



A dual luciferase assay for evaluation of skin sensitizing potential of medical devices

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Received: 20 March 2019 / Accepted: 2 July 2019 / Published online: 30 July 2019
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Abstract

According to standing regulations animal tests are still state of the art for the evaluation of the sensitization potential of medical devices. The aim of our study was to develop an in vitro method that can be used for testing of extracts of medical devices. The novel MDA-ARE assay is a cell based reporter gene assay focused on the ARE-Nrf2 pathway, which is involved in the dermal sensitization process. Optimization of the reporter construct and the cell line resulted in an improvement of the detection limit and a reduction of the incubation time to 6 h, which lowers cytotoxic side effects of the extracts on the cells. Using the assay, 21 out of 22 pure chemicals were identified correctly as skin sensitizers or non-sensitizers. All sensitizers could be detected at far lower concentrations compared to the local lymph node assay, the state-of-the-art animal test. To evaluate the assay's suitability for the testing of medical devices, medical grade silicone containing 0.1% of known skin sensitizers was prepared as positive controls and extracts of these positive controls were tested in comparison to extracts from pure silicone samples. All silicone samples were correctly and reproducibly identified as sensitizing or non-sensitizing demonstrating that the MDA-ARE assay is a sensitive and reliable tool for the detection of skin sensitizers in extracts of medical devices. The developed and validated test protocol was used for medical device extracts and showed its applicability for real samples and thus can contribute to reduce or even to replace the need for animal tests.

Keywords In vitro · Skin sensitization · Medical devices · Luciferase · ARE · Nrf2

Introduction

Humans are exposed to a variety of toxic substances, many of which can lead to irritating and sensitizing reactions. In particular, medical devices like implants exhibit long-term contact to human tissues. In order to guarantee safety for the

end-users of medical devices, products have to be tested for adverse reactions on the skin. Equipment that gets in contact with the patients' skin has to be assessed at least for cytotoxicity, irritation and sensitization potential [1]. According to international standards (ISO 10993), only cytotoxicity can be evaluated with in vitro methods, while the assessment of an irritating or sensitizing potential of medical devices is still based on animal tests [2, 3]. Substitution of these in vivo tests for assessment of skin irritation and skin sensitization are driven by the Organisation for Economic Co-operation and Development (OECD) and the European Union Reference Laboratory for alternatives for animal testing (EURL ECVAM) [4–6]. The aim of this work was focused on the development and validation of a suitable in vitro testing method to estimate the skin sensitization potential of medical device extracts. Skin sensitization is defined as a cutaneous reaction triggered by immunological responses to a substance, which can manifest in the form of allergic contact dermatitis [7, 8].

Elisabeth Mertl and Elisabeth Riegel have contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11033-019-04964-8>) contains supplementary material, which is available to authorized users.

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First attempts for predicting sensitization of chemicals as well as of medical devices were based on guinea pig tests [9, 10] and later on the mouse local lymph node assay (LLNA) [8]. Socio-political pressure focused on the 3R principle [11] has led to various attempts in the development of alternative methods [12–14]. In 2015 the first methods became accepted as OECD guidelines as validated *in vitro* assays to assess skin sensitization (DPRA and KeratinoSens) [4, 5]. In 2016 the h-Clat assay was added [6]. However, these assays have only been validated for pure chemicals, whereas extracts of medical devices are still undergoing animal testing [15].

Extracts of medical devices show different challenges for *in vitro* testing [16]. On the one hand, matrix effects might disturb the tests, on the other hand cytotoxic substances leaching from the medical device can interfere with cell culture assays. Furthermore, medical devices should be extracted with polar and non-polar solvents. The use of non-polar solvents in cell culture assays bears risk of cytotoxicity if they are not diluted, however a dilution reduces the chance of detecting sensitizers at low concentrations. Therefore, most *in vitro* tests focus on the assessment of pure chemicals and so far no *in vitro* test for detection of the skin sensitizing potential of extracts of medical devices has been established.

However, *in vitro* assays are not the only available information source for the evaluation of the safety of substances but form part of a weight of evidence approach in an integrated testing strategy (ITS) [17]. As sensitization itself is the result of various complex events, many researchers believe that no single test alone can predict all human sensitizers [15]. While animal tests screen the whole sensitization process, alternative *in vitro* testing methods are focused on various different key events of the adverse outcome pathway (AOP) starting with an initial binding of haptens to proteins leading to activation of keratinocytes and dendritic cells and proliferation of T-cells and finally the manifestation as allergic contact dermatitis [18]. In this context, approaches combining various *in vitro*, *in chemico* and *in silico* tests have been established [19]. Following procedures of integrated approaches to testing and assessment (IATA), prediction models are already used in the evaluation of chemicals in the cosmetic industry and in the EU regulation for registration, evaluation, authorization and restriction of chemicals (REACH) [20]. Through IATA, already available data, together with newly generated results from different tests and computer based models (for structural similarities or mechanistic categories), is assessed for the characterization of chemical hazards [21–25].

Although risk assessment through prediction models has already gained wide acceptance, assessment of sensitization of complex formulations of medical devices is still a more difficult challenge. Especially impurities, degradation and decomposition products which form part of the final

product, but were not intentionally added during manufacturing, cannot be identified easily. These substances might influence the toxicity of the product, which makes *in silico* hazard assessment challenging [26, 27]. The main advantage of *in vivo* and *in vitro* tests is that no information about the composition is necessary for risk assessment.

The antioxidant response pathway was found to be a good sensor for the prediction of skin sensitizing potential [28]. The pathway activity is upregulated in skin models as well as in various keratinocyte cell lines in a quantitative way after treatment with skin sensitizing chemicals [17, 29] and was successfully used to create the KeratinoSens cell culture assay which has been published as an OECD testing guideline for pure substances [5]. In non-activated cells, the cytoplasmic protein Keap1 (Kelch-like ECH-associated protein 1) usually binds to Nrf2 (nuclear erythroid 2-related factor 2) and leads to its rapid degradation via ubiquitination. After reaction of thiol-groups with chemicals, Keap1 undergoes conformational changes and dissociates from Nrf2, leading to its accumulation and nuclear translocation. In the nucleus Nrf2 heterodimerizes with small Maf proteins and binds to antioxidant response elements (ARE) in promoter regions of cytoprotective and antioxidant genes, resulting in activation of these target genes (reviewed in [30]).

We used the antioxidant pathway to detect skin sensitizers as pure substances and in complex formulations. The major goal was to enable testing of medical device extracts and provide an alternative to animal testing.

Materials and methods

Test materials

Sensitizing chemicals were benzylideneacetone (CAS 122-57-6, Sigma), oxazolone (15646-46-5, Sigma), 1,4-benzoquinone (CAS 106-51-4, Sigma), 2,4-dinitrochlorobenzene (CAS 97-00-7, Sigma), 4-nitrobenzylbromide (CAS 100-11-8, Sigma), glyoxal (CAS 107-22-2, Sigma), methyl-dibromoglutaronitrile (CAS 35691-65-7, Sigma), cinnamic aldehyde (CAS 104-55-2, SAFC), isoeugenol (CAS 97-54-1, Sigma), ethylene glycol dimethacrylate (CAS 97-90-5, Sigma), 2-mercaptobenzothiazole (CAS 149-30-4, Sigma), tetramethyl thiuram disulphide (CAS 137-26-8, Sigma), eugenol (CAS 97-53-0, Sigma), cinnamic alcohol (CAS 104-54-1, Sigma) and non-sensitizing substances include sodium dodecyl sulfate (CAS 151-21-3, Carl Roth), salicylic acid (CAS 69-72-7, Carl Roth), glycerol (CAS 56-81-5, Carl Roth), lactic acid (CAS 50-21-5, Carl Roth), methyl salicylate (CAS 119-36-8, Thermofisher), diethylphthalate (CAS 84-66-2, Thermofisher), sulphanilamide (CAS 64-74-1, Thermofisher), chlorobenzene (CAS 108-90-7, VWR) and isopropanol (67-63-0, CHEM-LAB).

