



Evaluation of in vitro assays for the assessment of the skin sensitization hazard of functional polysiloxanes and silanes

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ABSTRACT

The skin sensitization potential of chemicals has traditionally been evaluated in vivo according to OECD testing guidelines in guinea pigs or the mouse local lymph node assay. There has lately been a great emphasis on establishing in vitro test methods reflecting the key biological events in the adverse outcome pathway (AOP) for skin sensitization as published by the OECD. Against this background, a group of 8 polysiloxanes and silanes, seven of them aminofunctionalised, for which in vivo data were already available, has been tested in vitro in the direct peptide reactivity assay (DPRA), the KeratinoSens™ and the human cell line activation test (h-CLAT) and in the modified myeloid U937 skin sensitization test (mMUSST) as far as technically feasible. The main objective of the programme was to determine the utility of these systems for this heterogeneous group of silicone-based substances, recognizing that some substances are outside the assays applicability domains. The presented data provided some interesting mechanistical insights into the performance of these assays for functionalised siloxanes and silanes. The data also allow for a preliminary evaluation of proposed integrated testing strategies (ITS) to determine the skin sensitization potential of chemicals which were not considered in the training sets of the respective ITS.

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1. Introduction

Skin sensitization is the toxicological endpoint associated with substances that are considered to have the intrinsic ability to cause skin allergy in humans, termed allergic contact dermatitis (ACD). This adverse effect results from an overreaction of the adaptive immune system and thus involves two phases, the induction of sensitization, which is followed upon further contact with the sensitizing substance by the elicitation of allergy symptoms (Kimber and Dearman, 2002; Basketter and Kimber, 2010). Skin sensitization or ACD is considered to be one of the key human health endpoints of toxicological assessments of a substance both in the occupational and consumer setting (Kimber et al., 2011).

The key mechanistic events underpinning the chemical and biological pathways leading to ACD in humans are well understood and have been summarised in the OECD report titled 'The Adverse Outcome Pathway (AOP) for Skin Sensitization Initiated by Covalent Binding to Proteins' (OECD, 2012). It describes the molecular initiating and key events leading to skin sensitization. The knowledge of the AOP for skin sensitization elicited by covalent binding of substances to proteins has evolved rapidly over the past decade and can be summarised as eleven steps including four key events (KEs). These include the covalent binding of the chemical to the protein (haptenation) ('molecular initiating event' (MIE) or the 'KE1'); the activation of keratinocytes resulting in release of pro-inflammatory cytokines and chemokines and activation of antioxidant responses ('KE2'); the activation of skin resident dendritic cells (DC) and/or Langerhans cells via innate immune pathways leading to maturation and mobilisation of these cells to the draining lymph nodes

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(‘KE3’); and the DC-mediated antigen presentation to hapten-specific T-cells in the draining lymph nodes, resulting in activation and proliferation of T-cells (‘KE4’).

There is general agreement within the scientific community that it is unlikely that in the near future one single non-animal test method will be able to provide sufficient information to replace the use of animals for the skin sensitization endpoint (Adler et al., 2011). Instead, information from non-testing methods (e.g., ‘in silico’-data, analogue-based read across) and in vitro approaches addressing the key events in the AOP in an integrated testing manner will be used in combination to address this health endpoint in a Weight of Evidence (WoE) framework (Patlewicz et al., 2014; Tollefsen et al., 2014; EC, 2016).

In terms of methods addressing the key events in the skin sensitization AOP, three assays have been endorsed at OECD level until now: the direct peptide reactivity assay (DPRA) (Gerberick et al., 2004, 2008; OECD, 2015a) which measures in chemico peptide reactivity of chemicals, the ARE-Nrf2 luciferase test method KeratinoSens™ assay (Emter et al., 2010; OECD, 2015b) which measures in vitro keratinocyte activation and the human cell-line activation assay (h-CLAT) (Sakaguchi et al., 2006, 2010; Ashikaga et al., 2006, 2010; OECD, 2016a) which measures in vitro dendritic cell activation.

Besides these, many other assays are in development and at different stages of the evaluation process (EURL-ECVAM, 2015). Out of these, two in vitro keratinocyte assays, the LuSens assay (Bauch et al., 2012; Ramirez et al., 2014, 2016; Urbisch et al., 2015), a test method similar to KeratinoSens™, and an assay determining the sensitizing potential and potency of chemicals using a panel of genes on an Episkin® RhE model, the so-called SENS-IS assay (Cottrez et al., 2015, 2016) are in the process of being evaluated by EURL-ECVAM. Also, data on over a hundred substances is available for the myeloid U937-based skin sensitization assays: U-SENS™ (formerly known as MUSST - myeloid U937 skin sensitization test) (Ade et al., 2006; Piroird et al., 2015; Alépée et al., 2015) and the modified MUSST (mMUSST) (Bauch et al., 2011; Urbisch et al., 2015), which similarly to the h-CLAT address KE 3. The U-SENS™ test is currently being peer-reviewed by EURL-ECVAM and a proposal for the development of an OECD Test Guideline has been included in the OECD work program (EURL-ECVAM, 2015; Ezendam et al., 2016).

Several in silico, in chemico and in vitro battery testing approaches have been proposed for the complete replacement of in vivo testing protocols. Recently, the OECD has developed a guidance document which describes around eleven proposed approaches and individual information sources that are available for testing and assessment of skin sensitization along with number of case studies (OECD, 2016b). The majority of these approaches are mechanism-based. They combine results from multiple test methods and/or computational tools that address different KEs of the AOP to estimate skin sensitization potential and occasionally potency. The testing approaches are categorised as either integrated testing or decision strategies (ITS or IDS), or sequential testing strategies (STS), which apply a fixed data interpretation procedure to the data generated with a defined set of individual information sources or as integrated approaches to testing and assessment (IATA), which follows an iterative process of information collection and the data gathered targets the generation of new data, when needed (OECD, 2008).

So far only one IATA has been proposed by Patlewicz et al. (2014). The remaining approaches are either ITS or STS based testing strategies including the ‘2 out of 3’ ITS by Bauch et al. (2012), Nukada et al. (2013), Natsch et al. (2013); the RIVM STS proposed by Van der Veen et al. (2014a,b); the IDS based approach by Strickland et al. (2016); decision tree based ITS by Macmillan et al. (2016);

potency prediction based on MIE and KE2 by Natsch et al. (2015); Kao STS and score-based ITS by Takenouchi et al. (2015); artificial neural network based statistical approach by Tsujita-Inoue et al. (2014); Bayesian ITS by Jaworska et al. (2015) and the probabilistic hazard assessment based approach by Luechtefeld et al. (2015).

Review of the different test approaches indicated that the ‘2 out of 3’ approach showed higher accuracy in predicting skin sensitizers than the approaches based on only two KEs (Ezendam et al., 2016). The ‘2 out of 3’ testing approach which was initially proposed by Bauch et al. (2012) and later updated by Natsch et al. (2013) and Urbisch et al. (2015) is based on assays reflecting the first 3 KEs: DPRA (KE1), LuSens assay and the KeratinoSens™ (KE2) and the mMUSST and the h-CLAT (KE3). As per this approach, chemicals are identified as ‘non-sensitizers’ if they are negative in both the direct (i.e., DPRA) and indirect reactivity assays (i.e., LuSens or KeratinoSens™) and as ‘sensitizers’ if positive in the DC activation test mMUSST. If the results of the protein reactivity and DC activation assays are contradictory or in case the h-CLAT is used instead of the mMUSST, then a weight of evidence approach should be used. The 2 KE based ‘Kao STS approach’ suggested a tiered approach using the h-CLAT and the DPRA for the prediction of the skin sensitization potential of chemicals. As a first step, chemicals being positive in the h-CLAT are classified as having strong or weak sensitization potential on the basis of their minimum induction value (MIT). Then, to reliably predict weak and moderate sensitizers, which the h-CLAT fails to detect, the DPRA is performed as a second step. If the chemical of interest is negative in the h-CLAT, but positive in the DPRA, the chemical is classed as a weak sensitizer, regardless of the average depletion score. If the chemical is negative in both tests, it is not considered to be a skin sensitizer (Nukada et al., 2013; Takenouchi et al., 2015).

