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Investigating the surfactant antagonism with the Open Source Reconstructed Epidermis (OS-Rep) model

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ABSTRACT

Under the current EU chemicals legislation, *in vitro* test methods became the preferred methods to identify and classify the skin irritation potential of chemicals and mixtures. Among these, especially *in vitro* skin models are widely used. For surfactants, a well-known group of typically irritating chemicals, it is a long-standing experience that the irritation potential of a mixture of surfactants is typically lower than the irritation potential of the single surfactants, an effect usually described as surfactant antagonism. In order to evaluate if this effect can be observed in skin model systems as well, the irritation potential of the surfactants and of their mixtures was determined in the Open Source Reconstructed Epidermis (OS-REp) models. Combinations of sodium dodecyl sulfate or linear alkylbenzene sulfonate with cocoamidopropyl betain and alkyl polyglycosid, respectively, resulted in a clear decrease of the irritation potential compared to the irritation exerted by the single surfactants. The effect appeared to be primarily driven by the mixture's lower ability to damage the skin model's barrier, as shown by a reduced fluorescein permeation.

1. Introduction

Surfactants are main constituents of different consumer products, e.g. detergents or cosmetic cleansing products. Due to the irritating properties of the surfactants, the products might show an intrinsic skin irritation potential as well.

According to UN GHS (United Nations, 2019), the irritation potential of a mixture is assumed to be reflected by the summation of the individual irritation potential of its ingredients if no test data on the mixture as such, or on similar mixtures, is available to assess the mixture's irritation potential.

However, it was generally observed for consumer products with contact to skin that in the case of surfactant mixtures, the resulting acute irritation potential can be much lower than expected from the summation of the individual surfactants' irritation potential, an effect called ‘surfactant antagonism’. This experience is supported by studies investigating the irritation potential of surfactant combinations *in vivo* as well as *in vitro* (Hall-Manning et al., 1998; Lee et al., 1994; Dillarstone and Paye, 1993; Rhein et al., 1990). The latest of these studies, published more than 20 years ago by Hall-Manning et al. (1998), investigated the irritation potential of the anionic surfactant sodium dodecyl sulfate (SDS), the nonionic alkyl polyglucosid (APG) and the amphoteric dimethyl dodecyl amido betaine (DDAB, a specific cocamidopropyl betaine) alone and in mixtures applying human patch tests. In that study, surfactant antagonism was demonstrated for the combinations of SDS with APG or DDAB. In addition, a relationship of skin irritation with the surfactants' critical micelle concentration (CMC), which is widely discussed as a physico-chemical determinant for the irritating properties of surfactants, was confirmed. However, the authors pointed out that the irritation potential of a mixture of surfactants cannot be predicted from its CMC because skin irritating effects usually occur at surfactant concentrations significantly exceeding the CMC. Therefore, the effect of mixtures of surfactants on the irritation potential still needs to be determined by dedicated irritation tests.

Due to the progress in the development of alternatives to animal testing, different *in vitro* methods are available nowadays to determine the skin irritating properties of substances. Methods like the OECD TG 431 and 439 (OECD, 2019; OECD, 2021), which are based on human 3D epidermal models, aim at deriving a classification for skin irritation or corrosion effects according to UN GHS (United Nations, 2019).

Abbreviations: SDS, Sodium dodecyl sulfate; LAS, Linear alkylbenzene sulfonate; CABP, Cocamidopropyl betaine; APG, Alkyl polyglucosid; DDAB, Dimethyl dodecyl amido betaine; CMC, Critical micelle concentration.

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However, even though these methods became the preferred *in vitro* test methods for skin irritation testing, to our knowledge the surfactant antagonism has not been systematically investigated in such test systems yet. As skin models in general are suitable for testing complex mixtures, they may provide a useful test system to identify potential changes in the irritation potential of surfactant mixtures. Because the skin models closely mimic the structure and function of *in vivo* human skin, they allow a detailed investigation of the possible mechanism that underlies the combination effects of surfactants.

