



Assessment of the skin sensitisation hazard of functional polysiloxanes and silanes in the SENS-IS assay

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ABSTRACT

In the context of a larger testing programme that aimed at assessing the skin sensitisation potential of functional polysiloxanes and silanes, this investigation complements the available *in vitro* and *in vivo* data with data in the SENS-IS assay, a human *in vitro* 3D skin-based model. The SENS-IS assay allowed testing of all functional polysiloxanes and silanes without any solubility issues or limitations related to the multiconstituent nature of the commercial grade test substances. It appeared to encompass skin metabolism, a factor which we considered important for the skin sensitisation hazard assessment particularly of aminofunctionalised siloxanes and silanes. These three technical aspects posed significant challenges in the first part of the *in vitro* programme with the OECD-validated *in vitro* assays. The SENS-IS assay delivered promising results for this group of substances. On its own, it was the best performing model, as it did not pose any technical issues with the assay and it matched all *in vivo* outcomes. Considering its performance and avoidance of any limitations due to lack of solubility or chemical composition aspects, we concluded that the SENS-IS assay to be a suitable starting point for an integrated testing strategy for skin sensitisation for the group of functional polysiloxanes and silanes.

1. Introduction

In two previous articles, we reported the results of a testing programme that aimed at assessing the skin sensitisation potential of a heterogeneous group of functionalised polysiloxanes and silanes (Petry et al., 2012, 2017). The programme served three purposes:

- (1) To elucidate the discordant results of polymeric functional polysiloxanes observed *in vivo* in the murine local lymph node assay (LLNA) and in the guinea pig maximisation test (GPMT)
- (2) To understand the performance of recently validated *in vitro* assays addressing key events along the adverse outcome pathway for skin sensitisation for functionalised polysiloxanes and silanes (OECD, 2012)
- (3) To evaluate the utility of recently published integrated testing strategies (ITS) as, for example, to determine the skin sensitisation hazard of the investigated group of functionalised polysiloxanes

and silanes.

Eight polysiloxanes and silanes, seven of them aminofunctionalised, were tested for their potential to cause skin sensitisation *in vitro* and *in vivo*. *In vitro*, the chemicals were evaluated in the direct peptide reactivity assay (DPRA), the KeratinoSens™, the human cell-line activation assay (h-CLAT) and/or the modified myeloid U937 skin sensitisation test (mMUSST). *In vivo*, the substances were tested cfvghjuikoin the murine local lymph node assay (LLNA) and in guinea pig assays according to the maximisation test (GPMT) or Buehler protocol.

In our first report, we focussed on elucidating the discordant results which were obtained for five polymeric and aminofunctionalised polysiloxanes in the LLNA and the GPMT (Petry et al., 2012). While all five silicone materials were consistently negative in the GPMT, the testing revealed weak to moderate skin sensitisation potential in the LLNA. Neither known chemical factors nor study quality factors could explain the discordant results. Only in the case of one polymeric and

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aminofunctionalised siloxane, the occurrence of an excessive level of irritation may have contributed to the positive LLNA response. A weight of evidence analysis, considering all existing information including physico-chemical factors, predicted structure activity, animal data and human experience from exposures at the workplace or via chemicals' use in cosmetics, concluded that none of the five substances represented a significant skin sensitisation hazard to humans.

In the second publication in early 2017, we discussed the responses of polysiloxanes and silanes in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assay. Additional non-polymeric functionalised siloxanes and silanes were introduced to allow an evaluation of the *in vitro* assays for silicone-based substances that were shown to be positive for skin sensitisation *in vivo* in the LLNA and the GPMT. The '2 out of 3' ITS approach by Bauch et al. (2012) was utilised to evaluate if the skin sensitisation hazard of polysiloxanes and silanes could be established on the basis of the outcomes in the respective *in vitro* assays. The silicone-type test substances were neither represented in the original *in vitro* assay validation work nor in the training set which led to the ITS proposed by Bauch et al. (2012) or Nukada et al. (2013).