The purpose of this manuscript is to report and assess the responses of a group of 8 polysiloxanes and silanes, seven of them aminofunctionalised, which differ in chemical characteristics, molecular weight and physico-chemical characteristics in the DPRA, the KeratinoSens™, the h-CLAT and/or the mMUSST relative to their in vivo outcomes. In vivo, the substances have been tested in the murine local lymph node assay (LLNA) and/or in guinea pig assays according to the maximization test (GPMT) or the Buehler protocol. Some of the in vivo data have been published before (Petry et al., 2012), but all in vitro data are unpublished so far. The data presented provide insights into technical challenges encountered and limitations when testing such type of polysiloxanes and silanes for skin sensitization in the selected in vitro models and allow a preliminary evaluation of current proposals for the hazard assessment and classification of chemical substances for the skin sensitization endpoint solely on the basis of in vitro data.

2. Materials and methods

2.1. Test substances

A total of eight polysiloxanes and silanes, seven of them aminofunctionalised, were tested for their skin sensitization potential in vitro and in vivo:

- Two propylpiperidyl aminofunctionalised silicones, **PS-1** and **PS-2** (CAS-number 171543-65-0); complex condensation products of differing molecular weights composed of a largely methylated siloxanes ((CH₃)₂SiO units) chain and a propylpiperidyl group at an approximate mole ratio of 1:96 (piperidine: (CH₃)₂SiO₂) described by a single generic CAS number;

- Aminofunctional polysiloxane, methoxy and hydroxy terminated ('**PS-3**'; CAS-numbers 102782-92-3 for methoxy-terminated and 75718-16-0 – hydroxy-terminated);
- 3-(2-Aminoethylamino) propyltrimethoxysilane (**PS-4**; CAS-number: 1760-24-3);
- Aminosilsesquioxane, methoxy terminated (**PS-5**; CAS-number 145775-27-5);
- Aminoethyl aminoisobutyl methyl dimethoxysilane (**PS-6**; CAS-number 23410-40-4);
- Reaction products of vinyltriacetoxysilane and glycidoxypentyltrimethoxy silane (**PS-7**; CAS-number 154518-41-9);
- Methylaminosiloxane with glycidyl trimethylammonium chloride (**PS-8**; CAS-number 495403-02-6).

Exemplary, generic and representative structures for polymeric PS-1 and PS-2 as well as PS-3 are provided in Fig. 1a and b and those for monomeric PS-4 and PS-6 in Fig. 1c and d:

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. In vivo methods

2.2.1. Murine local lymph node assay (LLNA)

Except for test substance PS-5, all functionalised polysiloxanes and silanes considered in this investigation were investigated in the mouse local lymph node assays (LLNA) according to OECD test guideline 429 (OECD, 2002). The specific study protocol followed that described by Petry et al. (2012).

In brief, nine week old female CBA/J mice were used for the LLNA studies with the test substances. For each test substance, a preliminary experiment was conducted with three animals to determine the highest tolerable exposure concentration under the conditions of the LLNA. Subsequently, five different concentrations (including solvent control) were applied daily to the ears of the

animals, which were observed for systemic effects and local skin irritation. Local irritant effects expressed as ear swelling were assessed by measuring ear thickness on Days 1, 2, 3, and 6 (after sacrifice).

In the main study, groups of 4 or 5 mice were treated by topical applications of 25 μ L test or control solutions to the entire dorsal surface of each ear. The topical applications were performed once daily over three consecutive days. In each study, a positive control was included. The negative control group was treated with the vehicle alone. On Day 6, all mice were dosed with 20 μ Ci ^{3} H-methyl thymidine by intravenous injection into the tail vein. Approximately 5 h later, all mice were sacrificed, the draining auricular lymph node excised, weighed, individually pooled for each animal (i.e., 2 lymph nodes per animal) and collected in PBS. Following further processing steps, the incorporation of ^{3} H-methyl thymidine into the lymph nodes was measured in a scintillation counter and expressed as the number of disintegrations per minute (DPM). The proliferative response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph node (i.e., DPM/lymph node). For each dose group, the stimulation index (SI) was calculated as the ratio of the arithmetic mean DPM/lymph node values (corrected for background) of the test and negative control groups. The EC3 value (theoretical concentration resulting in a SI value of 3) was determined by linear interpolation of points on the dose-response curve, immediately above and below the 3-fold threshold.

In addition to determining potential lymph node proliferative responses, ear thickness measurements and visual local skin reactions assessments were performed for all test substances except PS-7 (visual local skin reaction only). In the studies in which irritation was evaluated by ear thickness measurements and/or visual skin grading, the thickness of the left ear of each animal group and irritation (erythema or oedema) was recorded in parallel. According to OECD testing guideline 429, excessive local skin irritation is indicated by an erythema score ≥ 3 and/or an increase in ear

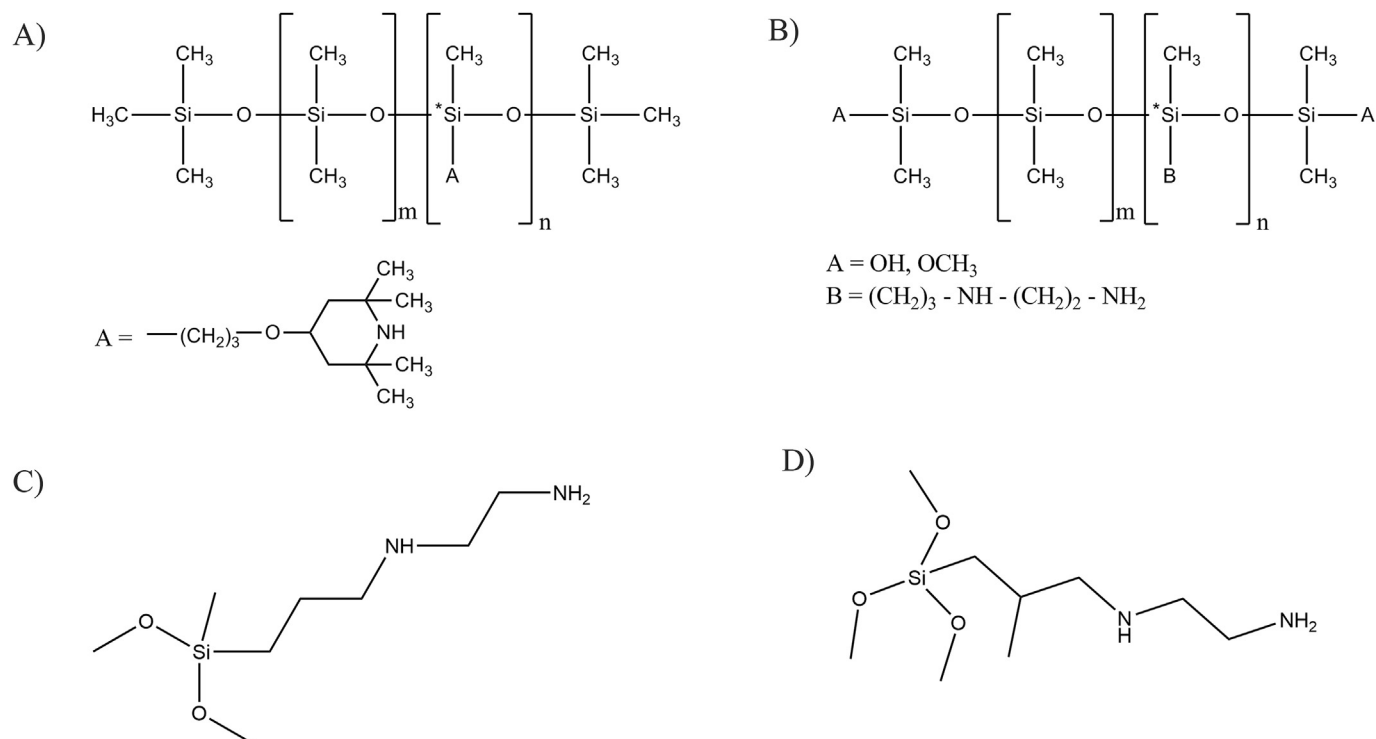


Fig. 1. a: Structure PS-1 and PS-2; b: Structure PS-3; c: Structure of PS-4; d: Structure of PS-6.

Table 1
Identity and chemical nature of the test substances.

