In Situ Alkylation of Reconstructed Human Epidermis by Methyl Methanesulfonate: A Quantitative HRMAS NMR Chemical Reactivity Mapping

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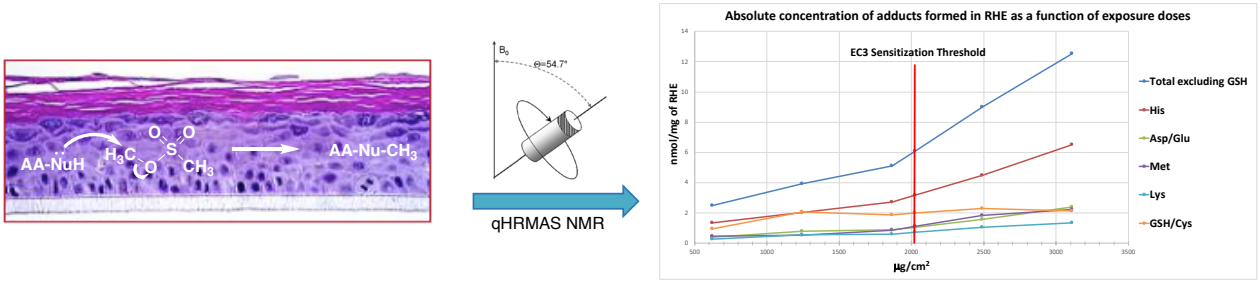

Abstract

Allergic Contact Dermatitis (ACD) is a reaction of the immune system resulting from skin sensitization to an exogenous hazardous chemical and leading to the activation of antigen-specific T-lymphocytes. The adverse outcome pathway (AOP) for skin sensitization identified four key events (KE) associated to the mechanisms of this pathology, the first one being the ability of skin chemical sensitizers to modify epidermal proteins to form antigenic structures that will further trigger the immune system. So far, these interactions have been studied in solution using model nucleophiles such as amino acids or peptides. As a part of our efforts to better understand chemistry taking place during the sensitization process, we have developped a method based on the use of high resolution magic angle spinning (HRMAS) NMR to monitor *in situ* the reactions of ^{13}C substituted chemical sensitizers with nucleophilic amino acids of epidermal proteins in reconstructed human epidermis. A quantitative approach, developed so far for liquid NMR applications, has not been developed to our knowledge in a context of a semi-solid non-anisotropic environment like the epidermis. We now report a quantitative chemical reactivity mapping of methyl methanesulfonate, a sensitizing methylating agent, in reconstructed human epidermis by qHRMAS NMR. First, the haptenation process appeared to be much faster in RHE than in solution with a maximum concentration of adducts reached between 4 to 8 hours. Second, it was observed that the concentration of cysteine adducts did not significantly increase with the dose (2.07 nmol/mg at 0.4 M and 2.14 nmol/mg at 1 M) nor with the incubation time (maximum of 2.27 nmol/mg at 4 h) compared to other nucleophiles indicating a fast reaction and a potential saturation of targets. Third, when increasing the exposure dose we observed an increase of adducts up to 12.5 nmol/mg of RHE, excluding

Cystein adducts, for 3112 μg/cm^2 (1M solution) of **(^{13}C)MMS**. This methodology applied to other skin sensitizers could allow to better understand the potential links between the amount of chemical modifications formed in the epidermis in relation with exposure and the sensitization potency.

INTRODUCTION

Skin allergy is a reaction of the immune system resulting from sensitization to an exogenous hazardous chemical and leading to the activation of antigen-specific T-lymphocytes.[1,2] People suffering from skin allergy develop clinical symptoms ranging from mild redness to intense itching and severe pain affecting their quality of life. The prevalence is increasing due to the widespread of hazardous chemical sources and in the western world, 15-20% of the population are allergic to at least one chemical present in their environment.[3, 4] As there is no treatment other than symptomatic, the prevention skin allergy relies on an early identification of sensitizing chemicals prior to their introduction into the market. This was done for years on the basis of animal methods such as the guinea pig Magnusson-Kligman test (OECD TG 406) or the murine local lymph node assay (LLNA - OECD TG 429) but since the implementation of EU regulations such as REACH,[5] and the Cosmetics Regulation,[6] there has been huge efforts to develop non-animal methods to assess skin sensitization.

The skin sensitization adverse outcome pathway (AOP) identified four key events (KE) associated to the mechanisms of this pathology. [7,8] The first one (KE1) is related to the ability of sensitizers to penetrate the skin through the *stratum corneum* and modify epidermal proteins to form antigenic structures triggering the immune system. This molecular initiating event, already postulated by Landsteiner and Jacobs 80 years ago,[9] is now widely accepted by toxicologists and immunologists. A wide range of experimental approaches using small nucleophilic chemicals,[10] short peptides containing one or more nucleophilic amino acids,[11,12] and proteins[13,14] were developed to improve our understanding of this molecular step. The main outcome was the

development of the direct peptide reactivity assay (DPRA)[15,16], a non-animal method validated by ECVAM[17] and OECD.[18] In DPRA, allergenic properties of chemicals are assessed by measuring their reactivity towards two peptides in solution containing a lysine (Lys) or a cysteine (Cys) residue and used as models for epidermal proteins. DPRA has a good accuracy of 80% in discriminating between chemicals (sensitizer or not compared to LLNA data) but less efficiency in terms of potency classification (weak, moderate, strong). Assays in solution seem thus to be far from providing accurate data for quantitative risk assessement of chemicals. Insurprisingly, reactions of chemicals with short peptides in solution (buffer or semi-organic) and epidermal proteins in a complex heterogeneous tissue (living epidermis) do not exhibit the same kinetic profiles and/or the same chemospecificities.

