to compartmentalization or binding to macromolecular structures. Using a standard solution of known concentration of lactate, the amplitude of the Eretic signal was calibrated to correspond to 1.93 μmol of protons. In order to accurately calculate molecule concentration in each sample, a correction factor taking into account the difference in pulse widths between the standard lactate sample and the different skin samples was also applied.[53] One-pulse and CPMG experiments were acquired with the following parameters: sweep width, 14.2 ppm; number of points, 32k; relaxation delay, 2 s; and acquisition time, 2.3 s. A total of 256 FID (free impulsion decay) were acquired resulting in an acquisition time of 19 min. All spectra were recorded in such a manner that only a zero phase order correction was necessary to properly phase the spectrum. The FID was multiplied by an exponential weighing function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation. All spectra were processed using automatic baseline correction routines. Spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm in ^{1}H and to 22.7 ppm in ^{13}C.

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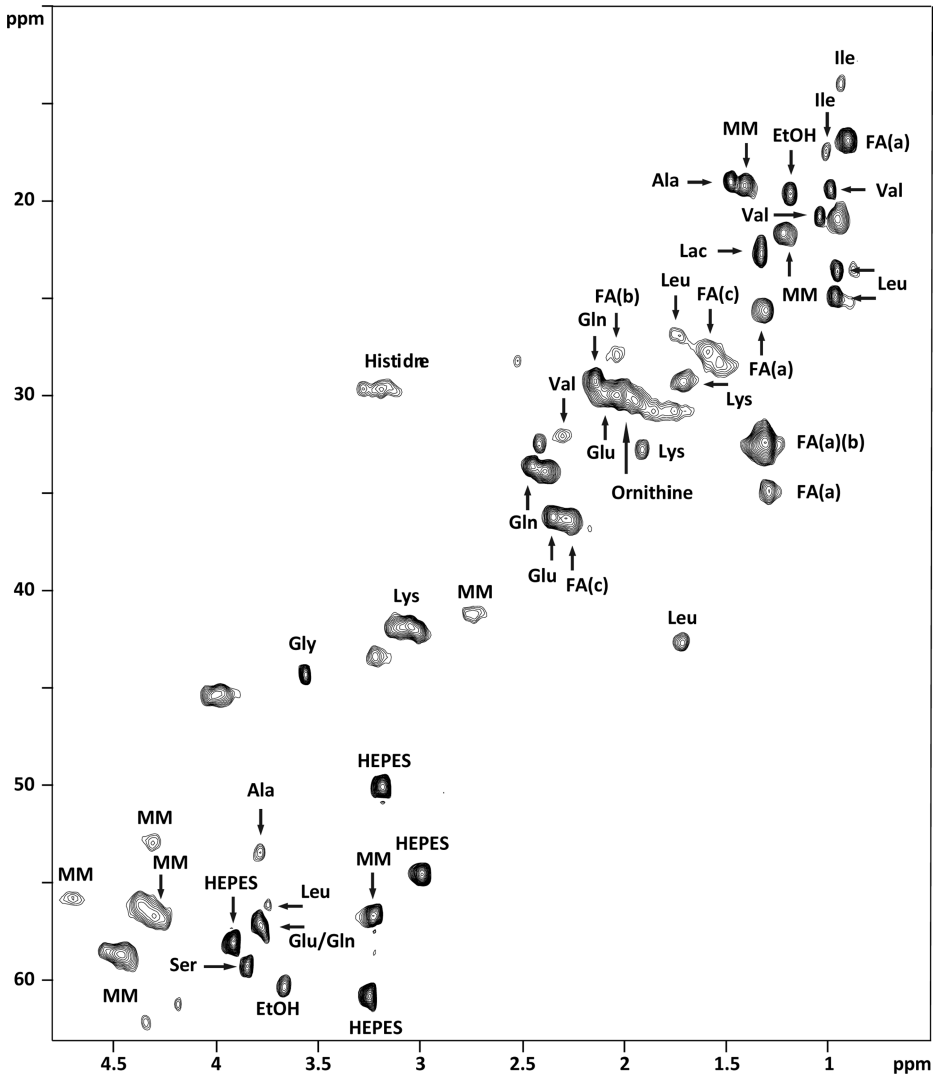

Figure 2. Representative 2D HR-MAS $^{1}H-^{13}C$ g-HSQC spectra of reconstructed human epidermis with metabolite assignment.