	PS-1	PS-2	PS-3	PS-4	PS-5	PS-6	PS-7	PS-8
Substance name	Propylpyridyl aminofunctionalised silicone (Type I)	Propylpyridyl aminofunctionalised silicone (Type II)	Poly[3-(2-aminoethyl) amino] propyl-trimethoxy methyl (di-methyl) siloxane	3-(2-Aminoethyl-amino) propyl-trimethoxy silane	Aminosilses-quioxane, methoxy terminated	Aminoethyl aminoisobutyl methyl dimethoxysilane	Reaction products of vinyltriacetoxysilane and glycidioxypropyltrimethoxy silane	Methylaminosiloxane with glycidyl trimethylammonium chloride
Chemical nature	Aminofunctional polysiloxane	Aminofunctional polysiloxane	Aminofunctional polysiloxane	Aminofunctional alkoxy silane	Aminofunctional alkoxy silane	Aminofunctional alkoxy silane	Alkoxy silane (non-aminofunctional)	Aminofunctional siloxane
CAS-number	171543-65-0	171543-65-0	10282-92-3	1760-24-3	145775-27-5	23410-40-4	154518-41-9	495403-02-6
Molecular weight	Polymer 54,000 g/Mol	Polymer >100,000 g/Mol	18,000 g/Mol	222 g/Mol	Multi-constituent confidential	220 g/Mol	Multi-constituent confidential	Multi-constituent confidential
Water solubility	<0.81 mg/L	<0.81 mg/L	<0.81 mg/L	>1 g/L; hydrolysis upon water contact	>1 g/L; hydrolysis upon water contact	>1 g/L; hydrolysis upon water contact	>1 g/L; hydrolysis upon water contact	Poorly soluble
Purity	>97%	>96%	97.6% ^a	96.4%	70 - 90%	85 - 100%	90 - 100%	70 - 90%

^a Polymer content (100% minus volatiles).

thickness of $\geq 25\%$ on any day of measurement.

The experimental details of the LLNA's including solvent, doses and positive control are presented in Table 2.

2.2.2. Guinea pig assays

2.2.2.1. Maximization test (GPMT). Guinea pig maximization tests (GPMT) were conducted with each silicone material except for PS-8 according to OECD test guideline 406 (OECD, 1992). The specific study protocol followed with the PS substances is that described by Petry et al. (2012).

In brief, for each test substance, a range finding study was performed to determine the adequate dose concentrations for the intra-dermal and topical induction as well as for the topical challenge. In the main study, the animals received two induction treatments, an intra-dermal injection on Day 1 and a topical application on Day 8. Both induction treatments were conducted with the highest systemically tolerated concentrations that caused mild to moderate irritation as determined in the preliminary experiment.

The intra-dermal injection comprised three pairs of injections of 0.1 mL each of the test compound in vehicle, the test compound in a 1:1 (v/v) mixture of Freund's Complete adjuvant (FCA)/physiological saline and a 1:1 (v/v) mixture of FCA/physiological saline. The control animals received applications without test substance. 24 h before the topical application of the test substance on Day 7, the skin of the animals treated with PS-1 was pre-treated with 0.5 mL of sodium lauryl sulphate at a concentration of 10% (w/w) in vaseline. No such pre-treatment was conducted with the other test substances. Topical challenge was conducted on Day 22 by applying the test substances at the highest non-irritating concentration either via an occlusive patch systems (for test substances PS-1, PS-2, PS-4 and PS-5) or via a saturated filter paper (for substances PS-3 and PS-7) for 48 h.

Following patch removal, the skin of the animals was graded and evaluated for skin responses indicative of skin sensitization after 24 and 48 h. A test substance was considered positive for skin sensitization if at least 30% of the treated guinea pigs revealed positive skin reactions after challenge. The exact experimental details including solvent, doses and positive control are presented in Table 3.

2.2.2.2. Buehler test. Test substance PS-8 was evaluated in a guinea pig test following the Buehler protocol according to the methodology described in OECD test guideline 406 (OECD, 1992).

In this study, twenty albino guinea pig females were treated topically with the undiluted test substance under occlusive conditions once weekly for a three week induction phase. Two weeks after the application of the final induction patches, the animals were challenged with the test substance at the highest non-irritating concentration of the test substance which has been determined in an irritation screen prior to the induction phase (i.e., 75% PS-8 in PEG 300). Ten animals served as control group and were treated with PEG 300 only during the induction phase and at challenge with 75% PS-8 in PEG 300, the highest non-irritating concentration.

The same patching method was used for irritation screen, induction and challenge. The patches (25 mm Hill Top Chamber) were loaded with 0.5 mL of the test substance or solvent and firmly applied on the flank of the animals. They were secured by an elastic plaster wrapped around the trunk of the animal and an adhesive tape. The occlusive dressing was left in place for six hours.

Prior to skin grading, the test item skin area of the animals used for irritation screen and challenge were depilated approximately 21 h after the patches had been removed, using an approved depilatory cream (VEET Cream, Reckitt & Colman AG, CH-4123

Table 2
Study design of LLNA studies.

	PS-1 (CIT, 2006a)	PS-2 (CIT, 2006b)	PS-3 (BSL, 2005a)	PS-4 (BSL, 2005b)	PS-6 (DC, 2011a)	PS-7 (DC, 2011b)	PS-8 (DC, 2011c)
No. of Animals							
Preliminary	4	4	5	3	3	3	3
Per dose/Control	4/4	4/4	5/5	5/5	5/5	5/5	5/5
Positive Control	4	4	5	5	5	5	5
Induction pre-test	5; 10; 25; 50% AOO (4:1)	5; 10; 25; 50% MEK	50; 100% AOO (3:1)	50; 100% AOO (3:1)	25; 50; 75; 100% DMF	10; 25; 50; 100% AOO (4:1)	25; 50; 75; 100% AOO (4:1)
Induction main test	2.5; 5; 10; 25; 50% AOO (4:1)	0.5; 1; 2.5; 5; 10; 25% MEK	5; 10; 25; 50% AOO (3:1)	5; 10; 25; 50% AOO (3:1)	10; 25; 100% DMF	25; 50; 75% AOO (4:1)	5; 10; 25% AOO (4:1)
Ear thickness measurements	Yes; Days 1,2,3, and 6	Yes; Days 1,2,3, and 6	Yes; Days 1,2,3, and 6	Yes; Days 1 and 6	Yes; Days 3 and 6	—	Irritation screen only
Positive control	HCA	HCA	p-Phenylene-diamine	p-Phenylene-diamine	HCA	HCA	HCA

Table 3
Study design of guinea pig studies.

Type of assay	PS-1 (CIT, 2009a)	PS-2 (CIT, 2009b)	PS-3 (RCC, 1999)	PS-4 (Hazleton, 1992)	PS-5 (DC, 1996)	PS-6 (DC, 1985)	PS-7 (DC, 1999)	PS-8 (DC, 2003)
	GPMT	GPMT	GPMT	GPMT	GPMT	GPMT	GPMT	Buehler
No. of Animals								
Preliminary	4	4	5	4	2	—	8	4
Treatment/Control	20/10	20/10	10/5	20/20	20/10	20/20	20/10	20/10
Positive Control	10/5	10/5	10/5	—	10	—	5/5	—
Induction Injection	10% w/w Corn oil	5% w/w Corn oil	5% w/w Olive oil	0.1% v/v Liquid paraffin	5% Dow Corning® 360 Medical Fluid	5% Dow Corning® 344 Fluid	0.5% v/v Alembicol D	—
Pre-treatment Induction Patch	10% SLS 100% Olive oil	No 25% Olive oil	No 75% Olive oil	10% SLS 10% Codex liquid paraffin	No 100% 10% Codex liquid paraffin	10% SLS 5% Dow Corning® 344 Fluid	No 25% v/v Alembicol D	No 100%
Challenge patch	100% Olive oil	25% Olive oil	15% Olive oil	10% Codex liquid paraffin	75% Dow Corning® 360 Medical Fluid	5% Dow Corning® 344 Fluid	First challenge: 5 and 10% v/v Second challenge: 1 and 5% v/v Alembicol D	75% PEG 300
Positive control	Yes Mercaptobenzo- thiazole	Yes Mercaptobenzo- thiazole	Yes Mercaptobenzo- thiazole	No	Yes 1-chloro-2,4- dinitrobenzene	No	Yes HCA	No

Allschwil, Switzerland). The depilatory cream was placed on the patch sites and surrounding areas, and left on for up to 3–5 min. It was then thoroughly washed off with a stream of warm, running water. The animals were then dried with a disposable towel and returned to their cages.

Skin grading was performed by visual assessment of erythema, oedema and other clinical changes of skin condition 24 h after removal of the patches for the irritation screen, induction and challenge and repeated 24 h later (48-h grades) for the irritation screen and challenge.

The exact experimental details of the guinea pig assays including solvent, doses and positive control are presented in Table 3.

2.3. In vitro methods

2.3.1. Direct peptide reactivity assay (DPRA)

Peptide reactivity data were generated using the method developed by Gerberick et al. (2004, 2007) which measures the reactivity of a test chemical with model hepta-peptides containing lysine or cysteine. In this test system, the test substances are

incubated with the synthetic proteins for 24 h at room temperature. The peptide reactivity is reported as percent depletion based on the decrease in non-reacted peptide concentration in the sample relative to the average concentration measured in the control.