As part of our efforts to better understand the chemistry taking place during KE1, we have developped a new method based on the use of high resolution magic angle spinning (HRMAS) NMR to monitor *in situ* the reactions of carbon-13 substituted chemical sensitizers with nucleophilic amino acids of epidermal proteins in a 3D reconstructed human epidermis (RHE)[19-21] as well as their metabolism.[22] Commercially available RHE, very close in terms of histology and metabolic activity to real human epidermis,[23,24] can be used as suitable biological tissue models. Advantages are to test chemicals under conditions close to human use (topical application), take advantage of the metabolizing enzymes present and benefit from the 3D microenvironment. Using HRMAS NMR we accessed the chemospecificity of nucleophilic amino acids involved in the formation of antigenic adducts, showing that chemical sensitizers can modify other amino acids than the conventional Lys/Cys residues used in the DPRA, such histidine and methionine. Though, it is still unknown whether a higher sensitization potency of a

chemical is related to its interaction with a larger number of nucleophilic sites on epidermal proteins or correlated with a higher modification of a specific type of amino acid. Ideally, identification and quantification of antigenic adducts formed in the epidermis could be a nice possibility to develop a new approach for risk-assessment based on the level of epidermal proteins modification.

Proton (^{1}H) NMR spectroscopy was first reported as analytical tool for quantitative analysis in 1963 by Jungnickel and Forbes.[25] In the past decades there has been growing interest in quantitative NMR (qNMR)[26,27] with the significant contribution of Turczan et al.[28] For complex mixtures with overlapped peaks or very weak amounts of molecules, 1D q^{1}H-NMR spectroscopy was not sufficient giving rise to the development of 2D qNMR spectroscopy with heteronuclear HSQC and homonuclear TOCSY experiments,[29,30] and combination of both.[31] This kind of approach, developed so far for liquid NMR applications, has not been developed to our knowledge in a context of a semi-solid non-anisotropic environment like the epidermis.

In this study, (^{13}C)-methyl methanesulfonate (**(^{13}C)MMS**) was selected as a model for the development of an HRMAS NMR quantitative approach. Methyl alkanesulfonates, identified as strong sensitizers in mice and guinea pigs, are capable of transferring a methyl group to nucleophilic amino acids *via* a nucleophilic substitution (Scheme 1). Indeed, these compounds have been shown to react with a variety of nucleophilic residues in RHE.[19] Herein, we report for the first time the *in situ* absolute quantification of allergen-epidermal proteins adducts in RHE using quantitative HRMAS NMR sequences. This methodology could help to refine and optimize current *in chemico* predictive strategies and be the basis for a future development of new tests using a more *in vivo* like integrated model.

EXPERIMENTAL PROCEDURES

Caution: Skin contact with methyl methanesulfonate must be avoided. As a potential sensitizing substance and human carcinogen, this compound must be handled with care.

Chemicals and reagents

(^{13}C)methanol and deuterated solvents were purchased from Euriso-Top (Saint Aubin, France). Acetone (99.8%, for analysis) used for the preparation of ^{13}C-methyl methanesulfonate solutions was purchased from Carlo Erba (Val de Reuil, France). All other chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) unless otherwise noted and used without further purification.

Synthesis of (^{13}C)methyl methanesulfonate (^{13}C)MMS

To a solution of ^{13}C-methanol (1.1 g, 33 mmol) in dry dichloromethane (18 mL) was added dry triethylamine (3.4 g, 33 mmol). The mixture was cooled to -10°C and methanesulfonyl chloride (3.8 g, 33 mmol) was added dropwise. The resulting solution was further stirred at 0°C for 1 hour then water (20 mL) was added. The aqueous layer was extracted with dichloromethane (2 × 15 mL). The organic extracts were washed with 3M HCl (30 mL) then with a saturated $NaHCO_3$ solution (2 × 15 mL) and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure to yield the crude product. The remaining methanesulfonyl chloride was removed by distillation (76 °C at 50 mmHg) to yield the pure product as a colorless oil (2 g, 55%).

^{1}H NMR ($CDCl_3$; 300 MHz, ppm): δ 3.00 (s, 3H), δ 3.89 (d, $^{1}J_{CH}$ = 150.15 Hz, 3H). **^{13}C NMR** ($CDCl_3$; 125 MHz, ppm): δ 36.72 (CH_3), δ 55.55 ($O^{13}CH_3$). GC-MS: 111 $(M)^+$

Reconstructed Human Epidermis (RHE)

Cell Culture: The EpiSkin™ RHE samples, large model (thirteen days old, 1.07 cm^2), were obtained from EpiSkin (Lyon, France). These models are differentiated from human keratinocytes isolated from healthy donors and include a human collagen (Type I) matrix, coated with a layer of Type IV human collagen, and a fully stratified and differentiated epidermis covered with a *stratum corneum*. The EpiSkin™ cultures are shipped onto a nutritive gel for transportation to maintain viability. Upon reception, the reconstructed epidermises were kept under sterile conditions in a 12-well culture dishes containing 2 mL/well of supplied maintenance medium (DMEM/Ham's F12) pre-warmed at 37°C. The medium was changed (2 mL) each 24 h.