In order to confirm resonance assignments, two-dimensional homonuclear and heteronuclear experiments were also recorded on six samples. 2D DIPSI2 spectra[54] were acquired with 170 ms acquisition time, 60 ms mixing time, 14.2 ppm spectral width, and 1.5 s relaxation delay. Thirty-two transients were averaged for each of the 512 increments during t1, corresponding to a total acquisition time of 8 h. Data were zero filled to a 2k·1k matrix and weighted with a shifted square sine bell function prior to Fourier transformation.

2D $^{1}H-^{13}C$ g-HSQC experiments using echo−antiecho gradient selection for phase-sensitive detection[55] were acquired using a 73 ms acquisition time with GARP ^{13}C decoupling and a 1.5 s relaxation delay. A total of 136 transients were averaged for each of 256 t1 increments, corresponding approximately to a total acquisition time of 15 h. Two 1 ms sine-shaped gradient pulses of strength 40 and 10.05 G/cm were used in the experiment. Data were zero-filled to a 2k·1k matrix and weighted with a shifted square sine bell function before Fourier transformation. These conditions of acquisition and processing were also used in the achievement of 2D $^{1}H-^{13}C$ g-HSQC multiplicity-edited experiments using echo−antiecho gradient selection for phase-sensitive detection.[56] These experiments that provide multiplicity information were particularly useful to characterize, track, or monitor the reactivity of ^{13}C substituted compounds as will be shown in the following.

Reaction of (^{13}C)Methyl Dodecanesulfonate with HSA Used as a Model Protein. To a solution of HSA (100 mg, 1.5 μmol, 1 equiv) in phosphate buffer (10 mL, 0.1 M, pH 7.4) degassed for 15 min was added a solution of (^{13}C)methyl dodecanesulfonate (80 mg, 300 μmol, 200 equiv) in a minimal amount of ethanol (1 mL). The reaction mixture was incubated at 37 °C for 9 days, and the solution was dialyzed against water (5 × 5 L), then lyophilized to give a yellow solid (181 mg), which was dissolved in 0.3 mL of H_2O, 0.1 mL of D_2O, a drop of CH_3CN as internal reference, and filtered into an NMR tube for further analysis.

RESULTS AND DISCUSSION

HR-MAS NMR Analysis of Native RHE. HR-MAS NMR spectroscopy experiments were performed on several samples belonging to two different SkinEthic RHE batches. 1D and 2D NMR spectra were found to be quantitatively and qualitatively quite similar. Freeze−thaw cycles and sample spinning effects on HR-MAS spectra were also studied.[57] The results showed that the effects on skin sample were much less important compared to those observed on organ samples.[57] Indeed, the spectra changed only slightly under the effects of the temperature variations and sample centrifugation (CPMG HR-MAS spectra of RHE after 30 min and 16 h in the spectrometer are provided as Supporting Information). The observed reproducibility and stability of RHE sample are indeed essential to ensure reliable and systematic studies by NMR spectroscopy.

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Table 1. ^{1}H and ^{13}C Resonance Assignments of Metabolites, Fatty Acids, and Macromolecules Present in a Reconstructed Human Epidermis[a]