Technical issues limited the ability to evaluate all functionalised polysiloxanes and silanes in the four *in vitro* assays. As at the time of programme initiation, assays were not yet accepted OECD guidelines and specific applicability domains were not fully defined yet, not all substances could be tested in all four *in vitro* assays. In particular, it is now known that the polymeric and multiconstituent-type test substances were out of the applicability domains of some of the *in vitro* assays. Solubility issues were encountered particularly with the polymeric siloxanes. Others formed emulsions or colloidal suspensions with the assay solvents increasing the uncertainty if under such conditions a sufficiently high amount of test substance reached the target cells. Another challenge was related to the multiconstituent nature of some of the commercial grade test materials. For those substances, gravimetric approaches were required resulting in test concentrations below the assay recommended levels. In these cases, negative results were considered inconclusive. Nevertheless, most tests were conducted at concentrations permitted by the guidelines. Overall, the results obtained in this first *in vitro* programme were heterogeneous. Particularly for the non-polymeric siloxanes and silanes, the *in vivo* outcomes were only inconsistently predicted across the four selected *in vitro* assays. While *in vivo* these substances were positive, the *in vitro* studies largely suggested an absence of skin sensitisation properties. Applying the non-animal based '2 out of 3' ITS approach by Bauch et al. (2012) would have correctly identified two, but would also have missed three *in vivo* sensitisers (Petry et al., 2017). Since our structure-activity assessment suggested that some of the test substances may be pro-haptens and therefore require metabolism to produce a positive skin sensitisation response, we speculated that the inconsistent results were also because of a limited metabolic capacity of the KeratinoSens™, the h-CLAT or the mMUSST assay.

In light of these technical difficulties, we selected the SENS-IS assay, an *in vitro* 3D skin model developed by Cottrez et al. (2015, 2016) to see how it would perform for the heterogeneous group of functionalised polysiloxanes and silanes. The use of a 3D skin model allows avoiding any test substance solubility and bioavailability limitations, and is considered to encompass skin metabolism more realistically than 2D skin models (Wiegand et al., 2014; Götz et al., 2012). The SENS-IS measures the gene expression of sensitisation or irritation markers at the KE-2 level of the skin sensitisation adverse outcome pathway (AOP). Furthermore, as ARE-activated genes are included, it may also provide information on protein reactivity of a test chemical (Reisinger et al., 2015). Clouet et al. (2017) proposed and evaluated a '2 out of 3' ITS in which the SENS-IS addresses KE-2, the DPRA KE-1 and the h-CLAT KE-3 and concluded that it provided very similar accuracy levels as the '2 out of 3' ITS approaches proposed by Bauch et al. (2012) and later updated by Natsch et al. (2013) and Urbisch et al. (2015) utilising the KeratinoSens™ or the LuSens to address KE-2. The SENS-IS assay was

submitted to ECVAM in September 2011 and enrolled into the OECD guideline work in 2015.

The SENS-IS assesses the potential of chemicals to specifically induce the expression of two sets of genes relevant during the sensitisation process described as 'REDOX' and 'SENS-IS' genes and another gene set which reflects the skin irritation potential (IRR genes) in the 3D EpiSkin® tissue model. The REDOX group includes a selection of 17 genes with an antioxidant responsive element in their promoter and monitor the redox protective signals induced through the interaction of sensitisers binding to cysteine amino acids of the Keap1-NRF2 complex (Urano and Motohashi, 2011). The SENS-IS group includes a selection of 21 genes involved in inflammation, danger signals and cell migration to address the complex cascade of events leading to activation of Dendritic Cells (DCs) by a sensitising substance.

The SENS-IS assay classifies a substance as a skin sensitiser if, at a given concentration, a test substance induces at least 7 genes measured by qPCR in either the REDOX or the SENS-IS group of genes. To take into account non-specific gene overexpression due to cell stress, the induction of more than 20 genes in the irritation ('IRR') gene set classifies a result as inconclusive and the test substance is re-analysed at a lower concentration. The potency of a sensitiser is determined by the minimal concentration needed to induce the over-expression of ≥ 7 genes in either the REDOX or the SENS-IS groups. A compound is classified as an extreme, strong, moderate or weak sensitiser if detected in the assay at a test concentration of 0.1%, 1%, 10% or 50%, respectively.