Treatment protocol: RHE samples were treated with a fixed volume (30 µL) of freshly prepared **(^{13}C)MMS** solutions in acetone or with 30 µL of acetone for control purposes. Different concentrations of **(^{13}C)MMS** varying from 0.4 to 1 M and different incubation times varying from 1 to 24 h were tested. To ensure the reproducibility of the process, experiments were performed in triplicate. Three different batches of EpiSkin™ RHE were treated in three independent experiments with **(^{13}C)MMS** (0.4 M) and incubated for the same durations. Once the incubation time was reached, the epidermis was separated from its collagen support using sterilized forceps, placed in an Eppendorf tube and frozen at -80°C. Freezing the samples

ensured to stop any metabolic activity within the epidermis and to maintain them stable until HRMAS NMR analysis.

Rotor preparation and data acquisition by HRMAS NMR

The rotor preparation and the data acquisition by HRMAS NMR were carried out according to the methodology already described. Briefly, each sample was prepared at -20 °C by introducing 15 to 20 mg of frozen RHE completed with D_2O into a disposable 30 µL KelF insert. Shortly before HRMAS analysis, the insert was placed into a standard 4 mm ZrO_2 rotor and closed with a cap. The HRMAS experiments were performed at 4 °C. Upon completion of the analysis, the insert was taken out of the rotor and stored back at -80 °C for further complementary NMR analysis at a later stage.

HRMAS spectra were recorded on a Bruker Avance spectrometer operating at a proton frequency of 500.13 MHz, equipped with a 4 mm triple resonance (^{1}H, ^{13}C, ^{31}P) gradient HRMAS probe. The temperature was maintained at 277 K throughout the acquisition time in order to reduce the effects of tissue degradation during the signal acquisition.

In order to detect and to quantify the different adducts formed by the reaction of **(^{13}C)MMS** with the RHE, we used the first increment of a 2D ^{1}H-^{13}C g-HSQC (gradient Heteronuclear Single Quantum Coherence). This type of acquisition is the best compromise between the sensitivity of the HSQC experiment for characterizing the ^{13}C labelled adducts and a reasonable period of time for quantifying these products.

A full 2D g-HSQC using echo-antiecho gradient selection was performed in order to assign unambiguously the different ^{13}C labelled products. All spectra were referenced by setting the lactate peak chemical shift to 1.33 ppm in ^{1}H and to 22.7 ppm in ^{13}C.

For the 1D HSQC increments, the settings were the following: 512 scans, a spectral width of 7002.801 Hz, a data size of 32 k points, an acquisition time of 2.33 s, and a relaxation delay of 13s. Free induction decays were multiplied by an exponential window function of 5 Hz prior to Fourier transformation and were corrected for phase and baseline distortions using TopSpin 3.5 (Bruker GmbH, Bremen, Germany). It must be emphasized that with a long relaxation delay of 13s, we can be relatively confident in terms of quantification.

Quantification procedure

Adducts were quantified using the PULCON method, which is a very accurate quantification method (with a very limited percentage of error).[32] Adduct quantification was performed using an external reference standard of **(^{13}C)MMS** (7 mM), scanned under the same analytical conditions as the RHE samples. This standard solution (26 µL placed in a kelF insert) was analyzed before each series of experiments and evidenced a remarkable stability of the analytical setup.

Spectra were normalized according to sample weight. Peaks of interest were quantified by an in-house program using MATLAB 7.0 (MathWorks, Natick, MA, USA) and based on the *J* scalar coupling constant. A correction of the peaks's intensities taking into account this *J* scalar coupling effect on HSQC spectra was applied in particular.[33] Peak integration was corrected according to the number of equivalent protons and also to the *J* scalar coupling effect on HSQC spectra, the overlapping existing between different adjacent peaks and finally on the multiplicity due to the

scalar coupling. Then in order to reach the absolute values for the concentration, this corrected peak's intensity was compared to the one obtained with the **(^{13}C)MMS** reference, corrected itself by the *J* scalar coupling effect and analyze under the same analytical conditions. Quantification results were expressed as nanomoles per milligram of tissue.

Skin sensitization thresholds derived from LLNA data

Skin sensitization thresholds can be derived from LLNA dose-response studies. The EC3 value is calculated by interpolating between two points on the stimulation index (SI) axis, one immediately above and the other immediately below, the SI value of 3. Based on the LLNA OECD protocol[34] using a defined volume of 25 μL of test solution (%) applied on an ear surface of 1 cm^2, it is possible to convert EC3 values originally expressed in % into EC3 values expressed in $\mu g/cm^2$ by applying a conversion factor of 250.[35]

RESULTS

Synthesis of (^{13}C)methyl methanesulfonate

The synthesis of (^{13}C)methyl methanesulfonate **(^{13}C)MMS** was carried out in a single step according to a previously described procedure[36] however with some modifications. The reaction between methanesulfonyl chloride and (^{13}C)methanol in the presence of triethylamine afforded the expected **(^{13}C)MMS** with a yield of 55%.