Tested chemicals were chosen according to the “Performance Standards for Assessment of Proposed Similar or Modified In Vitro Skin Sensitisation ARE-NRF2 Luciferase Test Methods” published by the OECD and according to Casati et al. [5, 31].

For extraction the solvents dimethylsulfoxide (DMSO) (CAS 67-68-5, Merck) and phosphate buffered saline (PBS) (Biochrom, L1825) were used.

Preparation of sensitizing medical device samples

The silicone Silpuran[®] 2400 was purchased from Wacker Chemie. The silicone consisted of two parts: A and B, whereby part A included a Pt-based catalyst and part B referred to liquid silicone. Pure silicone samples (negative control) were prepared and furthermore, the skin sensitizers cinnamic aldehyde, 1,4-benzoquinone and 2,4-dinitrochlorobenzene were added to the silicone at a concentration of 0.1% w/w. Sensitizing chemicals were added to aliquots of component B and mixed with a glass rod. Subsequently, part A and part B (containing sensitizers) were mixed in a ratio of 1:1 and again homogenized with a glass rod. The mixture was then transferred to glass test tube and cured overnight. After breaking the glass, the silicone containing skin sensitizers could be recovered and was cut into smaller pieces.

Extraction of samples and positive control

All samples were extracted with DMSO and PBS using a surface volume ratio according to ISO 10993-12:2012 [32]. 3 cm²/mL were applied to solid samples with a defined surface that were thicker than 0.5 mm. Samples thinner than 0.5 mm were extracted with 6 cm²/mL and textiles like sutures were extracted using 0.2 g/mL. Extraction was performed at 37 ± 1 °C for 72 ± 2 h with agitation. DMSO extracts were diluted 1:100 in PBS and PBS extracts 1:100 in PBS + 1% DMSO. Further dilution steps were made. Extracts and dilutions were applied to the cells within 7 days. Samples were stored at 5 °C until testing. As a positive control cinnamic aldehyde (32 µM) was added to the extracts to detect false negative results derived from sample matrices interacting with the testing system.

Sample preparation of pure chemicals

Chemicals were dissolved in DMSO or PBS 1% DMSO to a concentration of 200 mM. For the highest concentration in the assay (2 mM) the stocks were further diluted 1:100 in PBS or PBS 1% DMSO resulting in a final DMSO concentration of 1%. Samples were further diluted 1:2 in PBS 1% DMSO from 2 mM to 0.98 µM [5].

Plasmids for reporter optimization and generation of stable cell lines

For optimization of the ARE reporter (ARE sequence, multimerization of ARE binding site, reporter gene) we used transient transfection experiments in HEK293 cells. The pMlucM reporter plasmids were derived from pMlucF backbone [33] by changing the minimal Fos promoter to the artificial minimal promoter (TATAAAATTCTCATTCCAG CCGATACCGTCTCACTCT). Four different ARE binding sites were inserted with oligo cloning into the multiple cloning site and multimerized up to 12 times. The AREa sequence is derived from the human *AKR1C2* gene [34]. For AREb only the consensus core sequence of the *AKR1C2* gene was used. For AREc the AP-1 binding site within the AREb were mutated and AREd is the ARE sequence of mouse *Gst1* [35]. The constructs for generation of stable cell lines (pGVL8 backbone) in HaCat and MDA-MB486 cells, contained multimerized ARE binding sites upstream of the artificial minimal promoter and NanoLuc with protein and mRNA destabilizing sequences (NlucP) [36, 37]. Further the plasmid contained a constitutively expressed firefly luciferase (Fluc) for internal viability measurement, a puromycin resistance gene for selection and piggy bac terminal repeat sequences for genome integration [38]. For details concerning the single plasmids and stable cell lines used in the results section see S1 Table.

Cell culture, transient transfection and generation of stable cell lines

HEK293 (HEK293 T-Rex purchased from Invitrogen) and HaCat cells (purchased from Cell Line Service) were cultivated in DMEM (PAN-Biotech) supplemented with 10% (Fetal Calf Serum) FCS (PAN-Biotech) and Penicillin/Streptomycin (HyClone). Medium for MDA-MB486 (purchased from ATCC) was further supplemented with non-essential amino acids (HyClone). Cells were kept at 37 °C, 5% CO₂ in a humidified atmosphere. For transient transfection experiments 1 × 10⁴ HEK293 cells per well were seeded into polyethyleneimine (PEI)-coated [39] transparent 96-well plates, incubated for 24 h at 37 °C, and transfected with TurboFect (Thermo Fisher Scientific) according to the manufacturer's instructions with 10 ng reporter plasmid per well. For the generation of stable cell lines, HaCat or MDA-MB486 cells were transfected with pGVL8 constructs using the piggy bac transposon system [38]. After 48 h cells were selected with 1.5 µM puromycin added to the normal growth medium. Positive clones were picked after puromycin selection. Clones were tested for induction with 100 µM benzylideneacetone and selected for low basal NlucP levels and high induction rate. For more information on stable cell lines see S1 Table.

Luciferase and viability measurement

For all experiments except for Table 1, dual luciferase and resazurin measurements were performed as described previously [33]. For single luciferase measurement, only two dispensers (1 × substrate, 1 × H₂SO₄) were used, and luciferase activity was measured directly after substrate dispensing.

MDA-ARE assay used to perform experiments shown in Table 1

100 μL of a cell suspension (10⁵ cells/mL) were added to 96 well plates and incubated for 24 ± 4 h at 37 °C in a humidified atmosphere with 5% CO₂. Afterwards, the medium got replaced by 50 μL of the sample or dissolved chemicals. The plates were covered with sealing film and incubated for 6 h at 37 °C and 5% CO₂.

For measurement of the viability, Steady-Glo[®] substrate (Promega) was dissolved in the corresponding buffer and further diluted 1:10 with RO-H₂O to a final concentration of 1.54 mg/mL. 50 μL of the Steady-Glo[®] solution were added to the wells and luminescence measured with a plate reader (Victor³, Perkin Elmer).

Afterwards, for measurement of sensitization, the Nano-Glo[®] Luciferase substrate was diluted 1:50 in the corresponding buffer and further diluted 1:10 in PBS containing 1.5% EDTA. 50 μL of this solution was added to the wells and luminescence measured with a plate reader (Victor³, Perkin Elmer).

Fold inductions were calculated as the ratio of the relative light units of the samples to the relative light units of untreated PBS + 1% DMSO. A fold induction above 2 was counted as significant activation of the ARE-Nrf2 pathway.