The specific protocol followed in the DPRA's conducted with the PS substances is that described by Bauch et al. (2012), largely in accordance with OECD TG 442C (OECD, 2015a). The deviation relates to the use of a gravimetric procedure instead 100 mM test substance stock concentration due to the absence of substance specific molecular weights at study initiation. Depending on their solubility, the test substances were dissolved in propanol, acetone or acetonitrile. Test substances were freshly prepared prior to use. The cysteine model peptide was prepared as 0.667 mM stock solution in 100 mM phosphate buffer (100 mM NaH₂PO₄, 100 mM Na₂HPO₄, both Sigma Aldrich, Germany; pH 7.5) and lysine model peptide was prepared as 100 mM stock solution in ammonium acetate buffer (1.54 g ammonium acetate in 200 mL H₂O, Sigma Aldrich, Germany; pH 10.2). Both peptides were obtained from Syniosci (Livermore CA, USA). Based on the molecular weight, the test substances were incubated (in triplicate) with the C-containing peptide in a ratio of 1:10 (0.5 mM peptide, 5 mM test substance)

and with the K-containing peptide in a ratio of 1:50 (0.5 mM peptide, 25 mM test substance). Since information on the molecular weight was not available at the test institute for the test substances at the beginning of the study, a gravimetric procedure was applied. This was done by preparing a test substance stock concentration of 3.76% instead of a 100 mM solution. This concentration was chosen on a presumed molecular weight of 375 g/Mol. Triplicates of the concurrent vehicle control (negative control) were also incubated with the peptides. Ethylene glycol dimethacrylate (CAS-number 97-90-5; Sigma-Aldrich, Germany) or p-benzoquinone (CAS-number 106-51-4; puriss., Sigma-Aldrich) were used as positive controls. The peptide/test sample solutions were incubated in the dark for 24 h at room temperature and analyzed in triplicates for each peptide. Analysis of free peptides was performed by RP-HPLC (Agilent 1200 Series) with UV detection at 220 nm using a C18 Phenomenex Luna[®] column (2.0 × 100 mm × 3 µm particle; Phenomenex). Separation of peptides was performed by isocratic flow with a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 min, followed by an increase to 90% acetonitrile. A co-elution control was performed in order to detect possible interference of the test substance with the peptides. This control consisted of the test substance, vehicle and the respective peptide buffer but without peptide. The samples were additionally analyzed by measuring UV absorbance at 258 nm and the area ratio 220/258 was calculated as a measure of peak purity. The peptide depletion of the test substance was compared to the peptide depletion of the negative control and expressed as relative peptide depletion. The mean peptide depletion as average of C- and K-peptide depletion was calculated and used for the evaluation of chemical reactivity according to the classification tree model described by Gerberick et al. (2007).

2.3.2. Keratinocyte activation assay - KeratinoSensTM

The KeratinoSensTM assay is a cell-based assay with a reporter cell line to detect potential skin sensitizers by their ability to induce the transcription factor activity Nrf2.

The assay was performed largely in line with the OECD TG 442D (OECD, 2015b) with test substance PS-3, PS-4, PS-5, PS-6, PS-7 and PS-8 according to the Givaudan protocol (Emter et al., 2010). Similar to the DPRA, the deviation relates to the use of a gravimetric procedure due to the absence of substance specific molecular weights at the test institute at study initiation. Substance PS-1 and PS-2 could not be tested in the KeratinoSensTM assay due to lack of solubility in water, DMSO or ethanol. KeratinoSensTM reporter cells, developed by Givaudan Laboratories, derived from the human keratinocyte culture HaCaT and containing a stable insertion of a Luciferase gene under the control of the antioxidant-response element (ARE) of the gene AKR1C2 were grown for 24 h in 96-well plates. The medium was then replaced by a medium containing a final level of 1% of the solvent containing the test substance. Test substances PS-4, PS-5, PS-6, and PS-7 were dissolved at 4% in DMSO. Eleven twofold serial dilutions of these test substances were then made to obtain 12 concentrations in the range from 0.00195% to 4%. These concentrations were 25-fold diluted in cell culture medium and an aliquot was added to the cells (1/4th of the final volume) to reach final concentrations between 0.2 and 400 ppm. Since no molecular weights were available at the time of the study conduct, all study data are reported in ppm (weight/volume). Test substances PS-3 and PS-8 were only poorly soluble in DMSO. These substances were first dispersed in ethanol, yielding a turbid dispersion. The ethanol dispersion was then further diluted in DMSO and these solutions were tested according to the protocol. Ethanol as control was added to DMSO and diluted similarly. Following this procedure avoided the cells to be in contact with ethanol concentrations greater than 1% at all test concentrations.

Each test plate contained 7 test substances, 6 wells with the solvent control, 1 well with no cells for background value and 5 wells with a dose response of the positive control cinnamic aldehyde (>99% pure, Sigma-Aldrich). In each repetition, three parallel replicate plates were run with this same set-up and a fourth parallel plate was prepared for cytotoxicity determination.

Two endpoints were measured: (i) cytotoxicity as determined by the MTT assay - recorded in a parallel plate with the same cell batch and made up with the same dilutions of the test chemicals -, and (ii) luciferase induction after a 48 h treatment with test chemicals. For cytotoxicity, IC₅₀ values were extrapolated. For luciferase induction, the maximal fold induction over solvent control (*I*_{max}) and the concentration needed to reach a 1.5 fold induction (EC_{1.5}) were calculated. Chemicals were rated positive if the following conditions were met: (i) the *I*_{max} indicates > 1.5 fold gene induction and this induction was statistically significant above the solvent control, (ii) the EC_{1.5} value was below 1000 µM in all three repetitions or in at least 2 repetitions if the *I*_{max} is exactly equal to 1.5, the chemical was still rated negative and no EC_{1.5} value was calculated by the evaluation sheet, (iii) at the lowest concentration with a gene induction above 1.5 fold (i.e. at the EC 1.5 determining value), (iv) the cellular viability was above 70% or (v) there was an apparent overall dose-response for luciferase induction, similar between the repetitions.

2.3.3. Modified myeloid U937 skin sensitization test (mMUSST)

The myeloid skin sensitization test is a dendritic cell activation test using the human pro-monocytic cell line U937 as surrogate for dendritic cells. In the specific protocol considered in this investigation, the change in the expression of the cell membrane marker CD 86 measured by flow cytometry after 48 h s of test substance exposure is determined. A test substance was considered to have a dendritic cell activation potential when the marker expression exceeded the threshold of 1.2 compared to the vehicle treated cells (vehicle control) at any sufficiently non-cytotoxic (i.e., cell viability ≥ 70%) concentration in two independent experiments.

The mMUSST was performed with test substances PS-3, PS-4, PS-5, PS-6, and PS-7 according to the protocol described by Bauch et al. (2011). U937 cells were obtained from the German Resource Centre for Biological Material (DMSZ; ACC 5) and cultured in suspension in complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 100 U/mL penicillin – 100 µg/mL streptomycin (Biochrom AG, Germany). 0.5 × 10⁵ cells were incubated for 48 h in a 96-well plate with five different concentrations of the test substances diluted either in culture medium or ethanol. The concentrations to be used were chosen on the basis of the results of the propidium iodide cytotoxicity test: the highest tested concentration was generally two fold the concentration inducing a cytotoxicity of 25% (CV75), while the remaining four concentrations were obtained by a two-fold dilution series of CV75. Each concentration was run in duplicate. The strong sensitizer ethylene diamine (EDA; 70 µg/mL; Sigma-Aldrich) was used as a positive control and lactic acid (LA, 200 µg/mL; Sigma-Aldrich) as a negative control. After incubation with the test substance, cells were analyzed for CD86 expression (with anti-CD86-FITC antibodies; BD Pharmingen, Germany) as well as cell viability (exclusion of propidium iodide; Sigma-Aldrich) by flow cytometry, using a BeckmanCoulter FC500MPL equipped with CXP software. The experiment was performed twice.

2.3.4. Human cell line activation test (h-CLAT)

In the h-CLAT, the pro-monocytic cell line THP-1 is being used as surrogate for dermal dendritic cells. It measures the change in the expression of the cell membrane marker CD 54 and CD 86 after 24 h

of test substance exposure. A test substance is predicted to have skin sensitization potential when the marker expression exceeds the threshold relative to the vehicle control.