	compd	group	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)
1	isoleucine	δCH3	0.94	13.99
		γCH3	1.01	17.45
2	leucine	δCH3	0.96	23.62
		δ′CH3	0.97	24.88
		γCH	1.72	42.64
		βCH2	1.73	26.93
		αCH	3.74	56.09
3	valine	γCH3	0.99	19.44
		γ′CH3	1.04	20.77
		βCH	2.30	32.06
4	lactate	CH3	1.33	22.70
		CH	4.13	71.31
5	lysine	γCH2	1.40	23.00
		δCH2	1.71	29.30
		βCH2	1.91	32.76
		εCH2	3.05	41.82
6	alanine	βCH3	1.48	19.01
		αCH	3.78	53.42
7	ornithine	γCH2	1.85	25.53
		βCH2	1.95	30.25
8	acetate	CH3	1.92	25.86
9	proline	γCH2	2.01	26.80
		δCH2 (u)[b]	3.33	48.99
		δCH2 (d)[c]	3.41	48.98
		αCH	4.13	64.30
10	glutamate	βCH2	2.04	29.98
		γCH2	2.35	36.22
		αCH	3.78	57.09
11	glutamine	βCH2	2.14	29.23
		γCH2	2.45	33.62
		αCH	3.78	57.09
12	aspartic acid	βCH2(u)[b]	2.70	
		βCH2(d)[c]	2.80	
13	histidine	CH2	3.19	29.67
		αCH	4.00	58.74
		CH-arom	7.22	120.12
		CH-arom	8.38	137.04
14	glycine	αCH2	3.56	44.31
15	threonine	αCH	3.60	63.30
		βCH	4.26	68.98
16	serine	αCH	3.85	59.26
		βCH2	3.97	63.06
17	tyrosine	CH 3,5	6.89	118.55
		CH 2,6	7.19	133.52
18	phenylalanine	CH 2,6	7.32	132.17
		CH 3,5	7.43	131.70
19	arginine	γCH2	1.73	26.71
		βCH2	1.95	30.03
		δCH2	3.22	43.16
FA(a)	fatty acids (a)	CH3	0.91	16.90
		(2) CH2	1.29	34.89
		(1) CH2	1.31	25.59
		(n) CH2	1.31	32.40
FA(b)	fatty acids (b)	(n) CH2	1.31	32.40
		(2) CH2	2.04	27.92
		(2) CH	5.33	132.42
FA(c)	fatty acids (c)	(2) CH2	1.59	27.74
		(1) CH2	2.29	36.30
MM	macromolecules	CH3	0.88	23.52
		CH3	1.41	19.20
		CH2	2.73	41.24
		CH	3.23	56.69
		CH	4.30	56.69
		CH	4.31	52.93
		CH	4.46	58.64
		CH	4.70	55.76
EtOH	ethanol	CH3	1.19	19.65
		CH2OH	3.66	60.33
HEPES	hepes	CH2	2.99	54.55
		CH2	3.01	50.07
		CH2	3.17	54.31
		CH2	3.18	50.05
		CH2	3.25	60.78
		CH2	3.91	58.01

[a]Ethanol and HEPES signals are not metabolites but technical residues. [b](u) upfield. [c](d) downfield.

Representative HR-MAS one-pulse and CPMG spectra of RHE are presented in Figure 1a,b. Metabolites were assigned using standard metabolite chemical shift tables available in the literature.[58] $^{1}H-^{13}C$ g-HSQC standard and edited spectra as well as $^{1}H-^{1}H$ DIPSI spectra were also used to confirm some assignments. A representative g-HSQC standard spectrum is presented in Figure 2, and Table 1 presents detailed assignments of metabolites detected in RHE. A total of about 19 different metabolites were thus identified: ornithine, choline, proline, aspartic acid, isoleucine, leucine, valine, lactate, lysine, alanine, acetate, glutamate, glutamine, glycine, threonine, serine, tyrosine, phenylalanine, and arginine. It is worth mentioning here that the majority of the metabolites detected in RHE are common to organs[48] such as the brain,[5,7−10] breast,[11,12] prostate,[13−15] kidney,[16−18] ovary,[22] and colon.[23,24]

However, ornithine was found to be specific to the RHE model used, and the lack of taurine, myo-inositol, and creatine usually detected in organ biopsies must be emphasized. Concerning taurine, previous studies reported its presence, measured by HPLC, in the epidermis of several animal species and in the human skin.[59,60] In addition, it should be mentioned that α-glucose and β-glucose were initially absent from the spectra but appeared under postincubation conditions.