Results

Reporter construction

The induction of the ARE-Nrf2 pathway was demonstrated to be a reliable indicator of the sensitizing potential of pure chemical substances [28]. To be able to use an ARE reporter approach for testing extracts with only low concentrations of actual sensitizers, but with a large number of other potential cytotoxic components in a complex matrix, the development of a reporter cell line reacting to low concentrations of sensitizers was essential. ARE sequences are present in a large number of cytoprotective genes and the ARE core consensus sequence as binding site for Nrf2 was defined as TManRTGAYnnnGCRw-www [40, 41]. We selected four different ARE sequences (termed AREa, AREb, AREc and AREd) and cloned them upstream of a minimal promoter (TATA box) and firefly

luciferase (Fluc) as a reporter gene (Fig. 1a). Using AREb as a model we multimerized the sequence up to 12 times and tested the reporter activation after treatment with the potent ARE inducer benzylideneacetone [28]. We found best reporter induction with four–eight AREb sequences

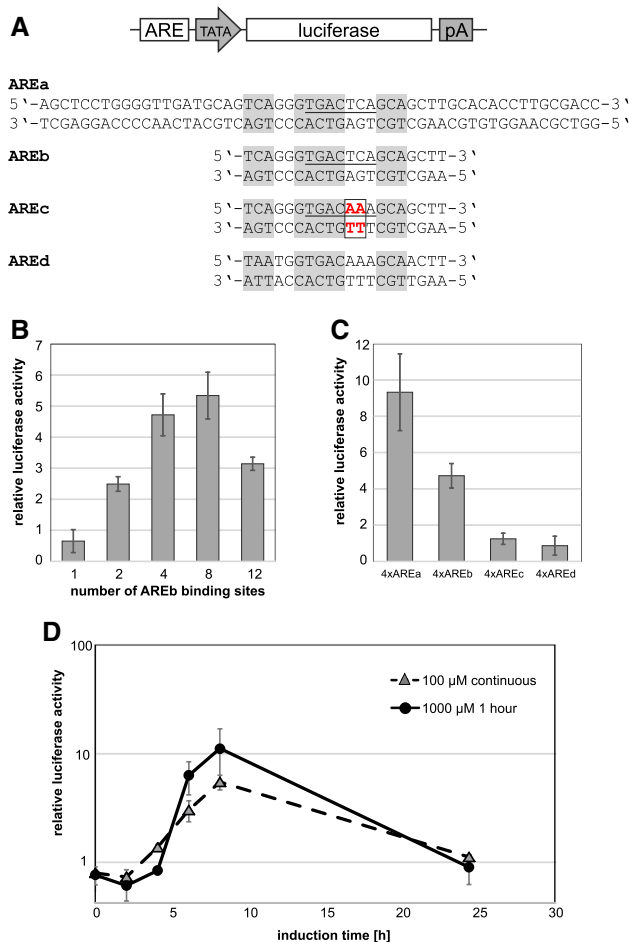


Fig. 1 ARE reporter construct optimization. Four different ARE sequences (AREa, AREb, AREc and AREd) were cloned upstream of a minimal promoter (TATA) and luciferase as reporter gene and a polyadenylation signal (pA) (a). Bases of the ARE consensus sequence are shaded and the overlapping AP1 site is underlined. Mutations of AREc compared to AREb (AP-1 site) are boxed and highlighted in red. For determination of optimal ARE numbers, AREb was multimerized 1–12 times and the different reporters (pMlucM 1 × –12 × AREb) were transiently transfected into HEK293 cells (b). For comparison of the different ARE sequences 4 times multimerized reporter constructs (pMlucM 4 × AREa,b,c,d) were transiently transfected into HEK293 cells (c) and for kinetics 4 times multimerized NlucP reporter (pGVL8 4 × AREa) was transiently transfected into HEK293 cells (d). 2 days after transfection cells were induced with 100 μM benzylideneacetone in DMEM + 10% FCS for 24 h before luciferase measurement [b–d (continuous)] or for 1 h with 1000 μM benzylideneacetone in DMEM + 10% FCS and measured after 0, 2, 4, 6, 8 and 24 h recovery time [d (1 h)]. Y-axis shows relative luciferase activity compared to uninduced control transfections. All values show means of at least 3 independent experiments with 12 replicates per plate. Error bars indicate SEM

(Fig. 1b). Due to lower background signals, we decided to concentrate on $4 \times$ ARE repeats and compared the different sequences after 24 h induction with 100 μ M benzylideneacetone (Fig. 1c). A direct comparison showed that AREa and AREb from human *AKR1C2* worked best (9.3-fold [± 2.1 SEM (standard error of the mean)] and 4.7-fold [± 0.8 SEM] induction, respectively). The loss of the AP-1 binding site in the AREc sequence completely abolished reporter activation under the tested conditions and also the AREd sequence from the mouse *Gst1* gene was not inducible in our system (1.2-fold [± 0.3 SEM] and 0.9-fold [± 0.5 SEM] induction respectively). We also looked at the pathway kinetics by using a short-lived reporter gene (NlucP). We induced the cells either for 1 h or continuously with 1000 μ M benzylideneacetone and found that

the reporter reacted with a rapid increase of reporter signal after a 4 h lag time and showed peak activation after 8 h (Fig. 1d).

Stable ARE reporter cell lines

For a first attempt to create a stable ARE cell line we used human keratinocytes (HaCat) as they are of skin origin and were shown to be a good host for ARE reporter constructs [34]. The constructs (Fig. 2a) that were stably integrated contained multimerized ARE sequences upstream of a minimal artificial promoter and NlucP as a reporter gene. The constructs further contain a constitutive minimal bidirectional beta actin promoter to drive

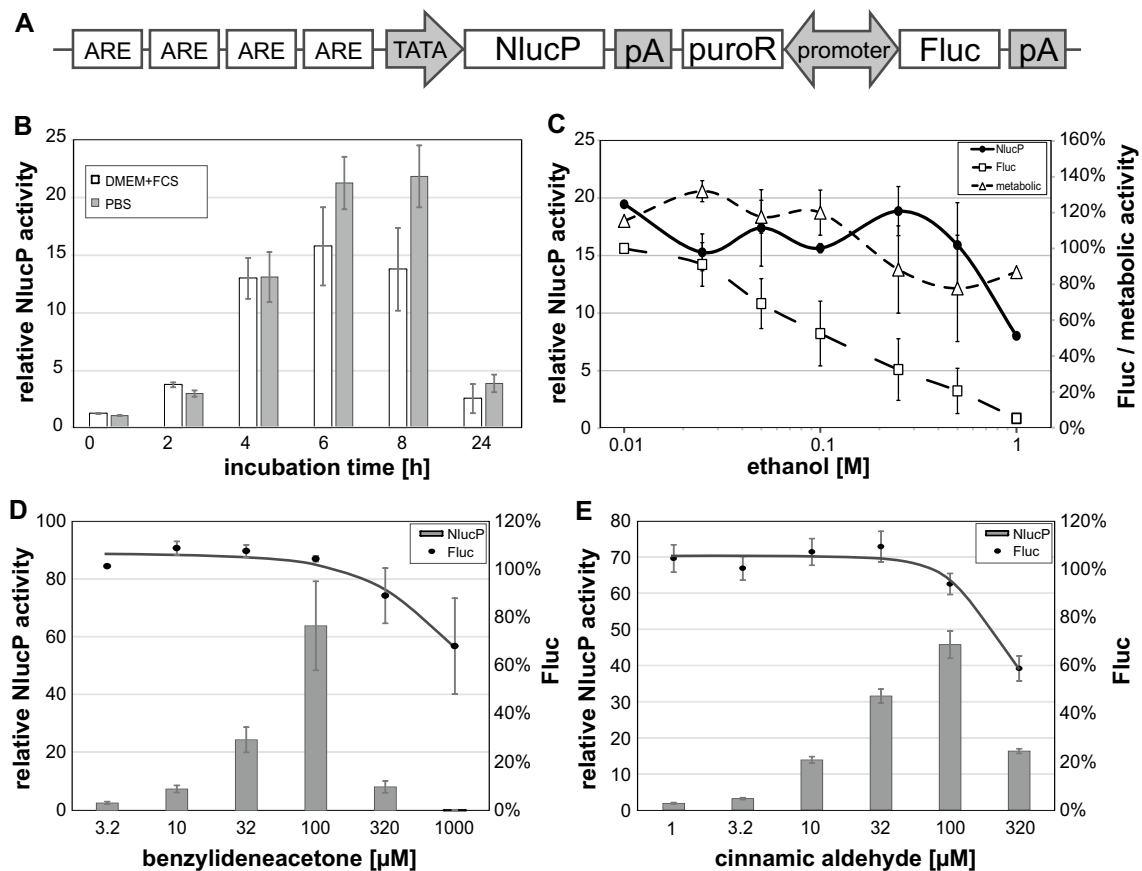


Fig. 2 MDA-ARE reporter cell line. The constructs for the creation of stable cell lines (a) contains multimerized ARE sequences upstream of a minimal promoter (TATA) and NanoLuc with protein and mRNA destabilizing sequences (NlucP) as reporter gene. A bidirectional minimal beta actin promoter is used to drive puromycin resistance gene (puroR) expression and constitutive expression of Fluc as internal reference (pA polyadenylation signal). For determination of optimal induction conditions cells were treated with 100 μ M benzylideneacetone in either DMEM including 10% FCS (DMEM+FCS) or PBS for 0–24 h (b). To demonstrate the function of the internal Fluc reference, cells were treated with increasing concentrations of ethanol