The assay was performed with silicone materials PS-4, PS-5, PS-6 and PS-7 followed the methodology described by [Bauch et al. \(2011\)](#). Briefly, THP-1 cells, obtained from the German Resource Centre for Biological Material (DMSZ; ACC 16) were cultured in complete RPMI 1640 medium with 25 mM HEPES buffer and 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Biochrom AG, Germany) and 0.05 mM of 2-mercaptoethanol (Invitrogen, Germany). The cells were kept in a humidified atmosphere at 37 °C and 5% CO₂. For the experiments, cells were seeded in 24 well plates by adding 1×10^6 cells in 500 µL per well. Treatment was performed by applying 500 µL of the test substance dilution (in medium) to each well for 24 h. A total of eight concentrations of each test substance were tested in duplicate. The concentrations used in the study were chosen on the basis of the results of the propidium iodide cytotoxicity test: the highest tested concentration was generally 1.2 fold higher than the CV75 concentration, while the remaining seven concentrations were obtained by 1:1.2 dilution series of the highest concentration. The strong sensitizer 1-chloro-2,4-dinitrobenzene (DNFB; 3 µg/mL; Sigma-Aldrich) and lactic acid (500 µg/mL) were used as positive and negative controls, respectively. After the incubation with the test substance, cells were analyzed for CD54 (with anti-CD54-FITC antibodies; DAKO, Denmark) and CD86 expression (with anti-CD86-FITC antibodies; BD Pharmingen) by flow cytometry, using a BeckmanCoulter FC500MPL equipped with MXP software. The experiment was performed twice.

3. Results

This study investigated the in vitro skin sensitization assays, i.e., the DPRA, the h-CLAT, the KeratinoSens™ and/or the mMUSST assays against known in vivo responses with seven different aminofunctional polysiloxanes and silanes and one non-aminofunctional alkoxy silane (i.e., PS-7).

Not all eight test substances could be tested in each in vitro assay, largely due to solubility issues. Parts of the in vivo data, the discordant results of polymeric polyaminofunctional silicones PS-1, PS-2 and PS-3 in the LLNA and the GPMT, have been published and discussed before ([Petry et al., 2012](#)). In previous report, the codes of test substances PS-1, PS-2 and PS-3 were PFS-45, PFS-50 and PAEMPS respectively. While the test substance batches of the in vivo and in vitro programme differed, the test substances met the same manufacturer specifications.

3.1. In vivo assays

[Tables 4 and 5](#) present the results of the test substances in the guinea pig assays as well as in the LLNA.

All seven tested polysiloxanes and silanes were positive in the LLNA. The guinea pig assays, however, provided a more heterogeneous picture. Similar to the LLNA, the GPMT's were of good quality (i.e., Klimisch score 1 or 2) and no signs of systemic toxicity were noted in any of the test materials. While test substances PS-1, PS-2 and PS-3 did not show any signs of skin sensitization in the GPMT assay, substances PS-4 to PS-8 were consistently positive in the GPMT or the Buehler assay, indicating a higher level of reactivity. In light of the discordant results for PS-1, PS-2 and PS-3 in the LLNA and the GPMT, a thorough weight of evidence analysis considering all existing information including physico-chemical, structure activity, animal and available human data, concluded that despite the positive findings in the LLNA, neither PS-1, nor PS-2 or PS-3 represented a significant skin sensitization hazard to humans ([Petry et al., 2012](#)).

3.2. In chemico/in vitro assays

3.2.1. Direct peptide reactivity assay (DPRA)

[Table 6](#) summarizes the reactivity of substances PS-1 and PS-3 to PS-8 in the DPRA. Solely test substance PS-2 was not evaluated in the DPRA as the substance was not soluble in any of the vehicles used in the DPRA assay (i.e., acetonitrile, ethanol, DMSO and propanol). Further, due to analytical interference, the lysine-peptide depletion could not be determined for test substance PS-6, preventing its categorization for overall protein reactivity according to the prediction model proposed by [Gerberick et al. \(2007\)](#).

It should further be noted that the classification tree model by [Gerberick et al. \(2007\)](#) has been proposed for substances for which a molecular weight is available. Since molecular weight information became only available after completion of the DPRA studies (see [Table 1](#)), the retrospectively determined concentrations were 0.7, 2.1, 168 and 170 mM for PS-1, PS-3, PS-4 and PS-6. Substances PS-5, PS-6, PS-7 and PS-8 were tested at concentrations less than 100 mM. For confidentiality reasons, no specific concentrations can be provided on a molar basis.

Relative to the guinea pig assays which were shown to determine more reliably the skin sensitization profile of polyaminofunctional silicones ([Petry et al., 2012](#)), the DPRA correctly predicted on its own right the absence of the skin sensitization potential of substances PS-1 and PS-3 as well as the skin sensitization potential of substances PS-4 and PS-7. The DPRA failed, however, to indicate a skin sensitization potential of substances PS-5 and PS-8 which were both positive in the GPMT and the LLNA. The deviations from the actual target concentration of 100 mM as a result of using a gravimetric procedure with assumed standard molecular weight need to be taken into account when interpreting the results from the DPRA. Particularly, the results of polymeric PS-1 and PS-3 are well below 100 mM rendering the DPRA for these substances inconclusive. While for PS-5 to PS-8 higher protein

Table 4
Overview of the results of guinea pig assays.

	PS-1 (CIT, 2009a)	PS-2 (CIT, 2009b)	PS-3 (RCC, 1999)	PS-4 (Hazleton, 1992)	PS-5 (DC, 1996)	PS-6 (DC, 1985)	PS-7 (DC, 1999)	PS-8 (DC, 2003)
Skin Grades Challenge							First challenge	Second challenge
24 h - Treated	4 ^a /20 ^b	0/20	0/10	9/20	20/20	9/20	17/20	0/20
24 h - Control	3/10	0/10	0/5	1/20	0/10	0/20	6/10	0/10
48-hr - Treated	2/20	0/20	0/10	0/20	19/20	9/20	18/20	11/20
48-hr - Control	2/10	0/10	0/5	0/20	0/10	0/20	7/10	1/10
Conclusion	Negative	Negative	Negative	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer

^a Number of animals showing skin reactions.

^b Total number of treated or control animals.

Table 5

Overview of the results for LLNA.

	PS-1 (CIT, 2006a)	PS-2 (CIT, 2006b)	PS-3 (BSL, 2005a)	PS-4 (BSL, 2005b)	PS-6 (DC, 2011a)	PS-7 (DC, 2011b)	PS-8 (DC, 2011c)
Vehicle	AOO (4:1)	MEK	AOO (3:1)	AOO (3:1)	DMF	AOO (4:1)	AOO (4:1)
Concentration	2.5/5/10/25/50%	0.5/1/2.5/5/10%	5/10/25/50%	5/10/25/50%	10/25/100%	25/50/75%	5/10/25%
Stimulation Index (SI) per dose level	1.1/0.9/1.6/0.7/12.2	1.8/1.8/2.5/6.4/22.3	1.6/3.5/18.9/22.8	1.7/2.5/4.7/7.9	2.6/6.1/12.7	7.9/16/18.9	2.8/2.6/3.1
Calculated EC ₃	20.1%	2.8%	8.7%	13%	<25%	<25%	<25%
Increased ear thickness (%)	4/3/-2/6/2/13	0/5/0/3/3/6	22/23/27/64/142	22/32/32/39/54	4/3.7/-4/24	—	—
Signs of irritation (dose)	No	No	Yes (25%/50%)	Yes (25%/50%)	Yes (100%)	Slight (75%)	—
Report conclusion	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer	Sensitizer

Table 6

Summary of the results of the DPRA assay.

Test substance	Solvent	Test substance concentration ^a	% depletion C- peptide	% depletion K-peptide	Mean % depletion	Reactivity ^b	Categorization ^b
PS-1	Propanol	0.7 mM	−0.8 ± 1.5	−1.5 ± 3.1	0.0	No to minimal	Non-sensitizer
PS-3	Propanol	2.1 mM	8.6 ± 3.0	−1.4 ± 0.7	4.3	No to minimal	Non-sensitizer
PS-4	Acetone	168 mM	30.7 ± 4.5	−0.4 ± 4.1	15.4	Low	Weak sensitizer
PS-5	Acetonitrile	N/P ^c	1.2 ± 0.7	0.7 ± 0.3	0.6	No to minimal	Non-sensitizer
PS-6	Acetonitrile	170 mM	16.0 ± 0.7	Interference	—	—	Not classifiable
PS-7	Acetonitrile	N/P ^c	62.0 ± 2.6	0.7 ± 0.5	31.3	Moderate	Moderate sensitizer
PS-8	Propanol	N/P ^c	−2.5 ± 1.9	−0.7 ± 2.1	0.0	No to minimal	Non-sensitizer

^a Determined gravimetrically based retrospectively obtained molecular weight information.^b According to Gerberick et al. (2007).^c This information cannot be provided for confidentiality reasons. The actual test concentrations were below the guideline recommended concentrations but still in a range permitted by the testing guidelines. Negative results were therefore considered as inconclusive.

reactivity has to be expected if tested at 100 mM, substance PS-4 would be less reactive.