The purpose of this communication is to report and assess the responses of 6 of the 8 polysiloxanes and silanes in the SENS-IS assay relative to their responses *in vitro* and *in vivo* as reported earlier (Petry et al., 2012, 2017). Unfortunately, two substances could not be included into the testing programme for test substance unavailability reasons.

2. Materials and methods

The SENS-IS method for the detection of sensitisers is based on the 3D EpiSkin® tissue model as a test system and on the analysis of the expression of a large panel of genes relevant during the skin sensitisation process. The modulation of these biomarkers is measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

EpiSkin® tissue models are obtained by culturing adult human keratinocytes on a collagen substrate in conditions permitting their terminal differentiation and the reconstruction of an epidermis with functional stratum corneum. After 3 days of immersed culture conditions, the epidermis is grown at the air/liquid interface during 10 days allowing differentiation and formation of the stratum corneum.

The human keratinocytes were obtained from healthy donors during plastic surgery. Various tests were carried out to confirm the bacteriological and fungal sterility of the cells and the absence of mycoplasma.

The test substance is applied onto the EpiSkin® tissue models at different concentrations in an appropriate vehicle. After exposure, the gene expression of three gene sets, one specifically reflecting the irritant potential (IRR genes) and two gene sets correlating with the sensitisation potential, i.e., the REDOX and the SENS-IS group, is measured as described by Cottrez et al. (2015).

2.1. Test substances

The following six functionalised polysiloxanes and silanes were evaluated in the SENS-IS assay. To allow comparison with the previous publication summarising all available *in vitro* and *in vivo* data, the same substance codes as those presented in Petry et al. (2017) have been used in this communication:

Table 1
Identity and chemical nature of the test substances.

	PS-1	PS-2	PS-3	PS-4	PS-7	PS-8
Substance name	Propylpyridyl functionalised silicone (Type I)	Propylpyridyl functionalised silicone (Type II)	Poly [3-((2-aminoethyl) amino) propyl]methyl (di-methyl) siloxane	3-(2-Aminoethyl-amino) propyl-trimethoxy silane	Reaction products of vinyltriacetoxysilane and glycidoxypopyl tri-methoxy silane	Methylaminosiloxane with glycidyl trimethylammonium chloride
Chemical nature	Aminofunctional polysiloxane	Aminofunctional polysiloxane	Aminofunctional polysiloxane	Aminofunctional alkoxysilane	Alkoxysilane (non-aminofunctional)	Aminofunctional siloxane
CAS-number	171543-65-0	171543-65-0	10282-92-3 75718-16-0	1760-24-3	154518-41-9	495403-02-6
Molecular weight	Polymer > 10,000 g/Mol	Polymer > 10,000 g/Mol	Polymer > 10,000 g/Mol	222 g/Mol	Multiconstituent	Multiconstituent
Purity	> 97%	> 96%	97.6% ^a	96.4%	90–100%	70–90%

^a Polymer content (100% minus volatiles).

- Two propylpyridyl functionalised silicones, **PS-1** and **PS-2** (CAS-number 171543-65-0);
- Aminofunctional polysiloxane, methoxy and hydroxy terminated (**PS-3**; CAS-numbers 10282-92-3 and 75718-16-0);
- 3-(2-Aminoethylamino) propyltrimethoxysilane (**PS-4**; CAS-number: 1760-24-3);
- Reaction products of vinyltriacetoxysilane and glycidoxypopyl-trimethoxy silane (**PS-7**; CAS-number 154518-41-9);
- Methylaminosiloxane with glycidyl trimethylammonium chloride (**PS-8**; CAS-number 495403-02-6).