and 32 μ M benzylideneacetone (c) for 24 h in PBS or induced with increasing concentrations of benzylideneacetone (d) or cinnamic aldehyde (e) for 6 h in PBS. For viability measurement a resazurin assay was performed before luciferase measurement (c). Left y-axes show NlucP reporter activity relative to uninduced control cells and right y-axes show relative viability as Fluc relative to uninduced control cells (c–e) or RFU of generated resorufin relative to uninduced control cells (c; metabolic activity). Curve fit of the Fluc values in (d) and (e) was achieved with the least squares method. All values show means of at least three independent experiments with 12 replicates per plate. Error bars indicate SEM

the expression of a puromycin resistance gene for selection and Fluc for the use as an internal viability assay (see below). We tested the stable cell lines (either containing AREa or AREb) with different concentrations of benzylideneacetone and sodium dodecyl sulfate (SDS) which was shown to lead to false positive results in the LLNA assay (S1 Fig). Both cell lines (AREa and AREb) showed similar results and were able to detect ARE pathway activation at concentrations of 32 μM benzylideneacetone [3.5-fold (± 0.6 SEM) and 2.8-fold (± 0.9 SEM) reporter activation respectively] whereas for SDS both cell lines stayed at background reporter activation. The similar induction levels of the AREa and AREb cell line showed, that the multimerized core sequence of *AKR1C2* (AREb) is a suitable ARE-Nrf2 reporter.

Although several adaptations were made (ARE sequence, multimerization, reporter gene) the detection limit for sensitizing substances still did not reach the desired low level (32 μM benzylideneacetone). To be able to test extracts with low concentrations of sensitizers, the sensitivity of the reporter assay still demanded adaptations. Wang et al. [42] already stated that higher levels of the transcription factor Nrf2 and Keap1 could lead to a stronger reporter activation. We therefore searched for cell lines with high mRNA expression levels of these two genes in the cBioPortal database [43] and identified the breast cancer cell line MDA-MB468 as a potential candidate. We therefore created a stable 4 \times AREb NlucP cell line (Fig. 2a) in MDA-MB468 (MDA-AREbn2; referred to as MDA-ARE cell line further on). To lower the detection limit, different induction protocols were tested. Cells were either induced with DMEM + 10% FCS or in PBS with 100 μM benzylideneacetone for 0–24 h (Fig. 2b). The maximum reporter activation was seen for both induction media at 6–8 h. But for the induction level the cells treated in PBS clearly showed higher reporter activation compared to cells treated in DMEM + 10% FCS [15.7-fold (± 3.4 SEM) maximum activation and 21.7-fold (± 2.7 SEM) maximum activation, respectively]. Although deprived of all nutrients, the cell viability was not obviously disturbed within the first 8 h (microscopic observation). The tolerance of PBS incubation further leads to the possibility to directly perform extractions in PBS as a polar solvent and add the concentrated extract directly to the cells.

MDA-ARE cell line

To test the reduced detection limit in the MDA-ARE cell line, benzylideneacetone (Fig. 2d) and cinnamic aldehyde (Fig. 2e) were selected and applied to the cells in PBS at different concentrations for 6 h. Indeed, the lowest concentration for reporter activation was seen at 3.2 μM benzylideneacetone (2.6-fold (± 0.4 SEM) reporter activation, compared to 32 μM in the HaCat cell line, (S1 Fig) and 1 μM for

cinnamic aldehyde (1.8-fold [± 0.2 SEM] reporter activation). Furthermore, even after a short induction time of 2 h, significant reporter activation could be detected [3.3-fold activation (± 0.5 SEM) for 100 μM benzylideneacetone]. An incubation for 24 h still resulted in high reporter activation however, no improvement over 6 h incubation concerning the detection limit was observed.

The MDA-ARE cell line also contains a constitutively expressed Fluc. This should provide information about cell viability and cell number. To test this, we detected both luciferases simultaneously with a dual luciferase measurement. The cells were treated with 32 μM benzylideneacetone (as a moderately inducing concentration) together with increasing amounts of ethanol as an unspecific toxic insult. Ethanol in this case represents any cytotoxic by-product that might be present in complex extracts of medical devices. In addition to NlucP and Fluc we further used a resazurin assay for determination of cytotoxicity by the means of their metabolic activity (resazurin assay, Fig. 2c). Fluc values started to decrease continuously at concentrations of 0.025 M ethanol indicating a reduced protein synthesis because of cytotoxicity. The resazurin assay on the other hand showed increased signals (> 100% compared to cells not treated with EtOH) at low concentrations of ethanol (0.01–0.1 M) indicating an induced metabolic activity of the cells and started decreasing at 0.25 M ethanol. The NlucP reporter showed reduced values starting from 0.5 M ethanol. Fluc values resulted in background levels at 1 M ethanol, whereas the resazurin assay still indicated around 80% metabolic activity. When tested with pure inducers we observed a drop of Fluc signals at 320 μM benzylideneacetone (Fig. 2d) and cinnamic aldehyde (Fig. 2e), which correlates with a drop in NlucP activity at the same concentrations. Therefore, monitoring of the internal reference Fluc provides accurate information about the cell viability on the level of protein homeostasis, in particular when complex mixtures of test substances are applied.

To further characterize the MDA-ARE cell line, we tested the known sensitizers ethylene glycol dimethacrylate (Fig. 3a), cinnamic aldehyde (Fig. 3b), 1,4-benzoquinone (Fig. 3c), and the negative control lactic acid (Fig. 3d) in a broad range of concentrations (2–2000 μM) according to the OECD guideline 442D [5]. For viability assessment, we measured the Fluc activity in parallel to the NlucP reporter signal. The MDA-ARE cell line reached maximum inductions (I_{max}) of 7.5–21.9-fold for the inducers. In all cases, the viability determined via Fluc was greater than 80% at the level of I_{max} , indicating that the substances show very specific ARE pathway activation without cytotoxic side effects. The threshold for the categorization of a chemical substance as skin sensitizer was set at 2-fold reporter activation compared to untreated control cells (effective concentration; EC₂) and was determined according to the data shown in

Fig. 3 Validation of the MDA-ARE reporter cell line. The MDA-ARE reporter cell line was treated with different concentrations of ethylene glycol dimethacrylate (a), cinnamic aldehyde (b), 1,4-benzoquinone (c) or lactic acid (d) for 6 h in PBS + 1% DMSO. Left y-axis shows relative NlucP activity relative to cells treated with PBS + 1% DMSO. Right y-axis (red) shows rel Fluc activity as indicator for cell viability. The blue broken line indicates the EC2 threshold. All values show means of at least three independent experiments with three replicates per plate. Error bars indicate SEM. (Color figure online)