3.2.2. KeratinoSens™ assay

Table 7 summarizes the results of the aminofunctional polysiloxanes and silanes in the KeratinoSens™ assay. Only six PS materials could be tested. Test substances PS-1 and PS-2 could neither be solubilized in DMSO nor in ethanol and were therefore not evaluated further in the KeratinoSens™ assay.

The KeratinoSens™ predicted the available in vivo skin sensitization data correctly for test substances PS-3 and PS-7. Test substance PS-7 resulted in a positive response in the KeratinoSens™ which matched the positive results obtained in the GPMT and the LLNA. Substance PS-3 was found to be negative in the KeratinoSens™ as it was in the GPMT. While PS-3 was positive in the LLNA, this finding was considered to be a false positive following a weight of evidence assessment on the basis of all available human and animal data (Petry et al., 2012).

Four out of six test materials (i.e., PS-4, PS-5, PS-6, PS-8) yielded in a negative KeratinoSens™ result while the same chemicals resulted in positive responses in the guinea pig assays. These mispredictions suggest that the KeratinoSens™ has only a limited predictivity for detecting the skin sensitization potential or lack of it in aminofunctional silicones.

Table 7

Overview of results for the KeratinoSens™ assay.

Test substance	Solvent	Cytotoxicity	Luciferase induction			
		IC ₅₀ (ppm)	Average I _{max}	Average EC _{1.5} (ppm)	Positive repetitions	Overall rating
PS-3	Ethanol (emulsion)	114.96	1.09	>400	0/3	Negative
PS-4	DMSO	>400	1.21	>400	0/3	Negative
PS-5	DMSO	>400	1.29	>400	0/3	Negative
PS-6	DMSO	>400	1.06	>400	0/3	Negative
PS-7	DMSO	>400	13.52	69.80	3/3	Positive
PS-8	Ethanol (emulsion)	63.23	1.03	>400	0/3	Negative

3.2.3. Modified myeloid U937 skin sensitization test (mMUSST)

Table 8 summarizes the results of the aminofunctional polysiloxanes and silanes in the mMUSST assay. Only the skin sensitization potential of test substances PS-3 to PS-7 could be assessed in the mMUSST assay. Substances PS-1, PS-2 and PS-8 were not soluble in any of the test substances used in the mMUSST assay (i.e., culture medium, DMSO, ethanol, acetonitrile).

All tested functionalised polysiloxanes and silanes yielded in positive results in the mMUSST assay, thereby predicting correctly the in vivo skin sensitization potential of substances PS-4 to PS-7 as determined in the GPMT and the LLNA. The skin sensitization potential of substance PS-3 was mispredicted when comparing it to the outcome in the GPMT. In an earlier weight of evidence based analysis, substance PS-3 was not considered to cause skin sensitization (Petry et al., 2012), leading to the conclusion that the mMUSST assay mispredicted the skin sensitization potential of test substance PS-3.

3.2.4. Human cell line activation test (h-CLAT)

Solubility issues limited also the types of test substances that could be evaluated in the human cell line activation test (h-CLAT). From the functionalised polysiloxanes and silanes considered in this investigation, only substances PS-4, PS-5, PS-6 and PS-7 could be tested. Substances PS-1, PS-2, PS-3 and PS-8 could not be solubilized in any of the vehicles used in the h-CLAT assay, leading to

Table 8
Summary of the results of mMUSST assay.

Test substance	Solvent	Concentration range (µg/mL) ^a	CD86 induction ^b (fold induction at highest non-cytotoxic concentration)		Conclusion ^c
			Run 1	Run 1	
PS-3	Culture medium	26.7–426.8	2.12	1.64	Positive
PS-4	Culture medium	125–2000	1.58	1.61	Positive
PS-5	Culture medium	125–2000	1.57	1.29	Positive
PS-6	Culture medium	125–2000	2.11	1.85	Positive
PS-7	Culture medium	63.1–1010	1.61	1.29	Positive

^a The highest tested concentration generally corresponds to 2× the CV75 concentration (estimated concentration that affords 75% cell viability).

^b The fold induction corresponds to the highest increase observed at a concentration where cell viability is ≥ 70%.

^c Substance is considered positive if it exceeded the threshold 1.2 relative to the vehicle control in two independent experiments.

the termination of the initiated studies. Table 9; summarizes the results obtained in the h-CLAT for test substances PS-4 to PS-7.

The h-CLAT yielded in a heterogeneous picture with regard to its ability to predict in vivo skin sensitization responses in vitro. While the in vivo skin sensitization hazard was correctly predicted with the h-CLAT for substances PS-6 and PS-7, it was not correctly predicted for substances PS-4 and PS-5. These two substances were positive for skin sensitization in the GPMT and the LLNA, but were not identified to have a sufficiently high dendritic cell activation potential to consider them positive in the h-CLAT.

4. Discussion

The identification of a chemical's potential to induce allergic contact hypersensitivity is an integral part of the hazard assessment process. In the regulatory context, the skin sensitization potential of a chemical has traditionally been evaluated using animal tests, in particular the guinea pig tests following maximization or Buehler protocol as described in OECD testing guideline (TG) 406 or the mouse local lymph node assay (LLNA) as described in OECD TG 429/442A/442B.

As a result of a combination of ethical considerations, societal expectations and regulatory pressures, there is an increased demand for reducing animal testing in chemical safety assessments. Regulatory-wise, this demand already manifested itself in the European Union (EU) in the complete animal testing ban under the EU Cosmetics Regulation (EC) No 1223/2009 (EC, 2009) or in the promotion of the 3R (Reduce, Refine, Replace) concept in the EU REACH Regulation (EC) No 1907/2006 (EC, 2006). The assessment of a skin sensitization potential is a requirement for all non-corrosive chemicals to be registered under REACH. Further, REACH stipulates that every effort must be made to avoid animal testing and that animal testing should only be the last resort. Accordingly, the use of validated and adequately documented in vitro methods for

the adaptation of the standard testing regime is explicitly stated in Annex XI of the REACH regulation (EC, 2006). Due to recent developments in the non-animal testing methods for skin sensitization, the REACH regulatory text and related REACH Guidance R7a on data requirements for the skin sensitization endpoint under Annex VII have been revised, entering into force in October 2016. As a result, the REACH requirements for skin sensitization are changing, making non-animal testing the default requirement (EC, 2016).

Although huge progress has been made in understanding the chemical and biological pathways leading to allergic contact dermatitis which ultimately led to the establishment of the adverse outcome pathway (AOP) for skin sensitization by the OECD (2012), there is consensus within the scientific community that on the basis of non-animal testing methods, the skin sensitization hazard can only be established by using a combination of in silico, in chemico and in vitro methodologies.

Recently, several battery approaches of in chemico and/or in vitro tests focusing on the key steps of the skin sensitization process along the AOP have been proposed for the complete replacement of animal testing. At its most basic level, any integrated testing strategy (ITS) should comprise methods which have a mechanistic rationale, e.g., their place in the AOP for skin sensitization could be identified (OECD, 2012; Basketter et al., 2013, 2015). Reisinger et al. (2015) provides an overview of assays being used to address a chemicals potential to cause skin sensitization along the AOP. Focusing specifically on those assays considered in this investigation, the DPRA evaluates key event 1 (i.e., protein/peptide reactivity), the KeratinoSens™ represent key event 2, giving a measure of keratinocyte activation and the h-CLAT, and mMUSST describe key event 3 (i.e., dendritic cell activation). These assays are already being used in a number of ITS in combination and cover the first 3 of the 4 key events in the skin sensitization process. These ITS are based on large data sets and include, but are not limited to the '2 out of 3' approach (Bauch et al., 2012; Natsch

Table 9
Summary of the results of h-CLAT assay.