Two substances, the aminofunctional PS-5 and PS-6, for which we presented *in vitro* and *in vivo* data in our 2017 publication, could not be included into the SENS-IS programme for test substance unavailability reasons.

The test substances were all commercial grade raw materials provided by the co-authors' companies. The identity and chemical nature of the test substances are summarised in Table 1.

2.2. The SENS-IS protocol

The SENS-IS test programme was conducted in the laboratories of Immunosearch (06130 Grasse, France). The individual experiments followed the protocol described by Cottrez et al. (2015, 2016).

The EpiSkin[®] tissue models (30 days old; 1.7 cm²), meeting the supplier's acceptability criteria, were purchased from Skinethic (Lyon, France). The tissues were transferred to sterile 12-well culture dishes containing 2 mL/well of pre-warmed (37 °C) maintenance medium (DMEM/Ham's F12) which was supplied with the kit. The medium was replaced every 24 h.

Following 14 days of culturing, 30 µL of neat as well as 50% (v/v) or 10% (v/v) dilutions of the test substances in solvent olive oil (i.e., PS-1, PS-2, PS-3, PS-4 and PS-8; supplier olive oil: Puget, France) or DMSO (i.e., PS-7; supplier DMSO: PAN Biotech GmbH, Germany) were applied with a micropipette onto the stratum corneum of the EpiSkin[®] tissues resulting in a skin surface concentration of 26.3 µL/cm². The test substances were gently spread over the epidermis surfaces to ensure total surface coverage. The tissues were incubated with the test substances or the controls for 15 min at 37 °C (5% CO₂) and then washed with 25 mL PBS per epidermis. After washing, the tissues were further incubated at 37 °C (5% CO₂) for 6 h.

Each skin model was lifted from the insert using forceps and transferred into a vial for snap freezing in liquid nitrogen. To extract the total RNA, the epidermis was submerged in 1 mL Qiazol reagent (Qiagen, France) and homogenised using two steel beads in a TissueLyser (Qiagen, France). Following addition of 0.2 mL bromochloropropane to the supernatant and centrifugation at 12000g for 15 min, 60 µL of 70% ethanol was added to the aqueous phase and immediately pipetted onto an RNeasy spin column (Qiagen, France) which is placed in a 2 mL collection tube. The total RNA is subsequently

extracted according to the manufacturer's instructions.

The reverse transcription of total RNA was performed using 1 µg total RNA in 20 µL volume using Random Primers and SuperScript III Reverse Transcriptase (Invitrogen, France) according to the manufacturer's instructions. Quantitative RT-PCR was conducted using SYBR Green Real-time PCR Master Mix (Roche Diagnostics, France) with 0.4 µM of each oligonucleotide primer in a total volume of 25 µL. The reaction was performed in an LC480 System (Roche Diagnostics). The amplification programme consisted of one cycle at 95 °C with a 1 min hold, followed by 40 amplification cycles (95 °C for 15 s and, annealing at 60 °C for 15 s) and completed by final 72 °C step for 40 s.

The genes expressed in the samples were measured through an absolute quantification analysis using the software provided by Roche Diagnostics. The level of tissue destruction was evaluated by determining Cycle Threshold (CT) value of the HSPAA1 gene. A sample CT value > 110% of the corresponding value obtained for the vehicle control indicated an unacceptable level of tissue damage. Irritation signals were analysed by measuring the expression level of 23 genes as described Cottrez et al. (2016). The samples were rejected if more than 20 genes were over-expressed by more than 1.25 of the control vehicle value. A test substance was considered a skin sensitizer when at least 7 genes from either the REDOX or the SENS-IS gene lists are over-expressed by a factor greater than 1.25 of the control value (Cottrez et al., 2015).

The test substances and the controls were tested in three different batches of the EpiSkin[®] model. The protocol foresaw further experiments if invalid results were obtained in the first two experiments. Each experiment included negative controls (i.e., Olive oil, DMSO, PBS), a positive irritation (5% SLS) and a positive sensitisation (1% 2,4,6-trinitrobenzene sulfonic acid 'TNBS' in PBS) control. An overview of testing conditions is presented in Table 2.