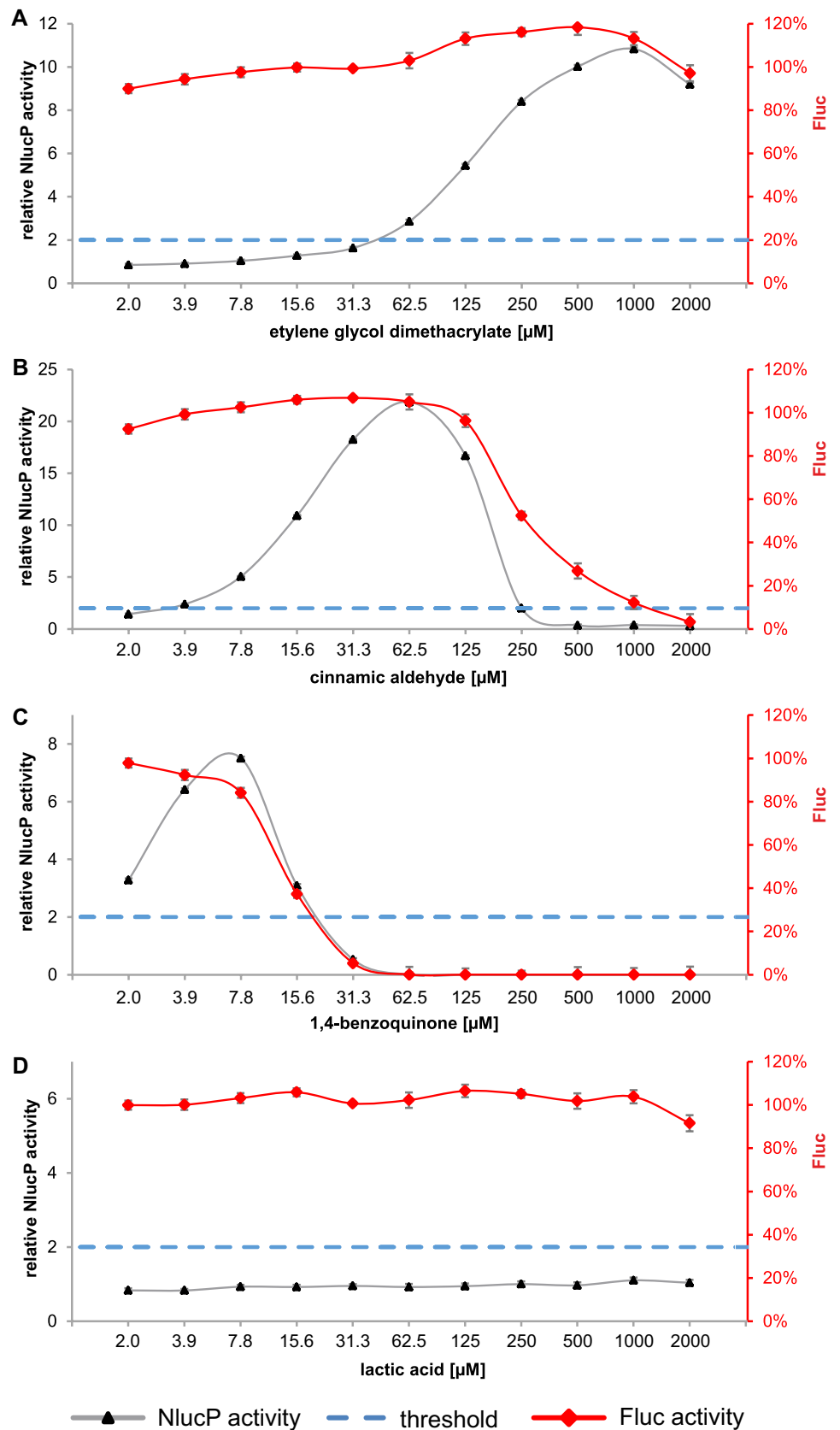


Fig. 3 and Table 1. Whereas ethylene glycol dimethacrylate shows no toxicity up to 2000 μM (Fig. 3a) the NlucP values and Fluc values of cinnamic aldehyde (Fig. 3b) and 1,4-benzoquinone (Fig. 3c) start to decrease simultaneously after Imax indicating cytotoxic effects. The negative control lactic acid (Fig. 3d) shows neither reporter activation, nor a drop of viability under the tested conditions.

Comparison of the MDA-ARE assay to the LLNA

The LLNA is still the gold standard when testing medical devices for their ability to cause skin sensitization [49]. To provide an alternative with our newly developed MDA-ARE assay we compared the results obtained from testing pure chemicals according to OECD guideline 442D [5] with data from the LLNA. 22 Different chemicals were screened in triplicate analysis for skin sensitization using the MDA-ARE assay. A 2-fold induction compared to the negative control was used as threshold above which a sample was classified as sensitizing. Using these criteria 21 out of the 22

substances could be correctly classified as sensitizers or non-sensitizers. Only methyldibromoglutaronitrile, which is a human sensitizer, did not show any induction above 2 in the MDA-ARE assay, although it was detected as a sensitizer in the LLNA (Table 1). In addition, we determined the lowest concentration of tested chemicals that give a positive result in the tests. In the MDA-ARE assay we chose an induction above the MDA-ARE threshold (EC2) and compared it to literature data for the LLNA (EC3). All positively detected chemicals showed an effective concentration considerably lower than the in vivo test (LLNA). Oxazolone was detected at ten times lower concentrations, however, all other tested sensitizers showed positive reactions at concentrations 10^3 – 10^5 times lower than the concentrations that caused a positive result in the LLNA (Table 1). SDS which is known to show a false positive response in the LLNA, was classified correctly as a non-sensitizing substance in the MDA-ARE assay, as did the non-sensitizing substances salicylic acid, glycerol and lactic acid.

Table 1 Comparison of the lowest concentrations of pure chemicals causing a positive test result in the MDA-ARE assay and the LLNA

	CAS no.	LLNA		MDA-ARE EC2 [μM]
		EC3 [μM]	Literature	
Sensitizers				
Oxazolone	15646-46-5	460	[44]	36.2
1,4-Benzoquinone	106-51-4	916	[45]	0.6
2,4-Dinitrochlorobenzene	97-00-7	2469	[45]	1.4
4-Nitrobenzylbromide	100-11-8	2314	[17]	1.4
Glyoxal	107-22-2	241,213	[17]	27.7
Methyldibromoglutaronitrile	35691-65-7	48,883	[46]	n.i.
Cinnamic aldehyde	104-55-2	226,998	[45]	2.0
Isoeugenol	97-54-1	91,352	[46]	33.8
Ethylene glycol dimethacrylate	97-90-5	1,765,715	[44]	59.1
2-Mercaptobenzothiazole	149-30-4	580,005	[44]	16.4
Tetramethyl thiuram disulphide	137-26-8	216,279	[45]	5.8
Eugenol	97-53-0	791,717	[45]	345
Cinnamic alcohol	104-54-1	1,565,179	[45]	74.5
Non-sensitizers				
Sodium dodecyl sulfate	151-21-3	485,484	[45]	n.i.
Salicylic acid	69-72-7	–	[45]	n.i.
Glycerol	56-81-5	–	[45]	n.i.
Lactic acid	50-21-5	–	[45]	n.i.
Methyl salicylate	119-36-8	–	[45]	n.i.
Diethylphthalate	84-66-2	–	[45]	n.i.
Sulfanilamide	63-74-1	–	[45]	n.i.
Chlorobenzene	108-90-7	–	[45]	n.i.
Isopropanol	67-63-0	–	[45]	n.i.