Since magnetic resonance is an intrinsically quantitative technique, it was possible to obtain metabolite levels for such samples. According to the protocol described elsewhere,[48] metabolite concentrations were measured by simply integrating the areas under individual peaks of the metabolites. For some metabolites presenting at least one resonance with sufficient spectral resolution, the obtained values in (μmol/mg of RHE) were isoleucine (0.6), valine (1.1), lactate (3.0), lysine (4.9), alanine (2.6), acetate (0.3), glutamate (4.1), glutamine (3.7), aspartic acid (1.3), glycine (5.2), threonine (3.5), serine (5.5), tyrosine (0.3), and phenylalanine (0.6). Note that these concentrations were obtained from about 15 mg of RHE within an experimental time of about 10 min. These concentrations are indicative since they were obtained on not fully relaxed systems. To obtain more reliable and accurate quantifications of metabolites, other methods[48] are available and suitable in the presence of overlapping signals.[61]

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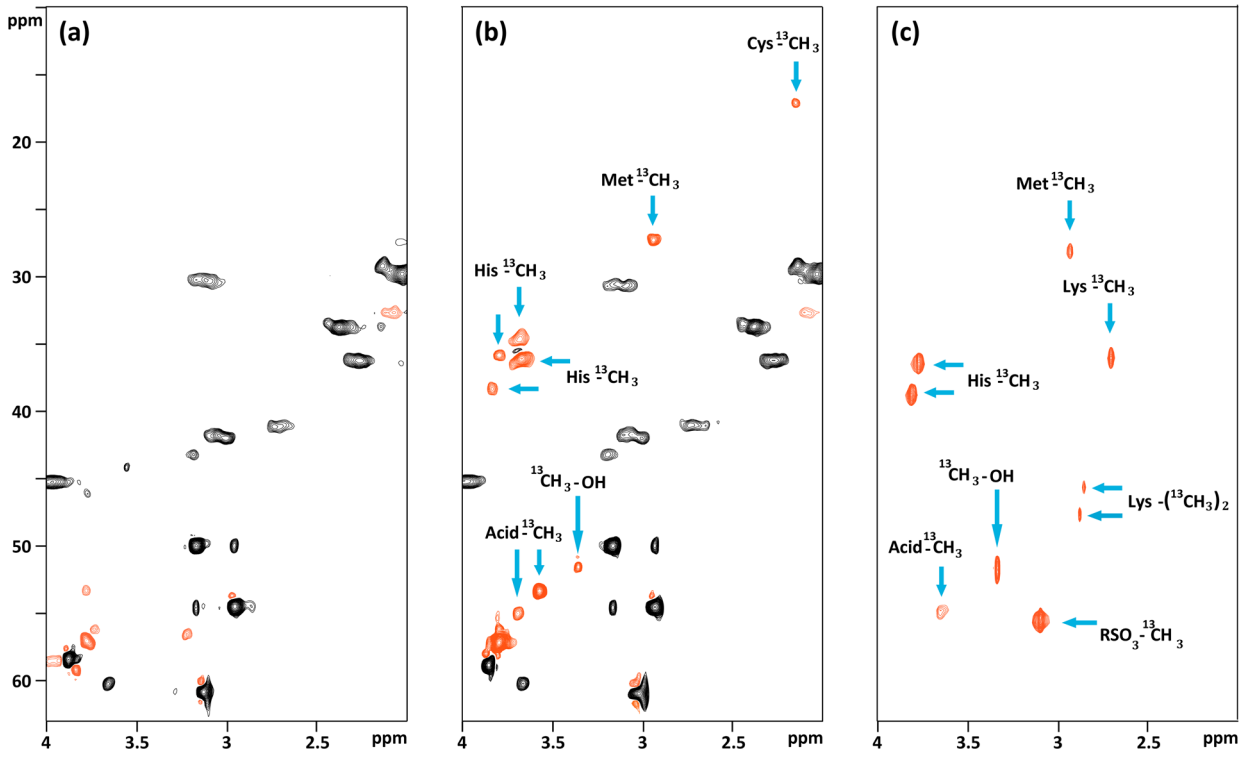