3. Results

In all 3 experiments conducted, the skin sensitizer positive control, 1% TNBS in PBS, was correctly identified as a skin sensitizer in both the SENS-IS and the REDOX-groups with more than 7 overexpressed genes

Table 2
Overview of testing conditions.

	PS-1, PS-2, PS-3, PS-4, PS-8	PS-7
Test concentrations	10%/50%/100%	10%/50%/100%
Vehicle	Olive oil	DMSO
Positive control (irritation)	5% SLS (Invitrogen, France)	5% SLS (Invitrogen, France)
Positive control (sensitisation)	1% TNBS (Sigma, France)	1% TNBS (Sigma, France)
Negative controls	Olive oil; PBS; DMSO	Olive oil; PBS; DMSO

Table 3
Control values measured in each of the three test substance experiments.

Controls	Number of overexpressed genes			Conclusion	
	SENS-IS	REDOX	IRR		
1% TNBS (positive sensitisation)	10/12/10	17/16/11	13/ 14/ 11	Positive (sensitisation)	Acceptance criteria reached
5% SLS (positive irritation)	4/4/2	1/5/2	18/ 22/ 23	Positive (irritation)	
100% DMSO (negative control)	2/4/4	4/1/1	8/ 8/8	Negative	

in each group. One percent TNBS in PBS was also correctly identified as negative for skin irritation in the IRR-group (i.e., < 15 overexpressed genes). Likewise, SLS at 5% was correctly identified as an irritant with a number of overexpressed irritant genes 'IRR' greater than 15. SLS at 5% was also correctly identified as a non-sensitiser, with a number of overexpressed genes in the SENS-IS and the REDOX-groups below 7. The negative control DMSO neither overexpressed equal or more than 7 genes in the SENS-IS and the REDOX-groups, nor more than 15 genes in the IRR-group.

Table 3 presents the overview of the results obtained for the positive (sensitisation, irritation) and negative controls across the three experiments. In conclusion, the outcomes achieved for the positive and negative controls met the SENS-IS acceptability criteria as defined by Cottrez et al. (2016) throughout the programme. Hence, the results obtained with the silicone-based test substances, as presented in the following, are considered to be valid.

Table 4 summarises the results obtained for the 6 six functionalised polysiloxanes and silanes in the SENS-IS assay.

Test substances PS-1, PS-2 and PS-3 induced less than 7 genes in either the SENS-IS or the REDOX gene groups when tested at concentrations of 10%, 50%, or 100%. Each of the PS-4 and PS-7 test substances induced seven or more genes of the REDOX group at concentrations $\geq 50\%$, while the PS-8 test substance induced eleven genes

Table 4
Overview of SENS-IS results for 6 six functionalised polysiloxanes and silanes.

	Number of overexpressed genes			Conclusion
	10%	50%	100%	
Test substance PS-1				
SENS-IS genes	4	4	3	Negative for Sensitisation
REDOX genes	1	3	2	Negative for Sensitisation
IRR genes	1	8	6	Negative for Irritation
Test substance PS-2				
SENS-IS genes	3	2	6	Negative for Sensitisation
REDOX genes	0	0	2	Negative for Sensitisation
IRR genes	1	10	17	Positive for Irritation
Test substance PS-3				
SENS-IS genes	4	2	3	Negative for Sensitisation
REDOX genes	3	2	2	Negative for Sensitisation
IRR genes	7	9	13	Negative for Irritation
Test substance PS-4				
SENS-IS genes	5	3	3	Negative for Sensitisation
REDOX genes	1	7	7	Positive for Sensitisation
IRR genes	4	4	5	Negative for Irritation
Test substance PS-7				
SENS-IS genes	3	5	<i>Skin destruction</i>	Negative for Sensitisation
REDOX genes	3	7	<i>Skin destruction</i>	Positive for Sensitisation
IRR genes	9	17	<i>Skin destruction</i>	Positive for Irritation
Test substance PS-8				
SENS-IS genes	3	6	11	Positive for Sensitisation
REDOX genes	3	6	5	Negative for Sensitisation
IRR genes	7	13	14	Negative for Irritation

Table 5
Summary and evaluation of test results against majority concept ('2 out of 3' approach).