22 Pure chemicals (qualified as sensitizers and non-sensitizers for humans according to [5, 47, 48]) were tested with the MDA-ARE ARE reporter cell line. The lowest concentration showing a fold induction above 2 was recorded (EC2) and compared to the lowest concentration showing a stimulation index of 3 in the LLNA (EC3). *n.i.* no induction > 2

MDA-ARE assay for testing extracts

To test if the MDA-ARE assay is suitable for detection of skin sensitizers in extracts of medical devices, we prepared silicone samples spiked with 0.1% cinnamic aldehyde, 1,4-benzoquinone or 2,4-dinitrochlorobenzene. We then performed extractions of these samples (silicone + 0.1% incorporated sensitizers) according to ISO 10993-12:2012 [32] with a polar (PBS) and a non-polar (DMSO) solvent. Results of the extracts of the spiked silicone samples and pure silicone samples (blank) as negative controls are shown in Fig. 4. For measurement, extracts were diluted in PBS from 1 to 0.031%. The viability (determined by Fluc measurement) and the reporter induction (determined by NlucP activity) of the sample extracts were recorded. To check if components of the extracts have a negative influence on the ARE reporter activation we used a parallel approach where 32 μM cinnamic aldehyde was added to all dilutions of the extracts as positive control. For all three tested skin sensitizers an elevation of the induction above 2-fold could be detected in at least one of the two extracts (PBS or DMSO). For extracts of silicone spiked with 0.1% cinnamic aldehyde an induction above 2 was observed for 1% PBS, resulting in a lowest ineffective dilution (LID) of 0.005 (Fig. 4a). The LID value refers to the lowest dilution of the sample which showed a negative result in the assay. The corresponding DMSO extract showed higher activities (LID 0.00125). The extracts of silicone spiked with 0.1% 1,4-benzoquinone showed activation of the NlucP signal from 1 to 0.25% in PBS (LID 0.005 and 0.00125, respectively), but not in DMSO (Fig. 4b). Silicone samples spiked with 0.1% 2,4-dinitrochlorobenzene activated the NlucP reporter for both PBS (LID 0.0025) and DMSO extracts (LID 0.00125). At higher concentrations of the DMSO extract (0.5–1% extract in the sample), cytotoxic effects of the samples were observed (Fig. 4c), which did not appear in any of the PBS extracts. Furthermore, PBS extracts did not affect the induction of the positive control, whereas DMSO extracts at high concentrations reduced these values (Figs. 4a', b' and c'). Extracts of the pure silicone did not show any significant change of either the Fluc or NlucP activity (Figs. 4d and d'). After the proof of concept with the silicone extracts, we tested polar and non-polar extracts of medical devices (Table 2). In 40 samples tested, 12 (30%) showed a fold induction higher than two in either the polar extract ($n=3$) or the non-polar extract ($n=9$). Materials that were classified as potentially skin sensitizing include acrylic resins, silicone, fluoroelastomers (FKM), silicone rubber–vinyl methyl silicone (VMQ), latex, thermoplastic polymers (TPE), metals and rubber. One sample was cytotoxic in the polar extracts (viability below 70%) and five samples were cytotoxic in the non-polar extracts. In three of these samples, adding cinnamic aldehyde (32 μM) to the extracts as

a positive control did not lead to a fold induction above 2 because of the cytotoxic effects.

Discussion

Recently, different in vitro assays for the detection of skin sensitization have been developed. In this context, OECD guidelines have been published and ECVAM validation of some methods has taken place. Still, most of these methods focus on the detection of pure chemicals and so far for state of the art testing of medical devices in vivo tests have to be used.

A major step towards in vitro testing was driven by the EU Cosmetic Directives 76/768/EEC, banning animal tests for assessment of cosmetics [20]. The first cell-based assay published by the OECD was the KeratinoSens assay followed by the LuSens [5]. Although not published by the OECD, other cell-based assays based on the ARE-Nrf2 pathway have been developed [12, 42]. These cell lines were selected for high induction levels and their ability to correctly discriminate between sensitizers and non-sensitizers [34, 35, 42]. In case of extracts of medical devices, the requirements are quite different and existing in vitro methods are not suitable so far. Non-polar solvents, as required for medical device extraction are either not soluble in cell culture medium (plant oils) or show cytotoxic effects on the cells even in low concentrations [32]. Through evaporation, the solvent can be replaced by a polar, non-toxic solvent, but within this step, crucial substances can get lost [50, 51]. To avoid losses during solvent exchange, DMSO was chosen in our approach as extraction solvent. DMSO is less toxic to cells than other organic solvents, and can be used in cell culture medium up to a final concentration of 1%. Nevertheless, a dilution step of the extracts by a factor of 100 is required. Therefore, the aim was to reduce the detection limit of sensitizers in complex solutions. The AREb sequence (ARE core sequence of *AKR1C2*) demonstrated that the core binding site for Nrf2 is sufficient for a robust reporter activation after induction, whereas minor contributions are mediated by the flanking sequences of AREa. The complete loss of reporter activation upon mutation of the AP-1 binding site within the ARE core sequence of *AKR1C2* was unexpected, as it does not interfere with the Nrf2 consensus binding site [40]. Nevertheless, the role of members of the Jun protein family was already demonstrated for other antioxidant response promoters [52–54]. Although the AREd sequence of mouse *Gsta1* has worked well in a liver cell line [35] it did not show induction in HEK293. This effect of different induction potential of ARE reports in different cell lines was also already observed for another reporter construct, which worked well in an MCF-7 context [42] but failed to give robust induction in HaCat cells [34]. Although MDA-MB468 cells are not involved in