Figure 3. Representative edited 2D HR-MAS $^{1}H-^{13}C$ g-HSQC spectra of reconstructed human epidermis treated by methyl dodecanesulfonate with (a) natural ^{13}C abundance or (b) ^{13}C-substituted methyl; (c) 2D $^{1}H-^{13}C$ g-HSQC of human serum albumin treated in solution by (^{13}C)methyl dodecanesulfonate. Arrows indicate the observed new signals.

HEPES, macromolecules (MM) and lipids were partially identified and reported in Table 1. Their corresponding signals are indicated in the HR-MAS one-pulse spectrum (Figure 1a). As can be seen, the intensities of macromolecules and lipids signals were comparable to those of the metabolites, and therefore, the attenuation of these large signals by the CPMG sequence led to high quality spectra without any deformations of the baseline. Likewise, this feature explains the quality of 2D experiments where the cross-peaks are not disturbed by the signals of lipids or macromolecules (Figure 2).

Tissue histology, ultrastructure, lipid composition, and lipid organization of the SkinEthic RHE tissues has been studied by Ponec et al.[62] They showed that this model was histologically similar to that of the *in vivo* human epidermis. Lipid analyses revealed that the major barrier lipids were synthesized *in vitro* but not exactly in the same proportions as those found in native skin, with a low content of free fatty acids and the incomplete profiles of glucosphingolipids and of ceramides. Moreover, due to the absence of sebaceous glands and adipocytes, respectively present in the dermis and hypodermis of human skin, NMR analysis of RHE showed a limited concentration of lipids.

HR-MAS NMR Analysis of RHE Treated with (^{13}C)-Methyl Dodecanesulfonate. First, we looked at potential metabolic modifications induced by methyl dodecanesulfonate, a potent skin allergen. A simple visual inspection (data not shown) of HRMAS CPMG spectra obtained on untreated RHE or on RHE treated by natural ^{13}C abundance methyl dodecanesulfonate followed by 24 or 48 h of postincubation clearly demonstrated changes over time in the metabolic profile of RHE. In particular, the levels of histidine increased significantly, whereas the levels of alanine, glycine, and serine decreased. More interestingly, signals corresponding to α-glucose and β-glucose, not present on RHE native samples, appeared after methyl dodecanesulfonate exposure and incubation. These modifications can have many origins such as cytotoxicity, cell activation, and oxidative stress but are still unexplained.

Then to characterize the reactivity of methyl dodecanesulfonate, the 2D $^{1}H-^{13}C$ g-HSQC edited sequence was used. This sequence provided additional multiplicity information with CH and CH_3 signals being phased up and CH_2 signals being phased down. Representative g-HSQC edited spectra that correspond to RHE treated with natural abundance and (^{13}C)methyl dodecanesulfonate are presented in Figure 3a and b, respectively. The spectral region was limited to the region where changes were observed. CH and CH_3 signals appear in red, whereas the CH_2 group appears in black.

A simple visual comparison between Figure 3a and b shows the appearance of several red signals (CH_3) arising from the transfer of (^{13}C) methyl groups to nucleophilic residues in RHE. The latter were identified and assigned on the basis of previous experiments carried out on single amino acids[63] or proteins.[45] Moreover, calculations of predicted spectra were performed to confirm the assignments of the different adducts using ACD/CNMR and ACD/HNMR Predictor software (version 6.0, ACD/Laboratories, Toronto, Canada). Table 2 presents detailed assignments of the products detected.