Quantitative HRMAS NMR study of the reaction of (^{13}C)MMS with RHE

One challenge to overcome was the potential overlap of NMR signals in a highly complex tissue such RHE. Indeed, many signals arising from the complex metabolome of RHE are present and could interfere with signals arising from the tested chemical and its further interactions with nucleophilic amino acids (Figure 1a and 1b). To isolate signals arising only from adducts, we used a carbon-13 substituted allergen and developed the use of quantitative 1D-HSQC sequences taking advantage of the ^{1}H-^{13}C spin coupling (Figure 1c and 1d). 1D-HSQC spectra clearly showed the appearance of new peaks (Figure 1d) compared to the control sample (Figure 1c) upon the exposure of RHE to **(^{13}C)MMS**. New peaks signing the transfer of methyl groups ($^{13}CH_3$) to nucleophilic amino acids residues (Scheme 1) could be easily assigned: the remaining starting material **(^{13}C)MMS** (4 ppm), histidine adducts signals (broad peak at 3.7-3.9 ppm), glutamic/aspartic acid adducts (3.7 ppm), methanol resulting from the hydrolysis of **(^{13}C)MMS** in the presence of water (3.36 ppm), methionine adducts (3 ppm, characteristic signal of sulfonium cation), lysine mono- and dimethylated adducts (2.6-2.8 ppm) and cysteine adducts (2.0-2.2 ppm). The assignment of these signals was confirmed by 2D HSQC NMR experiments showing the

correlation between the proton and carbon signals of each adduct (Figure S1). Notably, the peak of histidine adducts was overlapping with another peak corresponding to C terminal acid adducts as shown in the 2D spectrum. However, the relative percentage of C terminal acid adducts compared to that of histidine adducts was expected to be negligible due to their low probability factor. Noteworthy, no signals arising from a potential reaction of **(^{13}C)MMS** with other potential nucleophilic residues (arginine, tryptophane or tyrosine) were detected under our conditions of experiments.

To obtain quantitative signals, new 1D-HSQC experiments were set and optimized. The optimal acquisition time was found to be 150 min while maintaining a maximal stability of the samples. NMR experiments were run at 4 °C to minimize any enzymatic activity of the epidermis and reduce the potential reactivity of the remaining **(^{13}C)MMS** during acquisition. In order to test this reactivity**,** two 1D HSQC experiments were consecutively recorded for the same sample. Indeed, little change (< 3%) in the total amount of adducts was observed (Figure S2) and the spectra of RHE changed only slightly under the effects of the temperature variations and acquisition duration.

Time/response study

Based on our previous experiments on methyl alcanesulfonate,[19] an initial concentration of 0.4 M was chosen for the time/response study. In order to study the kinetics of the different reactions taking place concurrently within the epidermis, RHE samples were treated with 30 µL of **(^{13}C)MMS** in acetone corresponding to an exposure of 1244 µg/cm^2 followed by an incubation time of 1, 2, 4, 8 and 24 h, respectively. To evaluate the reproducibility of the

method, all experiments were performed in triplicates using different shipments of RHE. As shown in Table 1, which summarizes the absolute concentration of the different adducts observed in RHE, batch to batch reproducibility was very good. The results suggest that the reaction rate with lysine is much slower than that with the other amino acids the corresponding adducts being not observed at incubation times $<$ 4 h. Increasing the exposure time from 4 to 8 h had no significant effect on the formation of adducts as the reaction of **(^{13}C)MMS** with nucleophilic amino acids had already reached its optimum after 4 h. Interestingly, after 24 h the concentration of cysteine adducts dropped off dramatically from 2.27 to 0.65 nmol/mg of RHE. Such behavior could be associated to the detoxification process taking place in the epidermis especially with cysteine adducts of glutathione (GSH). The probability of GSH to be an easy target for **(^{13}C)MMS** is high as it is the most abundant non-protidic molecule containing thiol in living cells (12 mM) and with the least steric constraints.[37] Moreover, the role of GSH in cellular resistance to alkylating agents has been widely investigated.[38,39] Noteworthy, the total absolute concentration of covalent adducts increased with the exposure time to reach a maximum of 6.22 nmol/mg at 4 h then decreased dramatically to 3.66 nmol/mg after 24 h due mainly to the decrease in the absolute concentration of cysteine adducts. Table 1 also clearly shows the decrease in the concentration of **(^{13}C)MMS**, consistent with a progressive consumption yielding the corresponding adducts and the formation of methanol by hydrolysis. Analyzing the variation in concentration of **(^{13}C)MMS** and (^{13}C)methanol together showed that at time < 4 h, haptenation reactions were dominant while at time > 4 h, the hydrolysis reaction was the main process taking place in the epidermis as most of the nucleophilic residues within the epidermis had already reacted.

Dose/response study

The reactivity of chemical sensitizers with epidermal proteins is expected to be dependent on the applied dose and exposure time.[40] In order to evaluate the dose/response of the epidermis, EpiSkin™ RHE samples were topically exposed to different concentrations of **(^{13}C)MMS** (0.2, 0.4, 0.6, 0.8 and 1 M) in acetone (30 μL) as vehicle, that correspond to exposure of 622 to 3112 μg/cm^2, and then incubated for 8 h. We used an excess of sensitizer relative to the nucleophilic targets[14] to ensure an optimal haptenation reaction at levels that were detectable. An exposure time of 8 h was chosen based on our previous studies of chemical reactions with RHE and results of the time/response studies. In order to determine whether the chemical has completely diffused in the epidermis, the surface of RHE samples exposed to 1244 μg/cm^2 (0.4M solution) of **(^{13}C)MMS** during 1h and 8 h, respectively, were washed with d_6-acetone (500 μL) and the resulting solution examined by liquid ^{13}C NMR. As shown in Figure S3 comparing results after 1 h or 8 h of incubation only traces of **(^{13}C)MMS** remained on the surface at 8 h confirming our hypothesis that this duration is quite sufficient for a full absorption of the chemical within RHE.