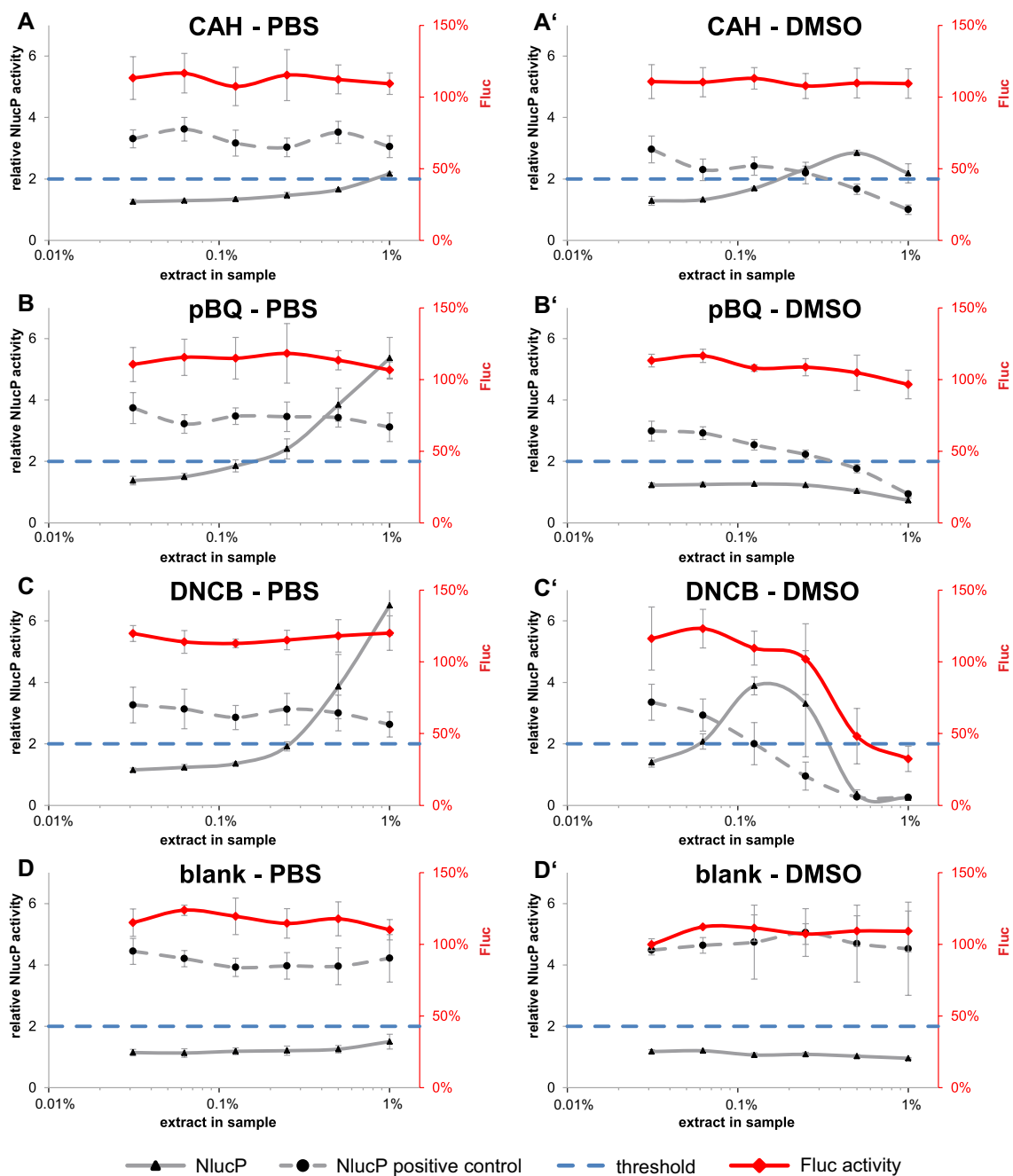


Fig. 4 Results for spiked medical silicone. Silicone with incorporated sensitizing substances (0.1% w/w) were extracted. Extract concentrations (0.031–1%) in PBS of these silicone samples of the indicated chemicals, cinnamic aldehyde (CAH) (**a** and **a'**), 1,4-benzoquinone (pBQ) (**b** and **b'**), 2,4-dinitrochlorobenzene (DNCB) (**c** and **c'**), or without addition (blank) (**d** and **d'**) were tested with the MDA-ARE assay. The left y-axis shows the fold induction of the samples (NlucP)

or the positive control (NlucP positive control, sample extract with 32 μ M cinnamic aldehyde added) relative to control cells (treated with PBS + 1% DMSO). The right y-axis shows viability of the cells relative to control cells (Fluc). The blue broken line indicates the EC2 threshold. All values are mean values from at least six wells treated with at least two independent extracts. Error bars indicate SD. (Color figure online)

the process of skin sensitization *in vivo*, they still represent a very sensitive model for the ARE-Nrf2 pathway. We chose the cell line because of its high levels of Nrf2 as well as Keap1. An overexpression of Nrf2 alone in our hands only resulted in increased background luciferase levels. However,

there are reports showing an increased level of Nrf2 leads to an increased background as well as increased pathway activation [42, 55], but this might again be cell line specific and dependent on endogenous Keap1 levels. In our hands, the MDA-ARE cell line reached a ~4-fold higher induction

Table 2 Testing of real sample extracts of medical devices

Type of device	Material	Polar extract			Non-polar extract			Classification
		Viability [%] ± SD	Fold induction	Pos ctrl	Viability [%] ± SD	Fold induction	Pos ctrl	
Hearing device	Acrylic resin	92 ± 12	2.88 ± 0.55	Yes	<i>67 ± 10</i>	0.52 ± 0.05	No	Yes
Hearing device	Silicone + coating	103 ± 2	3.95 ± 0	Yes	116 ± 10	1.39 ± 0.07	Yes	Yes
Dentistry device	FKM, VMQ,	99 ± 9	1.09 ± 0.07	Yes	<i>51 ± 2</i>	3.3 ± 0.39	Yes	Yes
Antimicrob. spray	Liquid	82 ± 13	1.08 ± 0.13	Yes	<i>52 ± 7</i>	1.34 ± 0.4	Yes	No
Implant-prototype	Epoxy resin	115 ± 13	1.25 ± 0.19	Yes	121 ± 10	0.87 ± 0.15	Yes	No
Implant	Silicone	117 ± 12	1.22 ± 0.1	Yes	102 ± 14	2.98 ± 0.33	Yes	Yes
Implant	Silicone	119 ± 13	1.26 ± 0.12	Yes	93 ± 5	1.39 ± 0.17	Yes	No
Needle	Stainless steel	88 ± 16	1.24 ± 0.26	Yes	104 ± 13	1.17 ± 0.11	Yes	No
Implant-prototype	Epoxy resin	91 ± 2	0.68 ± 0.08	Yes	126 ± 18	1.27 ± 0.08	Yes	No
Implant-prototype	Epoxy resin	78 ± 7	0.84 ± 0.1	Yes	129 ± 4	1.15 ± 0.03	Yes	No
Implant-prototype	Epoxy resin	94 ± 6	0.99 ± 0.12	Yes	82 ± 9	0.95 ± 0.14	Yes	No
Infrared device	Plastics	100 ± 6	1.24 ± 0.1	Yes	76 ± 7	1.06 ± 0.04	Yes	No
Implant-prototype	Carbone fibre	122 ± 6	1.39 ± 0.15	Yes	83 ± 19	0.81 ± 0.12	Yes	No
Implant	PE and PP	100 ± 15	1.01 ± 0.09	Yes	102 ± 10	1.26 ± 0.16	Yes	No
Gloves	Nitrile, purple	93 ± 10	1.19 ± 0.01	Yes	<i>31 ± 8</i>	0.79 ± 0.04	No	Ncc
Gloves	Nitrile, blue	108 ± 14	1.66 ± 0.13	Yes	<i>5 ± 6</i>	0.67 ± 0.01	No	Ncc
Gloves	Latex, white	91 ± 10	1.35 ± 0.28	Yes	113 ± 7	2.78 ± 0.2	Yes	Yes
Gloves	Latex, green	75 ± 2	3.54 ± 0.3	Yes	84 ± 17	1.38 ± 0.4	Yes	Yes
Laboratory device	Thermoplast	102 ± 7	1.28 ± 0.3	Yes	99 ± 8	1.69 ± 0.13	Yes	No
Hearing device	DLPA acrylate	114 ± 12	1.25 ± 0.11	Yes	95 ± 11	3.06 ± 0.57	Yes	Yes
Hearing device	DLPA acrylate	86 ± 7	1.65 ± 0.14	Yes	129 ± 9	2.31 ± 0.18	Yes	Yes
Hearing device	TPU	91 ± 16	1.05 ± 0.31	Yes	83 ± 17	0.87 ± 0.12	Yes	No
Prosthesis	Coating	80 ± 14	0.97 ± 0.17	Yes	91 ± 3	1.03 ± 0.1	Yes	No
Prosthesis	TPE, PC	114 ± 14	1.1 ± 0.06	Yes	109 ± 6	3.85 ± 0.31	Yes	Yes
Suture	Silicone	110 ± 7	0.89 ± 0.11	Yes	112 ± 13	1.17 ± 0.25	Yes	No
Suture	Silicone	81 ± 12	1.04 ± 0.13	Yes	88 ± 13	0.81 ± 0.09	Yes	No
Suture	PES	90 ± 11	0.66 ± 0.34	Yes	124 ± 14	1.12 ± 0.17	Yes	No
Active coal	Coal	62 ± 8	0.67 ± 0.14	Yes	92 ± 8	0.68 ± 0.19	Yes	No
Active coal	Coal	82 ± 9	0.77 ± 0.13	Yes	92 ± 12	0.76 ± 0.15	Yes	No
Dentistry device	Glass and metals	81 ± 9	1.17 ± 0.02	Yes	73 ± 5	2.76 ± 0.12	Yes	Yes
Hearing device	Silicone, parts	78 ± 3	0.76 ± 0.06	Yes	78 ± 5	0.6 ± 0.06	Yes	No
Hearing device	ABS	89 ± 3	0.8 ± 0.05	Yes	87 ± 6	0.96 ± 0.41	Yes	No
Hearing device	Rubber	95 ± 4	0.84 ± 0.08	Yes	98 ± 10	2.03 ± 0.08	Yes	Yes
Hearing device	TPE-A	107 ± 6	0.89 ± 0.1	Yes	90 ± 5	0.94 ± 0.08	Yes	No
Hearing device	TPE-A, brown	103 ± 7	1.73 ± 0.07	Yes	91 ± 10	1.02 ± 0.12	Yes	No
Hearing device	Silicone, parts	88 ± 12	0.7 ± 0.11	Yes	92 ± 10	0.92 ± 0.13	Yes	No
Hearing device	TPE-A	101 ± 9	1.04 ± 0.03	Yes	109 ± 10	1.09 ± 0.11	Yes	No
Menstrual cup	Latex	89 ± 10	0.88 ± 0.24	Yes	71 ± 12	2.46 ± 0.23	Yes	Yes
Menstrual cup	TPE	102 ± 6	1.01 ± 0.14	Yes	98 ± 12	1.29 ± 0.13	Yes	No
Menstrual cup	Silicone	82 ± 8	1.07 ± 0.04	Yes	111 ± 12	1.09 ± 0.2	Yes	No