First, the signal at 3.36(^{1}H)/51.57(^{13}C) ppm, with respect to the lactate chemical shift used as internal reference in RHE, was assigned to (^{13}C)methanol formed by the hydrolysis of methyl (^{13}C)dodecanesulfonate. Second, there was no residual signal of the starting material at 3.11(^{1}H)/55.62(^{13}C) ppm in both experimental conditions tested (Figure 3a and b) meaning that either the chemical causing skin sensitization reacted totally with nucleophilic residues of amino acids or was cleared by detoxifying/diffusion mechanisms. Third, the treatment of RHE

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with (^{13}C)methyldodecanesulfonate led to the appearance of 4 groups of well-defined signals. The signal at 17.27(^{13}C) ppm, correlated by g-HSQC data to proton at 2.15(^{1}H) ppm was interpreted as arising from a cysteine adduct, while the signal at 27.34(^{13}C) ppm correlated by g-HSQC data to the proton at 2.94(^{1}H) ppm was assigned to the methylsulfonium salt derived from an alkylation of methionine.[45] A large group of signals from 3.68(^{1}H)/34.76(^{13}C) to 3.84(^{1}H)/38.37(^{13}C) were characteristic of methyl adducts on histidine residues.[63] Finally, signals at 3.57(^{1}H)/53.35(^{13}C) and 3.70(^{1}H)/55.05(^{13}C) ppm were characteristic of methyl esters formed by the methylation of carboxylates such as glutamic or aspartic acid.

To compare the methylations observed in RHE with those potentially observed on a model protein, a g-HSQC standard spectrum of human serum albumin modified in solution by (^{13}C)methyl dodecanesulfonate is presented in Figure 3c. This spectrum was calibrated on $^{13}CH_3OH$ formed by the hydrolysis of (^{13}C)methyl dodecanesulfonate using the chemical shift value observed in the RHE (Table 2). NMR data obtained on

Table 2. ^{1}H and ^{13}C Resonance Assignments of Additional Signals Observed in Reconstructed Human Epidermis or HSA Treated by (^{13}C)Methyl Dodecanesulfonate

	RHE		HSA	
methylated products	δ^{1}H (ppm)	δ^{13}C (ppm)	δ^{1}H (ppm)	δ^{13}C (ppm)
$^{13}CH_3$–OH	3.36	51.57	3.36	51.57
acid-$^{13}CH_3$	3.57	53.35		
	3.70	55.05	3.69	54.73
RSO_3-$^{13}CH_3$			3.11	55.62
cysteine-$^{13}CH_3$	2.15	17.27		
methionine-$^{13}CH_3$	2.94	27.30	2.94	27.34
lysine-$^{13}CH_3$			2.71	35.45
lysine-($^{13}CH_3$)$_2$			2.86	45.32
			2.89	47.32
histidine-$^{13}CH_3$	3.68	36.15		
	3.70	34.76		
	3.81	35.93	3.80	35.94
	3.84	38.37	3.85	38.29

HSA showed the presence of similar adducts on methionine, histidine, and glutamic/aspartic acid residues. In HSA, no adducts with cysteine residues were observed, which is in accordance with the presence of only one single free cysteine residue (Cys 34), located in a sterically restricted hydrophobic environment. The presence of signals at 2.71(^{1}H)/35.45(^{13}C), 2.86(^{1}H)/45.32(^{13}C) and 2.89(^{1}H)/47.32(^{13}C) ppm were assigned to the formation of (^{13}C)methyl-lysine and (^{13}C)-dimethyl-lysine residues. Of the 58 lysine residues in HSA, it is well known that only a few are selectively targeted by sensitizers. Indeed, the physiological pH of incubation is not favorable for lysine reactivity, as lysine side chains are mainly protonated and therefore mostly unreactive. The presence of such signals could be due to the influence of the pH in local microenvironments surrounding the lysine residues of HSA that are most susceptible to modification.