As it can be seen in Table 2, we can observe an increase of adducts formed according to the exposure concentration except for cysteine that reached a plateau at 1244 μg/cm^2 (0.4M solution) indicating a potential saturation with a constant concentration of about 2 nmol/mg of RHE regardless of the applied dose. The total absolute concentration of all adducts including GSH formed in RHE increased from 3.47 to 14.66 nmol/mg of RHE as the exposure increased from 622 to 3112 μg/cm^2.

Skin sensitization threshold and adduct formation

Based on LLNA experiments, MMS has been classified as a moderate skin sensitizer with an EC3 value of 8.1%[41] which corresponds to an exposure dose of 2025 μg/cm^2. In our experiments RHE samples (1.07 cm^2) were treated with 30 μL of MMS solutions at concentrations ranging from 0.2 to 1M which correspond to exposures ranging from 622 to 3112 μg/cm^2 (Figure 2). We are therefore covering the sensitization threshold providing access to absolute concentrations of adducts formed in RHE, either individually or in total, at the exposure dose of 2025 μg/cm^2 needed to induce a sensitization (vertical line on Figure 2).

Discussion

The binding of a low molecular weight (< 500 g/mol) chemical sensitizer (electrophile) to a carrier protein (nucleophile) located in the epidermis to produce a macromolecular immunogenic complex is considered to be the first key event (KE1) in the multi-step skin sensitization process.[7,8] So far, our understanding of the different aspects (qualitative or quantitative) of this initiating key step was limited to data provided by *in vitro* tests involving the use of single nucleophiles (or peptides or proteins) in solution with fixed parameters (pH, stoichiometry…) to model the complex mixture of biological targets (epidermal proteins) within the complexity of a 3D viable epidermis.

For the first time the methodology that we propose, combining qHRMAS NMR and RHE, allows following and quantifying the reactivity of a skin sensitizer with nucleophilic residues

present in a complex 3D tissue. This mapping of RHE reactivity could give access to a deeper analysis on reactivity aspects taking place in the epidermis during the sensitization process.

First, the haptenization process appears to be much faster in RHE than in solution as the maximum concentration of adducts was often reached between 4 to 8 h. In solution more than one week was often required to observe significant modifications, detectable by NMR, following the reaction of human serum albumin with methylating agents (PBS; pH 7.4).[19] In a recent paper on protein modification with sensitizers, lysate of keratinocyte cells or *ex vivo* skin were incubated with a 1/100 molar excess of sensitizers at 37°C for 4 weeks to get a significant level of modifications.[14] Also, reactivity differences appeared between the nucleophilic amino acids with lysine being the less reactive with a detection only after 4 h of incubation and a maximum concentration of 0.55 nmol/mg of RHE and histidine showing adducts already at its maximum concentration of about 2 nmol/mg of RHE after 1 h only and then a stable profile.

Second, it was observed that the concentration of cysteine adducts did not significantly increase with the dose (2.07 nmol/mg at 0.4 M and 2.14 nmol/mg at 1 M, Table 2) nor with the incubation time (maximum of 2.27 nmol/mg at 4 h, Table 1) compared to other nucleophiles indicating a fast reaction and a potential saturation of targets. Indeed our data showed that the number of cysteine residues able to react with **(^{13}C)MMS** was limited to about 2 nmol/mg of RHE. Considering that cysteine is one of the less abundant amino acids in proteins[42] and often engaged in disulfide bridges, the rather high concentration of adducts observed (2.04 nmol/mg of RHE) and a saturation of modifications when increasing exposure are in good agreement with a GSH reactivity. This is also supported by results obtained when monitoring the reactivity as a function of exposure time, as we were able to detect a dramatic decrease in the concentration

of cysteine adducts between 4 h (2.27 nmol/mg) and 24 h (0.65 nmol/mg) in good agreement with a potential detoxification process of GSH adducts. This was not observed with adducts formed on other amino acids.

Third, when increasing the exposure dose we observed a significant increase of adducts formation (excluding potential GSH adducts) up to 12.5 nmol/mg of RHE for 3112 μg/cm^2 (1M solution) of **(^{13}C)MMS** (Figure 2). Based on LLNA experiments, MMS has been classified as a moderate skin sensitizer with an EC3 value of 8.1%[41] which corresponds to an exposure dose of 2025 μg/cm^2. As demonstrated by Basketter et al.[35] a linear correlation does exist between EC3 values in mice and sensitization thresholds in human derived from historical Human Repeated Insult Patch Test (HRIPT). In our experiments, RHE were exposed to doses ranging from 622 to 3112 μg/cm^2 and we are therefore covering the sensitization threshold of 2025 μg/cm^2. As shown on Figure 2 it is thus possible to get access to the amount of adducts formed in RHE corresponding to the sensitization threshold. In our case this could correspond to an "adduct threshold" of 6.09 nmol/mg of RHE (excluding GSH that would account to detoxication mechanisms) with a huge variation between Lys (0.72 nmol/mg) and His (3.18 nmol/mg). It is interesting to note that at the sensitization threshold we are above the exposure leading to a saturation of GSH (1244 μg/cm^2). It is the first time that it is possible to associate a quantitative amount of adducts formed *in situ* with a sensitizing dose.