Test Substance	In silico		In chemico/vitro studies				In vivo studies				SENS-IS vs In vivo/WoE		ITS '2 out of 3 approach' vs in vivo/WoE	
	OECD Toolbox	DPRA	KeratinSens™	h-CLAT	MUSST	SENS-IS	Guinea Pig	LLNA	WoE		DPRA/KeratinSens™/h-CLAT	DPRA/KeratinSens™/MUSST		
PS-1	Negative	Negative	– ^a	– ^a	– ^a	Negative	Negative	Positive	Negative	Consistent prediction ^b	NA ^f	NA		
PS-2	Negative	– ^a	– ^a	– ^a	– ^a	Negative	Negative	Positive	Negative	Consistent prediction ^b	NA	NA		
PS-3	Positive ^c (Metabolite)	Negative	Negative	– ^a	Positive	Negative	Negative	Positive	Negative	Consistent prediction ^b	Consistent prediction ^c	Consistent prediction ^c		
PS-4	Positive ^c (Metabolite)	Positive	Negative	Negative	Positive	Weak Positive	Positive	Positive	Positive	Consistent prediction	Inconsistent prediction	Consistent prediction		
PS-5	Positive ^c (Metabolite)	Negative	Negative	Negative	Negative	–	Positive	–	Positive	NT ^d	Inconsistent prediction	Inconsistent prediction		
PS-6	Positive ^c (Metabolite)	Negative	Negative	Negative	Negative	–	Positive	Positive	Positive	NT	Inconsistent prediction	Inconsistent prediction		
PS-7	Negative ^c	Negative	Positive	Positive	Positive	Weak Positive	Positive	Positive	Positive	Consistent prediction	Consistent prediction	Consistent prediction		
PS-8	Positive ^c (Metabolite)	Negative	Negative	– ^a	– ^a	Very Weak Positive	Positive	Positive	Positive	Consistent prediction	Inconsistent prediction	Inconsistent prediction		

^a Not tested due to solubility issues.

^b PS-1, PS-2 and PS-3 are not considered human skin sensitiser (Petry et al., 2012).

^c Representative molecule.

^d Not tested.

^e Representative sub-structure (i.e., Methylamino siloxane).

^f Not applicable.

of the SENS-IS group at the 100% concentration. From all 6 substances, PS-2 and PS-7 were considered positive for irritation at concentrations $\geq 50\%$ (PS-7) or 100% (PS-2).

Since the assay validity criteria were met in all experiments, the test substances PS-1, PS-2 and PS-3 were considered to be negative and the test substances PS-4, PS-7 and PS-8 positive for skin sensitisation in the SENS-IS assay.

4. Discussion

To allow a good overview, all information relevant to the skin sensitisation assessment of functionalised polysiloxanes and silanes as part of this programme has been compiled in Table 5. This includes QSAR predictions, *in vivo* and *in vitro* results as well as an assessment of the consistency of prediction depending on the data comparison approach being made.

Aminofunctionalised polymeric polysiloxanes were found to produce discordant results *in vivo* in the GPMT and LLNA and heterogeneous results when tested *in vitro* in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assay (Petry et al., 2012, 2017). *In vivo*, the aminofunctionalised polymeric silicone materials were consistently negative in the GPMT, but four of five substances were found to be positive in the LLNA. Neither known chemical factors nor study quality factors could explain the discordant results. Despite the positive findings in the LLNA, a thorough weight of evidence analysis taking into account physico-chemical factors, predicted structure activity, animal data and human experience from exposures at the workplace or via chemical's use in cosmetics, concluded that none of the five substances represented a significant skin sensitisation hazard to humans.