Chemical Reactivity of Skin Sensitizers toward Nucleophilic Amino Acids in RHE vs HSA in Solution. To induce skin sensitization, a chemical has to fulfill several steps: penetrate into the epidermis across the *stratum corneum*, form stable association with proteins in order to create an immunogenic complex, cause dermal trauma, and be inherently immunogenic. Indeed, the very first step of the sensitization process is not biological but chemical. The understanding of such chemical interactions is thus central to model the pathology and could be used for the development of alternative *in chemico* methods based on the assessment of hapten reactivity toward nucleophilic side chains of amino acids as predictive tools to prevent introducing new chemical sensitizers on the consumer market.[64] Thus, it has been shown that model nucleophiles/native amino acids, glutathione (GSH), or synthetic peptides containing nucleophilic residues could be very valuable tools for the detection of sensitizing molecules[65−70] and even their classification according to potency categories (weak, moderate, strong, and extreme sensitizers) as defined by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Task Force.[71] However, such studies were based on the reactivity observed in solution and not in the epidermis. Thus, nucleophilic amino acids most often cited as targets for skin allergens and therefore used as models of nucleophiles were cysteine and lysine. Using (^{13}C)methyl dodecanesulfonate, a very potent skin allergen, we have been able to demonstrate on RHE that the picture is somewhat different. First, a striking result was the reaction time needed to modify nucleophilic amino acids in a living epidermis compared to model reactions in solution. Thus, while it took several days to detect by NMR significant amounts of modification on N-acetylated amino acids, peptides, or proteins in solution, in RHE similar modifications of nucleophilic amino acids were observable in less than 24 h. Second, the chemioselectivity also appeared to be different with major modifications taking place on histidine, methionine, and cysteine residues in RHE, while on HSA, significant modifications were observed on lysine residues with the formation of methylated and dimethylated amino groups. Such modifications on lysine, a residue often cited as a target for chemical sensitizers based on *in vitro* experiments, were not observed in the reconstructed human epidermis. Third, a major drawback inherent to most *in vitro* methods is the poor water solubility of many organic molecules in aqueous media. Thus, semiorganic media based on buffer solutions and organic cosolvents such as ethanol or acetonitrile have been proposed, but a narrow equilibrium should be found between the amino acid/peptide and chemical solubilities. The use of RHE allows an epicutaneous application of the product to be evaluated and will therefore mimic a more conventional skin exposure.

High-resolution magic angle spinning is a NMR technique well adapted for the characterization of metabolites as shown in Figures 1 and 2. After exposure of RHE to (^{13}C)methyl dodecanesulfonate, we observed methylations of probably free amino acids even if we cannot exclude the modification of small peptides or mobile parts of proteins. In that respect, it is interesting to note that chemical shifts of methylated histidines for example appeared as a large group of signals from 3.68(^{1}H)/34.76(^{13}C) to 3.84(^{1}H)/38.37(^{13}C), indicating very different microenvironments.

■ CONCLUSIONS

This work demonstrated that HR-MAS NMR is well adapted for investigating the reconstructed human epidermis with spectra of good quality allowing qualitative as well as quantitative studies. Using this technique, we demonstrated that RHE could be used to investigate *in situ* chemical interactions taking place between carbon 13 substituted skin sensitizers and nucleophilic amino acids. In this work, we described the

optimal experimental conditions to study the effects of allergens on RHE.

We also described the preparation, storage, and analysis of such samples. The protocol described here could be used on different reconstructed epidermises to investigate various chemicals responsible of allergic contact dermatitis. In a general way, we believe that the possibilities offered by the combination of HR-MAS NMR and RHE are potentially important, opening perspectives for the evaluation of sensitizing chemicals but also for a molecular understanding of the immune system regulation by skin and respiratory chemical allergens.

■ ASSOCIATED CONTENT

Supporting Information

Additional NMR spectra for the effect of heating and spinning RHE skin samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACD, allergic contact dermatitis; CPMG, Carr−Purcell−Meiboom−Gill; DIPSI, decoupling in the presence of scalar interactions; GARP, globally optimized alternating phase rectangular pulse; HR-MAS, high-resolution magic angle spinning; HSA, human serum albumin; HSQC, heteronuclear single quantum correlation; RHE, reconstructed human epidermis

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