In this study, a set of surfactants similar to those previously used in the study by Hall-Manning et al. (1998) were applied in a skin irritation test using the Open Source Reconstructed Epidermis (OS-REp) model, an epidermal equivalent of the human skin (Mewes et al., 2016; Groeber et al., 2016). The irritation potential of binary mixtures of anionic SDS, the amphoteric cocamidopropyl betaine (CAPB), nonionic APG and the anionic surfactant linear alkylbenzene sulfonate (LAS) was compared with that of the individual compounds based on the cell viability measured by MTT as the primary indicator for the test samples' irritation potential. In addition, for SDS and CABP and their mixtures, the skin models' integrity after treatment was evaluated by immunohistochemistry, by determining fluorescein permeation (Yokota and Tokudome, 2015) and by measuring the release of LDH as a measure of membrane disintegration (Ka-Ming Chan et al., 2013). Finally, the CMC of SDS and CABP and their mixtures was quantified in order to evaluate the propagated relationship between the CMC and the irritation potential.

2. Materials and methods

2.1. Products and chemicals

The surfactants used in this study were sodium dodecyl sulfate (SDS; Sigma Aldrich, Germany; CAS 151-21-3), alkyl polyglucoside (APG, as APG 220 UPW from BASF SE, Germany; CAS 68515-73-1), cocamidopropyl betaine (CAPB, as TEGO Betain C60, Evonik Ind., Germany; CAS 97862-59-4) and linear alkylbenzene sulfonate (LAS, ISU chemical company, Germany; CAS 85536-14-7). APG, CAPB and LAS were obtained as solutions of 62%, 49%, and 96%, respectively, and diluted in demineralized water or cell culture medium for the experiments. SDS was obtained as a solid. A solution of 10% in demineralized water was diluted for the application in demineralized water or medium. The surfactant concentrations applied in the different tests refer to the concentration of active matter.

Epilife medium, dulbecco's phosphate buffered saline (DPBS), penicillin-streptomycin antibiotic supplement and human keratinocyte growth supplement (HKGS) were purchased from Gibco, USA. Cell culture inserts (12 mm diameter) were obtained from Millipore, Germany. Calcium chloride was purchased from Fluka, Germany. Keratinocyte growth factor (KGF), Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and fluorescein were purchased from Sigma, Germany. Rabbit anti-human aquaporin-1 antibody was obtained from Calbiochem, USA. Alexia Fluor® 568 goat anti-rabbit IgG antibody was purchased from Invitrogen, USA. DAPI was obtained from DAKO, Denmark. Normal goat serum was purchased from life technologies, USA.

2.2. Open source reconstructed epidermis

The OS-REp models were produced according to Mewes et al. (2016). Briefly, primary human keratinocytes were obtained from human foreskin biopsies and cultured in basal medium consisting of Epilife® medium with 60 μM calcium chloride (Fisher Scientific, Germany), supplemented with 1% HKGS (Fisher Scientific, Germany), and antibiotics (penicillin 50 U/ml, streptomycin 50 μg/ml) at 37 °C (5% CO_2). In the third passage, 3.15×10^5 cells were seeded on polycarbonate membranes of cell culture inserts (Millicell-PVF, pore size 0.4 μm, Ø12 mm, Merck Millipore, Germany) and cultured in submerse medium which was prepared by supplementing Epilife® medium with HKGS, antibiotics and calcium chloride (1.5 mM). After being cultivated for 24 h in submerse medium at 37 °C (5% CO_2), they were lifted to the air-liquid interface and cultivated for additional 19 days at 37 °C (5% CO_2) with ALI medium (Epilife® ALI medium supplemented with HKGS, keratinocyte growth factor, antibiotics, ascorbic acid phosphate (73 μg/ml) and calcium chloride).

The quality of each batch of OS-REp models was routinely assessed. For this, the quality control process followed partially the "General and Functional Conditions" as indicated in the OECD TG 439 (OECD, 2021). Each tissue batch was screened for appropriate tissue architecture based on histological sections, and tissue viability of untreated and SDS-treated models (negative and positive control, respectively), based on the MTT assay, was assessed. Only tissue batches were used in the present study for which both viability data fell within the applicability ranges for the OS-REp, which had been previously defined based on historical data.