Test substance	Solvent	Concentration range (µg/mL) ^a	CD54 induction ^b (highest non-cytotoxic concentration)		CD86 induction ^b (highest non-cytotoxic concentration)		Conclusion ^c
			Run 1	Run 2	Run 1	Run 2	
PS-4	Culture medium	558–2000	<i>Below threshold</i>	<i>Below threshold</i>	<i>Below threshold</i>	<i>Below threshold</i>	Negative
PS-5	Culture medium	560–2005	<i>Below threshold</i>	<i>Below threshold</i>	<i>Below threshold</i>	<i>Below threshold</i>	Negative
PS-6	Culture medium	49–1782	3.07	2.97	1.79	1.28	Positive
PS-7	Culture medium	219–785	2.70	2.28	1.31	1.06	Positive

^a The highest tested concentration generally corresponds to 1.2× the CV75 concentration (estimated concentration that affords 75% cell viability).

^b The fold induction corresponds to the highest increase observed at a concentration where cell viability is ≥ 50%.

^c Substance is considered positive if it exceeded the threshold 2.0 for CD54 and 1.5 for CD86 relative to the vehicle control in two independent experiments.

et al., 2013; Urbisch et al., 2015), a kind of weighted majority concept, a score-based battery system (Jowsey et al., 2006; Nukada et al., 2013) or an ITS based on Bayesian Networks (Jaworska et al., 2011, 2015).

Against this background, this investigation presents the outcome of a testing programme in which the skin sensitization potential of seven aminofunctional polysiloxanes and silanes and one non-aminofunctional alkoxysilane, for which in vivo data according to OECD TG 406 and 429 are available, were evaluated in the DPRA, KeratinoSens™, h-CLAT and MUSST. These assays were selected at the time of the programme initiation in 2013 as they were most advanced in the validation process, although applicability domains were not fully defined yet. The silicone-based test substances differed in chemical structures & reactivity, molecular weight (i.e., ranging from less than 1000 to greater than 10,000 g/Mol) and physico-chemical properties (e.g., from low to high water solubility). This type of silicone-based substances was neither represented in the assay validation data nor in the training set (i.e., applicability domain) which led to the most commonly used ITS ('2 out of 3') approach proposed by Bauch et al. (2012) or in those proposed by Nukada et al. (2013) or Jaworska et al. (2011, 2015). Although it is known today that some of the studies are in conflict with current testing guideline's applicability domain, this investigation provides additional in vitro information on the performance of a new group of chemical substances in these assays along with in vivo benchmark studies to the overall data set.

The close examination of the in vivo and in vitro skin sensitization data therefore serves 2 purposes: (1) to understand the performance of aminofunctionalised polysiloxanes and silanes in the DPRA, KeratinoSens™, h-CLAT and MUSST and (2) evaluate the '2 out of 3' approach for its utility in its current form to determine the skin sensitization hazard for chemicals outside its applicability domain. The evaluation was approached from the view point of how skin sensitization hazard assessments for this class of chemicals are to be conducted and GHS/CLP classifications derived once animal data are not available or allowed anymore. Therefore, in addition to providing insights into the technical challenges encountered when testing functionalised polysiloxanes and silanes, a group of silicone-based substances with varying reactivity, solubilities and thus toxicokinetic properties, for skin sensitization in currently proposed in vitro models, the data presented sheds light on the applicability domains of the current in vitro tests and their use in current ITS/IATA ('2 out of 3') proposals for the classification of chemical substances for skin sensitization under EU CLP or UN GHS classification systems solely on the basis of in vitro data.

Table 10 summarizes the outcome of the in chemico, in vitro/ in vivo tests as well the conclusions on the test substance's skin sensitization potential in vitro based on the majority concept ('2 out of 3') as proposed by Bauch et al. (2012) relative to their outcome in vivo. To complement the experimental data, Table 10 includes also structure activity information for each of the test substances using the OECD toolbox (OECD, 2016c). As depicted in Table 10, the silicone-based test substances provide a heterogeneous picture in terms of their predicted structure activity and responses in vitro and in vivo. When trying to interpret the results, a first distinction related to the chemical nature should be made at the outset between test substances PS-1 to PS-3 and PS-4 to PS-8: While test substances PS-1 to PS-3 are of polymeric aminofunctional nature with molecular weights larger than 10,000 g/Mol and poor water solubilities, substances PS-4 to PS-7 are of lower molecular weight and water soluble. PS-8 is less water soluble compared to PS-4 to PS-7. In addition, substances PS-4 to PS-7 contain alkoxy silicon groups ('Me-O-Si') which upon contact with aqueous medium hydrolyse quickly to a free hydroxyl group ('HO-Si'). Structure activity analysis using the OECD tool box (OECD, 2016c) results in the

same structural alerts for skin sensitization of the hydrolysis product compared to the parent compound and hence the hydrolysis is not expected to impact the outcome of in vitro studies. In this context it is emphasised that substances PS-1, PS-2, PS-3 and PS-8 are hydrolytically stable under the assay test conditions.

The polymeric or multiconstituent nature of most of the substances rendered a structure activity analysis difficult. Using the OECD toolbox (OECD, 2016c), the SAR analysis therefore focused on assessing the structure activity of representative structures (PS-4 to PS-7), substructures and/or individual functional groups (PS-1 to PS-3). For none of the molecular structures, alerts for skin sensitization could be directly identified. However, considering the conditions of skin metabolism, structural alerts for skin sensitization could be identified for the substituent 'B' of PS-3 (see Fig. 1b); substances PS-4, PS-5 and PS-6. The alert for each of the substances or substructures was related to the presence of a free terminal amino group, which under the conditions of metabolism or autoxidation, could be activated by oxidation of the NH₂ group followed by hydrolysis to an aldehyde group as described by Aptula et al. (2005) for 3-dimethylaminopropylamine or ethylenediamine. No structural alert was identified for substance PS-7, neither with nor without skin metabolism. Due to the complexity of the molecular structure of substance PS-8, the SAR analysis was neither feasible nor technically meaningful.

In vivo, the polymeric aminofunctional substances PS-1, PS-2 and PS-3 revealed discordant results between the LLNA and the GPMT. While the substances were positive in the LLNA, they consistently produced negative results in the GPMT. An earlier weight of evidence evaluation concluded that these substances should be considered as not sensitizing in humans (Petry et al., 2012). Thus, the LLNA appeared to produce false positive results for this type of silicones.

In vitro, solubility issues in the recommended assay solvents prevented the testing of individual substances in each of the assays: Polymeric substance PS-2 couldn't be tested in any of the assays, PS-1 could only be tested in the DPRA, and PS-3 could be tested in three of the four assays. The aminofunctional siloxane PS-8 could only be tested in the DPRA and the KeratinoSens™ assay. Substances PS-1 formed an emulsion with the vehicle propanol in the DPRA, as did the substances PS-3 and PS-8 in their respective vehicles in the KeratinoSens™. Test substances PS-4 to PS-7 could be tested in all four in vitro test systems without greater solubility issues.

However, relative to their in vivo outcomes (i.e., PS-4 to PS-7 were consistently positive in the GPMT/Buehler assay and the LLNA), the in vitro test systems when considered as an integrated and weighted '2 out of 3' approach provided mixed results for the aminofunctional silicone-based substances. Solely, the skin sensitization potential of test substance PS-7, a non-aminofunctional alkoxysilane, would have been correctly predicted on the basis of the in vitro test results. All in vitro tests confirmed the potential of PS-7 to cause skin sensitization as identified in the GPMT and the LLNA. The situation is somewhat more complicated for test substance PS-4: If a 3-study ITS would have used the DPRA, the KeratinoSens™ and the mMUSST assay to address KE's 1–3, the positive in vivo findings for skin sensitization would have been confirmed by the in vitro data. If instead of the mMUSST assay, the h-CLAT was used in a 3-study based ITS to address dendritic cell activation (KE-3), PS-4 would eventually not have been considered as a skin sensitizer if the assessment would have been entirely based on the in vitro data. Likewise, the positive in vivo findings for test substances PS-5 and PS-6 were not mirrored in vitro when considering a single assay for each KE in a majority ITS approach. For completeness, it should be noted that the tiered h-CLAT/DPRA approach proposed by Nukada et al. (2013) would have correctly