CONCLUSION

So far, our understanding of the different aspects (qualitative or quantitative) of KE1 was limited to data provided by *in vitro* tests involving the use of single nucleophile (or peptide, or protein) in solution with fixed parameters (pH, stoichiometry…) to model the complex mixture of biological targets (epidermal proteins) within the complexity of a 3D viable epidermis.

For the first time the methodology that we propose, combining qHRMAS NMR and RHE, allows following and quantifying the reactivity of a skin sensitizer with nucleophilic residues present in a complex 3D tissue. It is thus possible to associate a quantitative amount of adducts formed *in situ* with a sensitizing dose. This methodology applied to other skin sensitizers could allow to better understand the potential links between the amount of chemical modifications formed in the epidermis in relation with exposure and the sensitization potency.

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ABBREVIATIONS

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; DPRA, direct peptide reactivity assay; ECVAM, European Center for Validation of Alternative Methods; HRMAS, high-resolution at magic angle spinning; g-HSQC, gradient Heteronuclear Single Quantum Coherence; LLNA, local lymph node assay; qNMR, quantitative Nuclear Magnetic Resonance; RHE, reconstructed human epidermis;

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Table 1

Absolute concentrations* of adducts formed in RHE (nmol/mg) following application of 1244 μg/cm^2 **(^{13}C)MMS** in acetone (30 μL of a 0.4 M solution) as a function of exposure time.

Time (h)	(^{13}C)MMS	$^{13}CH_3$-OH	His-$^{13}CH_3$[a]	Asp/Glu-$^{13}CH_3$	Met-$^{13}CH_3$	Lys-$^{13}CH_3$	Cys-$^{13}CH_3$	Total[b]
1	9.2±1.90	0.32±0.08	2.09±0.42	0.51±0.22	0.33±0.10	ND	1.77±0.11	4.70±0.85
2	4.53±0.07	0.43±0.06	1.60±0.23	0.57±0.09	0.51±0.05	ND	2.0±0.10	4.68±0.47
4	2.49±0.54	0.56±0.10	2.18±0.65	0.76±0.10	0.46±0.15	0.55±0.14	2.27±0.55	6.22±1.59
8	1.86±0.43	0.91±0.23	1.95±0.10	0.87±0.08	0.41±0.12	0.49±0.07	2.04±0.12	5.76±0.49
24	0.58±0.07	1.13±0.36	1.51±0.26	0.75±0.07	0.38±0.06	0.37±0.11	0.65±0.07	3.66±0.57

* Mean ±SD results from three batches of RHE run as separate experiments. [a]: includes marginal contribution of the C-terminal acid adducts, [b]: the total absolute concentration of adducts including GSH/Cys.

Table 2

Absolute concentrations of adducts formed in RHE (nmol/mg) as a function of exposure to **(^{13}C)MMS** ranging from 622 to 3112 μg/cm^2 (30 μL of 0.2M to 1.0M solutions in acetone).

Exposure μg/cm^2	(^{13}C)MMS	$^{13}CH_3$-OH	His-$^{13}CH_3$[a]	Asp/Glu-$^{13}CH_3$	Met-$^{13}CH_3$	Lys-$^{13}CH_3$	Cys-$^{13}CH_3$	Total[b]
622 (0.2M)	0.56	0.32	1.35	0.40	0.46	0.29	0.97	3.47 (2.50)
1244 (0.4M)	1.62	2.36	2.05	0.79	0.53	0.56	2.08	6.01 (3.93)
1867 (0.6M)	1.84	2.74	2.74	0.90	0.87	0.60	1.89	7.00 (5.11)
2489 (0.8M)	4.58	4.49	4.49	1.59	1.84	1.07	2.31	11.31 (8.99)
3112 (1.0M)	5.71	6.53	6.53	2.39	2.23	1.37	2.14	14.66 (12.52)

[a]: includes marginal contribution of the C-terminal acid adducts, [b]: the total absolute concentration of adducts including GSH/Cys. In brackets are absolute concentrations of adducts excluding GSH/Cys.

Figure 1

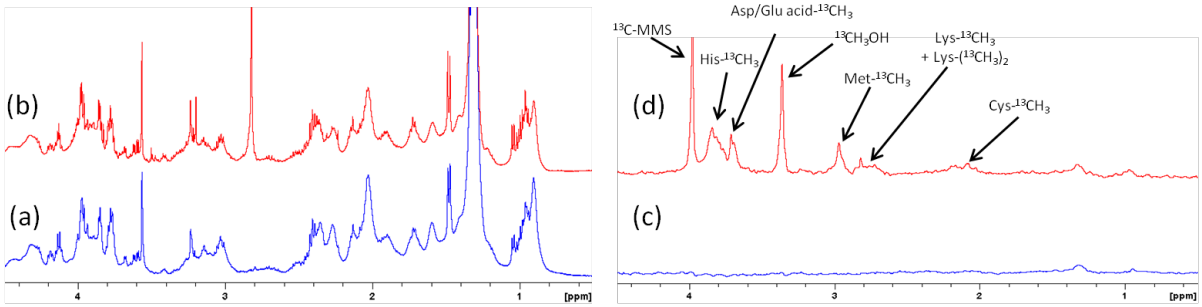

(b)
(a)
4
3
2
1
[ppm]
^{13}C-MMS
Asp/Glu acid-$^{13}CH_3$
$^{13}CH_3OH$
His-$^{13}CH_3$
Met-$^{13}CH_3$
Lys-$^{13}CH_3$
+ Lys-$(^{13}CH_3)_2$
Cys-$^{13}CH_3$
(d)
(c)
4
3
2
1
[ppm]