40 Medical devices were extracted with a polar (PBS) and a non-polar (DMSO) solvent and tested with the MDA-ARE assay. The table shows viability of each sample extract and its fold induction. Reduced viability is indicated by italics; fold induction above the threshold 2 is highlighted in bold. Furthermore, 32 µM cinnamic aldehyde was added to the extracts as positive control (pos ctrl) and measured with the MDA-ARE assay. If the fold induction of the positive control was above the threshold 2 “yes” is indicated in the table. Classification of the sensitizing potential is shown as “yes” if the fold induction of at least one of the two extracts is above the threshold of 2. ± Values represent SD. Classification is shown as “no” if the fold induction of both extracts is lower than two and the pos ctrl is higher than two (yes). For cytotoxic samples where the pos ctrl of at least one of the two samples was lower than the threshold of 2 no clear classification (ncc) was possible

compared to the HaCat cell line under the same induction conditions (compare Fig. 2b–S1 Fig A).

ARE pathway activation is mediated by the interaction of small reactive molecules (like haptens) with proteins.

The optimal growth medium for cultured cells contains a large number of proteins due to the supplemented serum. Therefore, the reactive molecules could bind to serum proteins before entering the cells and interacting with Keap1.

A serum reduction as done for other ARE cell lines [34] can therefore improve the pathway response. As we created a reporter cell line with a maximum induction after 6–8 h, we could reduce the time of compound incubation and therefore could completely deplete the medium of serum without a major impact on cell viability and proliferation. We decided to use PBS alone as general induction medium, because this provides the opportunity to use pure extracts of medical devices if they were performed in PBS, which further increases the sensitivity of the assay by a factor of 100. Another advantage when using shorter incubation time is that possible cytotoxic effects can be reduced compared to longer incubation times.

When testing substances and mixtures for their ability to induce the ARE-Nrf2 pathway, the cytotoxicity is always critical and was found to be the main limiting factor for the assessment of more complex formulations and plant extracts with the KeratinoSens assay [56, 57]. Substances were shown to give false positive results when cells suffer severe toxic effects [34] and complex formulations can give false negative results if cytotoxicity is reached before the pathway is activated [57]. The latter problem is solved in the MDA-ARE assay by reducing the incubation time to 6 h where often critical cytotoxic effects are not reached. For the problem of false positive effects, a parallel viability assay based on the metabolic activity of the cells (MTT or resazurin based assays) is already in use in other assays [34, 58]. Results gained from the luciferase measurement of an ARE reporter is considered to be significant, when the cell viability is greater than 70%. For our assay we compared a resazurin assay with a Fluc based internal reference. We found that especially the Fluc activity sensitively reacted to cytotoxic effects (Figs. 3b–c) and correlates well with the drop in NlucP reporter signal due to cytotoxicity. The Fluc measurement can be used as an alternative for metabolic based viability assays and prevents overestimation of cell viability that may be caused by metabolic hyperactivation induced by extract compounds.

One major goal of the project was that the developed assay should be able to detect skin sensitizers in complex mixtures at the same concentration levels or lower as the *in vivo* assay LLNA, which is currently used for the testing of medical devices for skin sensitization. We therefore performed full dose response curves with well-known sensitizers as well as non-sensitizers. In total, 21 out of 22 pure chemicals were correctly identified as sensitizers or non-sensitizers by the MDA-ARE assay. This results in 92% toxicological sensitivity and 100% specificity although, more pure substances need to be tested in the future to refine and secure these results. In extracts, low concentrations of sensitizers limit their detection. Except for two substances (oxazolone and methylidibromoglutaronitrile) all other chemicals were detected in the

MDA-ARE assay at concentration more than 1000-fold lower than with the LLNA. Therefore even a dilution step of 100 (for non-polar extracts in our procedure) still results in lower detection limits for the MDA-ARE assay compared to the LLNA.

To assess skin sensitization of medical devices, McKim and colleagues tested different vehicles spiked with sensitizers and showed responses of skin models (Epiderm and SkinEthik) towards the applied testing matrix [29]. Furthermore, Coleman and colleagues integrated skin sensitizers in silicone samples and could demonstrate a reaction of skin models (SenCeeTox) towards the extracts [15]. By assessing various endpoints they could detect skin sensitizers in silicone samples at a concentration of 10% w/w. However, a concentration of 10% w/w of a strong skin sensitizer seems unlikely in a medical device. Therefore, we prepared comparable silicon samples with 0.1% w/w and could easily detect the sensitizers by using an ARE-Nrf2 reporter gene assay. The assay allows application of both polar and non-polar solvents, which turned out to be critical depending on the solubility properties of the chemicals (Fig. 4; [59]).

The testing of extracts of medical devices showed that the MDA-ARE assay is sensitive enough to detect sensitizing agents in real world samples. The parallel assessment of the cell viability and the use of positive controls are essential to evaluate the results of the reporter assay because of the great variability in extract composition of the different materials. The strategy of using polar as well as non-polar extraction methods as in ISO 10993-12:2012 [32] is of great importance because several samples in our assay only tested positive in one of the extracts. Different reactivity in polar (saline) and non-polar (sesame oil) extracts was also described for the SenCeeTox assay [15].

Summarizing, the MDA-ARE assay is a suitable screening tool for detection of skin sensitizers in medical device extracts especially during product development. Both polar and non-polar extracts can be applied, it provides high sensitivity and low detection limits and its internal reference allows sensitive viability assessments. Furthermore, the assay time of only 6 h from induction to measurement delivers results of tested extracts within 1 single day. As a next step, we will compare the results of the medical devices from the MDA-ARE assay to the DPRA assay and animal tests.

As a conclusion, the MDA-ARE assay could be a useful tool for assessment of skin sensitizing potential of medical device extracts within an ITS combining various assays addressing the key events of the AOP for skin sensitization.

Acknowledgements Open access funding provided by FH Campus Wien - University of Applied Sciences. The work was supported by the Austrian Research Promotion Agency (FFG, Grant 839053) and the City of Vienna (MA23—project 18-18).

Author contributions EM, ER, NG, GE, GL, SA, MH, AW and CS were responsible for experimental design, analysis, execution and writing of the manuscript. CK and TC were responsible for experimental design, analysis, writing, communication, and supervision of the project. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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