Table 10

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		ITS '2 out of 3' approach' (Bauch et al., 2012)	
	OECD Toolbox (OECD, 2016c)	DPRA	KeratinoSens™	h-CLAT	MUSST	Guinea Pig	LLNA	DPRA/Keratino Sens™/h-CLAT	DPRA/Keratino Sens™/MUSST
PS-1	No structural alert	Negative Emulsion in propanol	NT ^a Not soluble	NT Not soluble	NT Not soluble	Negative	Positive	N/A ^b	N/A
PS-2	No structural alert	NT Not soluble	NT Not soluble	NT Not soluble	NT Not soluble	Negative	Positive	N/A	N/A
PS-3	Structural alert in functional group 'B' (skin metabolism)	Negative Soluble	Negative Emulsion in Ethanol	NT Not soluble	Positive Soluble	Negative	Positive	Consistent prediction ^c	Consistent prediction ^c
PS-4	Structural alert ^d (skin metabolism)	Positive Colloid solution in acetone	Negative Soluble	Negative Soluble	Positive Soluble	Positive	Positive	Inconsistent prediction	Consistent prediction
PS-5	Structural alert ^d (skin metabolism)	Negative Soluble	Negative Soluble	Negative Soluble	Positive Soluble	Positive	NT	Inconsistent prediction	Inconsistent prediction
PS-6	Structural alert ^d (skin metabolism)	Negative Soluble	Negative Soluble	Positive Soluble	Positive Soluble	Positive	Positive	Inconsistent prediction	Inconsistent prediction
PS-7	No structural alert ^d	Positive Soluble	Positive Soluble	Positive Soluble	Positive Soluble	Positive	Positive	Consistent prediction	Consistent prediction
PS-8	SAR analysis technically not feasible	Negative Soluble	Negative Emulsion in ethanol	NT Not soluble	NT Emulsion in ethanol	Positive	Positive	Inconsistent prediction	Inconsistent prediction

^a Not tested.^b Not applicable.^c PS-3 is not considered a human skin sensitizer (Petry et al., 2012).^d Representative structure.

identified the skin sensitization potential PS-6 and PS-7, but failed to identify that of PS-5.

Taking all together, this investigation identified some technical challenges associated with the testing of functionalised polysiloxanes and silanes in the in vitro assays covered in this investigation. This investigation was launched in 2013, two to three years before validation of the final protocols for the DPRA, KeratinoSens™ and h-CLAT and publication of test guidelines by the OECD. With current knowledge, the polymeric substances PS-1 to PS-3 are outside the test guideline's applicability domain due to their polymeric nature and poor water solubility. The multi-constituent nature going along with the difficulties in defining test substance purity and molecular weights of basically all test substances requiring the use of gravimetric approaches is a further limitation relative to the recently published testing guidelines. As a result, the actual test concentrations were below the guideline recommended concentrations but, except for the polymers PS-1 to PS-3, still in a range permitted by the testing guidelines.

The mixed results across the in vitro assays and occasional mismatches with the in vivo findings trigger the question how in a future non-animal data environment a weight of evidence (WoE) approach for the skin sensitization assessment for novel chemicals that are outside the applicability domain of the in vitro test systems should look like. However, notwithstanding that these relevant questions need to be addressed, these issues do not only apply to the in vitro systems. For the higher molecular weight hydrophobic silicones considered in this investigation (i.e., test substances PS-1 to PS-3), there was also a mismatch in vivo between the GPMT and the LLNA. In vivo studies are also not 100% predictive (ICCVAM, 2008). Thus, in the absence of human information, it is difficult to determine which in vivo study resulted in the correct outcome. In the case of PS-1 to PS-3, only a thorough WoE analysis of all available information, and here specifically the availability of human information like the absence of occupational allergic contact dermatitis in workers handling these materials with daily skin

exposures for more than 10 years or the absence of any evidence of increased allergic contact dermatitis in consumers being exposed to these substances in hair care applications at levels ≤2% indicated that the LLNA may not be suitable to evaluate the skin sensitization hazard of this class of chemicals and therefore supported the use of the GPMT data over those of the LLNA (Petry et al., 2012). Such human information does not exist for substances PS-4 to PS-8. However, for these substances, the LLNA and guinea pig data consistently suggested skin sensitization properties.

Specifically with regard to the types of polysiloxanes and silanes considered in this investigation, there is undoubtedly more work to be done to better understand at a chemical, toxicokinetic and mode of action level why certain assays may be more suitable than others. Chemically, more information is needed on the chemical reactivity of the aminofunctional groups contained in the substances and how these may interact with the in vitro systems under the respective assay conditions. Kinetically, it will be important to have reassurance particularly for those substances forming emulsions or colloidal solutions that test substances reached the target cells. Considering that the adopted assays will remain aqueous systems and solubility and/or hydrolysis have been identified to be present technical challenges for the group of aminofunctional polysiloxanes and silanes, new tissue-based models may be more appropriate to evaluate the chemicals' skin sensitization potential. In this context it should be noted that work has been initiated to assess the utility of EpiSkin tissue-based SENS-IS as described by Cottrez et al. (2015, 2016) for the assessment of the polysiloxanes and silanes covered in this investigation. Initial results look promising and will be reported upon completion of the work.

To identify and qualify more suitable new in vitro test systems, the substance's mode of action must be understood better: do these aminofunctional groups form under the conditions of metabolism for example aldehydes and thereby act as classical haptens or do these substances follow another MoA that is not captured in current test systems? If the aminofunctional polysiloxanes and silanes

require metabolism to exert their skin sensitization potential, in vitro assays with no or too limited metabolic capacity won't be capable of producing a positive skin sensitization response. Literature suggests that, except for the DPRA, the assays do have metabolic activity but it may not be enough to turn the prohapten into the electrophile that may cause the skin sensitization response observed in vivo (Natsch and Haupt, 2013). Interestingly, across all test substances, only PS-7, the only non-aminofunctional silane within the group of tested substances, was consistently tested positive in all 4 in vitro assays and thereby confirming the in vivo outcome. In comparison to the heterogeneous results obtained for the aminofunctional siloxanes and silanes, this observation paired with the knowledge that primary amines require oxidation to cause a skin sensitization response (Aptula et al., 2005) supports the role of the assay's metabolic capacity in producing consistent outcomes for these aminofunctional substances. However, in this context it should be noted that potent sensitizing amines such as ethylenediamine or 3-dimethylaminopropylamine have been reported to be detected in the KeratinoSens™ assay (Natsch et al., 2013). Moreover, more recent reviews suggest that large proportions of the pre- and pro-haptens are correctly identified by the non-animal methods (Casati et al., 2016; Urbisch D. et al., 2016). A better understanding of these aspects may help defining the most appropriate in vitro methods for aminofunctional polysiloxanes and silanes to improve the predictivity of the skin sensitization potential of this substance group on the basis of non-animal approaches.

5. Conclusions

This investigation aimed at understanding the performance of a total of eight polysiloxanes and silanes, seven of them aminofunctionalised, in the DPRA, the KeratinoSens™, and the h-CLAT and/or the mMUSST in vitro skin sensitization assays relative to their in vivo outcomes in the GPMT, the Buehler test and/or the LLNA. This type of silicone-based substances was neither represented in the original in vitro assay validation work nor in the training set which led to proposed ITS. The '2 out of 3' ITS approach proposed by Bauch et al. (2012) was utilised to evaluate if the skin sensitization hazard of the group of polysiloxanes and silanes could be established on the basis of the heterogeneous in vitro results obtained.

Due to lack of solubility, not all substances could be tested in all four in vitro assays. A second technical challenge was related to the multiconstituent nature of some of the commercial grade test substance which required using gravimetric approaches to establish the test concentrations. As a result, the actual test concentrations were largely below the testing guideline recommended concentrations rendering in the case of negative findings the results as inconclusive. Nevertheless, most tests were conducted at concentrations permitted by the guidelines. For those substances that could be tested in vitro, the results were heterogeneous. Particularly for non-polymeric siloxanes and silanes, the in vitro assays inconsistently predicted the in vivo outcomes. While in vivo these substances were positive, the in vitro tests largely suggested absence of skin sensitization properties. Across all test substances, only for one substance, the only non-aminofunctional silane, the in vivo outcome was correctly predicted by all four assays. This suggests an important role of the assay's metabolic capacity in producing a positive response for the aminofunctional polysiloxanes and silanes. Individual assays such as the h-CLAT appear to be less suitable for addressing the potential of polyaminofunctional siloxanes to activate dendritic cells (KE-3) than the mMUSST assay. Relative to the in vivo results, the non-animal based '2 out of 3' ITS approach proposed by Bauch et al. (2012) would have correctly identified 2 in vivo sensitizers (if MUSST instead of h-CLAT

is considered as KE-3 assay), but would have missed 2 in vivo sensitizers. The data set is too small to derive meaningful statistical conclusions, but the data presented here may be included in the larger data sets that led to recently proposed ITS/IATA approaches to increase the classes of chemicals included in the respective training sets.

Future work should focus on improving the understanding of the mode of action how these aminofunctional polysiloxanes and silanes cause skin sensitization and identify most suitable in vitro skin sensitization testing strategies accordingly.

Conflict of interest statement

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