To understand the suitability but also limitations of recently validated *in vitro* assays for assessing the skin sensitisation of functionalised polysiloxanes and silanes, an expanded group of test substances was tested *in vitro* in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assay. Due to lack of solubility in the assay media, not all substances could be tested in all four *in vitro* assays. Overall, heterogeneous results were obtained. Particularly for the non-polymeric siloxanes and silanes, the *in vivo* outcomes were inconsistently predicted *in vitro* across the four selected assays. While *in vivo* these substances were positive, the *in vitro* studies largely suggested absence of skin sensitisation properties. Applying the non-animal based '2 out of 3' ITS approach by Bauch et al. (2012), allowed the correct identification of two (i.e., PS-4 if mMUSST instead of h-CLAT is taken as KE-3 assay, PS-7), but missed the three *in vivo* (i.e., PS-5, PS-6, PS-8).

Despite some technical challenges and the heterogeneous outcome, this first *in vitro* programme provided some interesting insights into the utility and performance of these validated *in vitro* systems for the skin functionalised siloxanes and silanes. This may ultimately allow an adjustment of an *in vitro* skin sensitisation testing strategy for this class of materials depending on physico-chemical characteristics and predicted structure activity of the silicone-based substance. Besides assay specific limitations regarding the assay solvents, another important factor appears to be either the absence of metabolic activation in the case of the DPRA (Patlewicz et al., 2016) or the limited metabolic capacity of the KeratinoSens™, the h-CLAT or the mMUSST assay as reported by Natsch and Haupt (2013) to produce a positive skin sensitisation response with those functionalised siloxanes that are likely to require metabolism to act as skin sensitiser. In analogy to the knowledge that primary amines like 3-Dimethylpropylamine require oxidation to produce a positive skin sensitisation response (Aptula et al., 2005), a possible mode of action could be that amino groups in aminofunctionalised siloxanes or silanes may form, under the condition of metabolism, aldehydes and subsequently act as classical haptens. However, it should also be noted that recent reviews suggested that a large proportion of pre- and pro-haptens are correctly identified by the non-animal methods (Casati et al., 2016; Urbisch et al., 2016).

Considering the technical challenges encountered in the first *in vitro*

programme, we wondered how the SENS-IS assay, a human *in vitro* 3D skin based model, would perform for this heterogeneous group of functional polysiloxanes and silanes. The SENS-IS model is considered to encompass skin metabolism, allows potency assessment and importantly avoids any limitation regarding the solubility or the chemical nature of the test substances (Cottrez et al., 2016). Except test substances PS-5 and PS-6 which were unavailable for the testing programme, all functionalised polysiloxanes and silanes which were previously investigated *in vitro* in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assays and *in vivo* in the LLNA, GPMT or Buehler test, were also tested in the SENS-IS assay.

The SENS-IS delivered very promising results. It identified test substances PS-1, PS-2 and PS-3 to be negative and substances PS-4, PS-7 and PS-8 to be weakly positive for skin sensitisation. The testing of the PS substances thereby matched the results obtained *in vivo* in the GPMT and/or the LLNA. In case of discordant results between GPMT and the LLNA, like in the case of substances PS-1, PS-2 and PS-3, the weight of evidence as determined by Petry et al. (2012) was considered.

Qualitatively, the SENS-IS test was the one that predicted most consistently the *in vivo* results of the investigated polysiloxanes and silanes. Additionally, it also consistently predicted the *in vivo* results from a potency point of view when compared to available LLNA EC3 values. This includes test substances PS-4 and PS-8 for which the skin sensitisation potential was inconsistently predicted when evaluated on the basis of the results obtained in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assay using a '2 out of 3' approach as proposed by Bauch et al. (2012). We speculated that the limited metabolic capacity of the KeratinoSens™, the h-CLAT or the mMUSST may have been a reason why these assays suggested a false negative for substances PS-4 and PS-8. The SENS-IS appeared to have sufficient metabolic activation to turn the potential pro-haptens PS-4 and PS-8 into skin sensitisers. Unfortunately, for test substance availability reasons, we could not include the aminofunctional test substances PS-5 and PS-6 into the SENS-IS programme. This would have allowed further verification of our hypothesis that only *in vitro* assays with a sufficiently high metabolic capacity produce a positive skin sensitisation response with non-polymeric aminofunctional siloxanes and silanes.