Figure 2

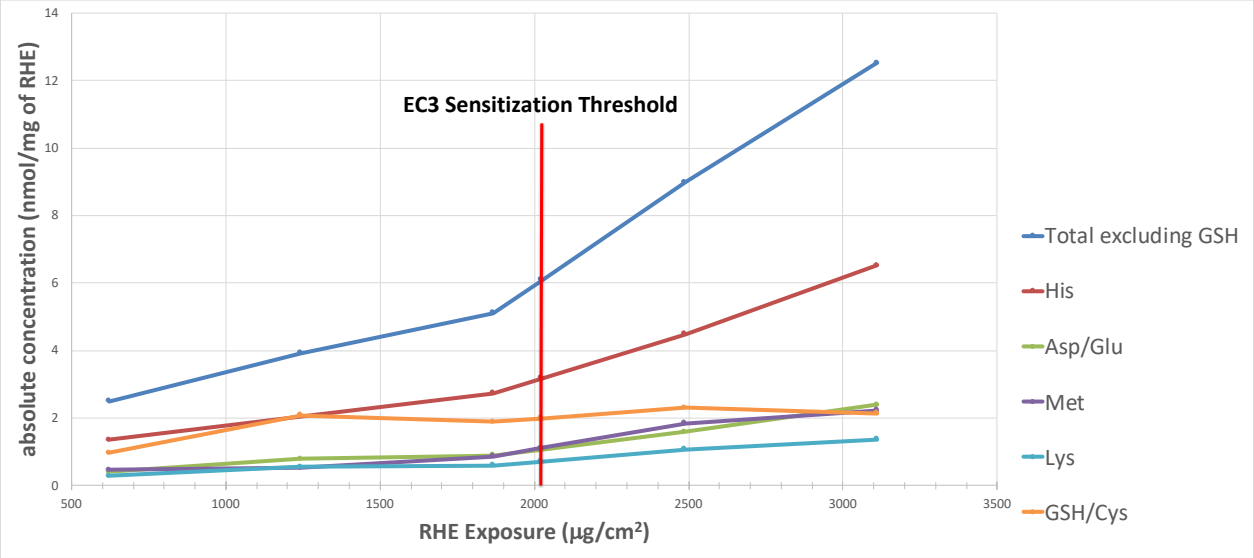

EC3 Sensitization Threshold
absolute concentration (nmol/mg of RHE)
RHE Exposure (µg/cm²)
0
2
4
6
8
10
12
14
500
1000
1500
2000
2500
3000
3500
Total excluding GSH
His
Asp/Glu
Met
Lys
GSH/Cys

Figure captions

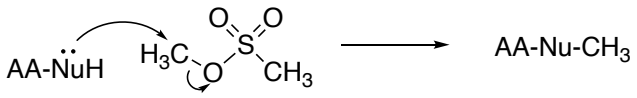
Figure 1: (a) ^{1}H NMR spectrum of a control RHE treated with 30 μL of acetone and post-incubated for 8 h. (b) ^{1}H NMR spectrum of RHE treated with 30 μL of (^{13}C)MMS (1M) in acetone and post-incubated for 8 h. (c) 1D HSQC spectrum of a control RHE treated with 30 μL of acetone and post-incubated for 8 h. (d) 1D HSQC spectrum of RHE treated with 30 μL of (^{13}C)MMS (1M) in acetone and post-incubated for 8 h.

Figure 2: Absolute concentrations of adducts formed in RHE (nmol/mg) as a function of exposure to **(13C)MMS** (μg/cm^2) after 8 hours of incubation. The vertical line indicates the sensitization threshold of 2025 μg/cm^2 derived from the EC3 value of MMS.

Scheme legends

Scheme 1: Structure and reactivity of methyl methanesulfonate (MMS) towards nucleophilic residues on side chains of amino acids.

Scheme 1



Supporting Information:

Figure S1: (a) Representative 2D HRMAS ^{1}H-^{13}C g-HSQC spectrum of control RHE. (b) Representative 2D HRMAS ^{1}H-^{13}C g-HSQC spectrum of RHE treated with **(^{13}C)MMS** and 8 hours of incubation. Arrows indicate the observed signals of adducts.