There is wide consensus that due to the complexity of the skin sensitisation process, no single non-animal method can replace animal testing. Instead, it will be necessary to develop integrated approaches to testing and assessment using combinations of assays representing the different key elements (KE) of the skin sensitisation adverse outcome pathway (AOP). While there appears to be a growing support of the '2 out of 3' approach in classifying or not for skin sensitisation [positive or negative outcome in any two of the three OECD validated *in vitro* assays (i.e., DPRA, the KeratinoSens™, the h-CLAT)], Roberts and Patlewicz (2018) argued, on the basis of an analysis of *in vitro* and *in vivo* (human, LLNA) data available on 271 chemicals, that a binary combination approach of the DPRA and the h-CLAT gives a better performance compared to the '2 out of 3' approach proposed by Bauch et al. (2012). They highlighted the importance of paying close attention to the reaction chemistry of the substances under evaluation and their physico-chemical characteristics, to conduct the most targeted testing necessary to address the specific decision under consideration (Roberts and Patlewicz, 2018). This is particularly important for the reactive silicone-based test substances considered in this investigation.

Given the small number of test substances, it is premature to draw a final conclusion regarding the most appropriate ITS for the functional polysiloxanes and silanes considered in this investigation. The fact that all substances were outside the applicability domain of at least one of the assays underlying either the binary approach proposed by Roberts and Patlewicz (2018) or the '2 out of 3' approach proposed by Bauch et al. (2012) or Clouet et al. (2017), prevents the direct application of these ITS approaches. However, considering its good performance and avoidance of any limitations due to lack of solubility or chemical composition aspects, suggests the SENS-IS to be a suitable starting point

for an ITS for skin sensitisation for functional polysiloxanes and silanes.

The exact chemical nature and understanding of the chemical reactivity of the respective siloxane or silane will determine which additional *in vitro* assays may be the most appropriate to confirm or disprove the findings in the SENS-IS. Collection of further data with the SENS-IS, particularly for substances with similar chemical and physico-chemical characteristics and also requiring skin metabolism to become reactive, is needed to decide which ITS is the most suitable for the skin sensitisation hazard assessment of functionalised polysiloxanes and silanes. While on its own the SENS-IS showed the best performance from all *in vitro* assays considered in this investigation, it remains to be shown that the SENS-IS is sensitive enough over a broader range of substances.

5. Conclusions

In the context of a larger testing programme that aimed at assessing the skin sensitisation potential of functional polysiloxanes and silanes *in vitro* against their *in vivo* outcomes, this investigation complements the available data in the DPRA, the KeratinoSens™, the h-CLAT or the mMUSST assay with data in the SENS-IS assay, a human *in vitro* 3D skin based model. The SENS-IS assay measures the gene expression of irritation and sensitisation biomarkers at the KE-2 level of the skin sensitisation AOP. Despite the fact that this assay is not approved at OECD level yet, it allowed testing of all functional polysiloxanes and silanes without any solubility issues or limitations related to the multi-constituent nature of the commercial grade test substances. It is further considered to encompass skin metabolism, a factor which may be important in the skin sensitisation hazard assessment of aminofunctionalised siloxanes and silanes. These three technical aspects posed significant challenges in the first part of the *in vitro* programme with the validated *in vitro* assays, leading to heterogeneous testing results which largely contributed to the inconsistent prediction of the *in vivo* outcomes.

The SENS-IS delivered promising results. Alone it was best performing as it did not pose any technical issues with the assay and it matched all *in vivo* outcomes. Considering its performance and avoidance of any limitations due to lack of solubility or chemical composition aspects, we concluded that the SENS-IS to be a suitable starting point for an ITS for skin sensitisation for the group of functional polysiloxanes and silanes.

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Transparency document

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Conflicts of interest

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