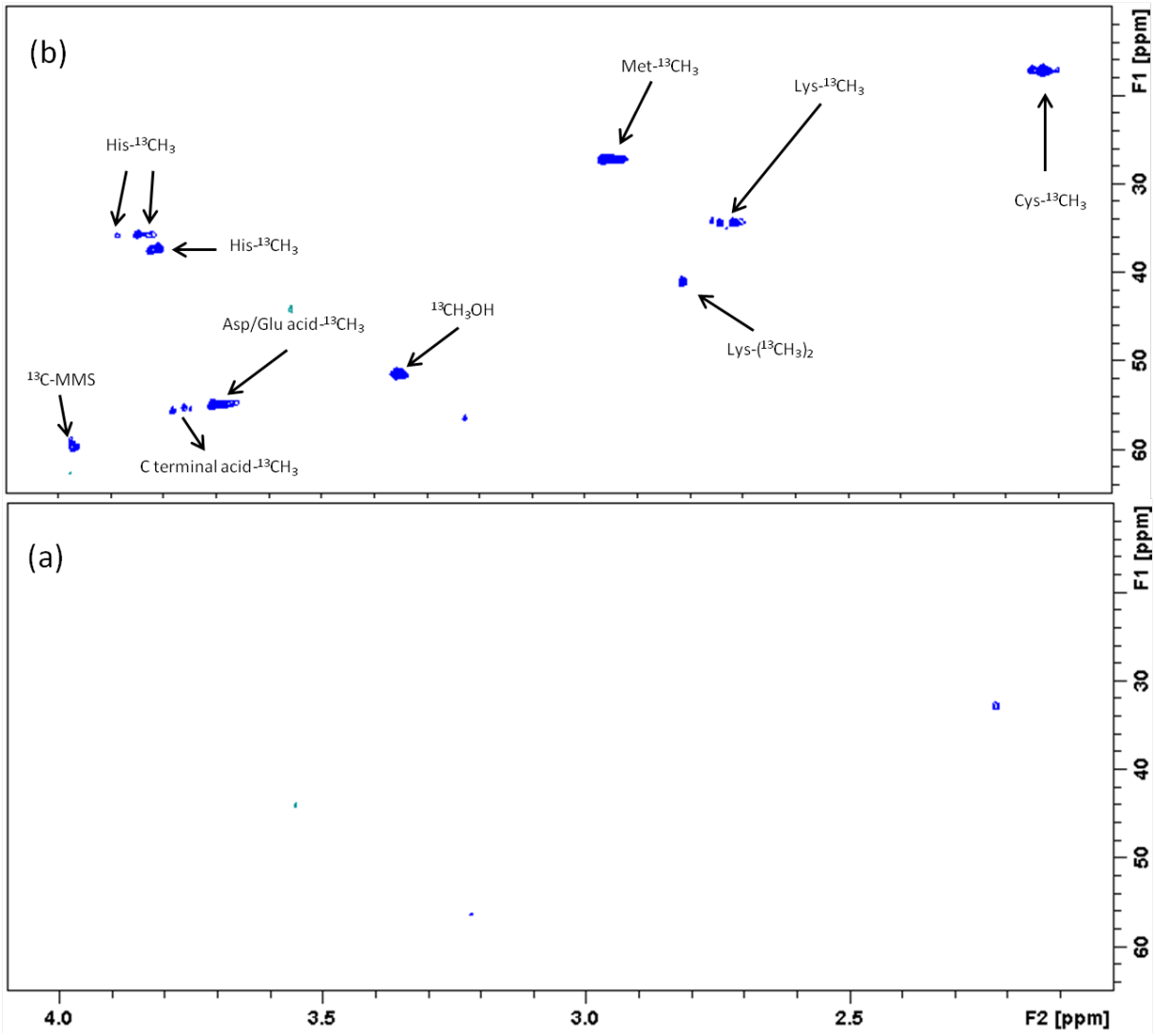
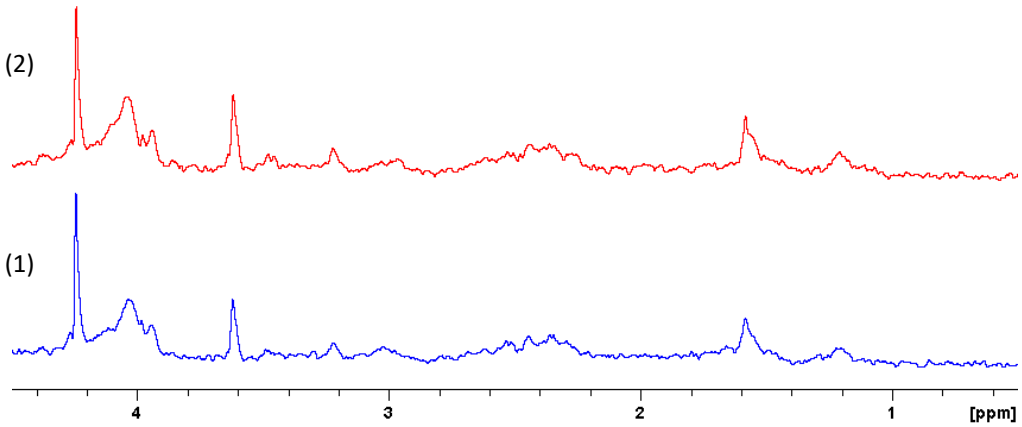


Figure S2: (a) Superimposed 1D HSQC spectra recorded consecutively, (1) then (2), for the same RHE sample following 8 h of exposure to 1244 µg/cm^2 **(^{13}C)MMS**. (b) Absolute concentration of the different resulting adducts (nmol/mg of RHE)

(a)



(b)

	His-$^{13}CH_3$	Asp/Glu acid-$^{13}CH_3$	Met-$^{13}CH_3$	Lys-$^{13}CH_3$	Cys-$^{13}CH_3$	Total
(1)	2.64	1.11	0.29	0.45	2.15	6.64
(2)	2.73	0.91	0.35	0.41	2.44	6.84

Figure S3: Superimposed ^{13}C spectra of the solutions resulting from the washing of RHE exposed to 1244 μg/cm^2 **(^{13}C)MMS** solution in acetone after 1 h **(1)** and 8 h **(2)**.