2.3. Viability testing

Tissue viability testing was carried out with the OS-REp *in vitro* Skin Irritation Test (SIT) as described in Mewes et al. (2016) and Groeber et al. (2016). This test method was developed and validated according to the Performance Standards as outlined in the OECD Environment, Health and Safety Publications: Series on Testing and Assessment No. 220 (OECD, 2015). The complete set of validation data, including reproducibility and predictive capacity of the SIT, are openly available in the aforementioned publications. The only modification to this protocol was that surfactants were not applied as pure compounds but as aqueous solutions. Briefly, 25 μl of solutions of surfactants in deionized water were topically applied onto the epidermis models for 35 min. Deionized water only was applied as control. Tests were carried out in triplicates for every condition. After exposure to the test compounds, the models were rinsed with Dulbecco's phosphate buffered saline (DPBS) and incubated at 37 °C (5% CO_2) for 42 h. For the determination of tissue viability, the models were incubated for three hours with a volume of 200 μl of a solution of 1 mg/ml MTT in DPBS. After removal of the solution, the generated formazane was extracted with 1 ml of isopropanol. The optical density was determined spectrometrically at 570 nm without reference wavelength (Spectrofluor Plus Spectrometer, Tecan, Switzerland). Viability of epidermis models exposed to deionized water was considered as 100% control and the results of the surfactant treated models were expressed as a percentage of the control (relative viability). According to the prediction model, a relative tissue viability at or below 50% corresponds to a classification as a skin irritant (GHS Category 2), substances resulting in relative tissue viabilities $> 50\%$ can be considered as non-irritating to the skin (Mewes et al., 2016; OECD, 2021).

However, in the present study the prediction model with its 50% viability threshold (according to OECD TG 439) was not applied to assess the impact of the surfactants and its mixtures on the OS-REp models. As the skin irritation tests were mainly intended to compare the impact of different surfactants and combination of surfactants with each other, the tissue viability data were used comparatively in a so-called "benchmark approach". Hence, the test items were considered being more or less irritating than a benchmark item instead of defining them as skin irritating or non-irritating based solely on the relative tissue viability and its distance to the 50% threshold.

2.4. Histology and immunofluorescence staining

Immunohistochemistry was performed on 5 μm cryostat sections. After treatment with surfactant solutions, washing and incubation for 42 h at 37 °C (5% CO_2), epidermis models were removed from the cell culture inserts by cutting the membrane. Excised membrane and epidermis models were embedded in tissue freezing medium (Leica, Germany). Histological sections were obtained by using a Cryo Star

NX70 microtome from Thermo Scientific. For immunofluorescence staining, sections were blocked with normal goat serum (Life Technologies, USA) and incubated with anti-aquaporin rabbit antibody KP 9301 (Calbiochem, USA). Sections were incubated with the corresponding secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG antibody, Invitrogen, USA) and nuclei were stained with DAPI.

2.5. LDH assay

The medium underneath the epidermis models was withdrawn 42 h after surfactant exposure and the release of LDH in the medium was measured using a lactate dehydrogenase assay kit (Cytotoxicity Detection Kit (LDH), Roche, Germany), according to the manufacturer's instructions. Results are expressed as the average fold-increase ($n = 3$) compared to the water treated negative control.

2.6. Fluorescein permeation

Analogous to the viability testing, epidermis models were exposed to surfactant solutions for 35 min, washed and incubated for 42 h at 37 °C (5% CO_2). After incubation, epidermis models were placed in 6-well plates containing 1.5 ml of ALI medium. 100 µl of ALI medium containing 0.05% fluorescein sodium salt was placed on the stratum corneum of the models. After 3 h of incubation at 37 °C (5% CO_2), the fluorescence intensity of the medium in the well underneath the epidermis models was determined by using a Tecan spectrofluor plus spectrometer (Excitation 485 nm, Measurement 535 nm). The x-fold increase compared to the medium in wells of models treated with water only was calculated for each of two epidermis models treated with the respective surfactant solution.

2.7. Determination of critical micelle concentration

The critical micelle concentration (CMC), which describes the concentration of surfactants in a solution above which micelles are formed, was derived from the change of the surface tension of surfactant solutions and mixtures. Surface tension was measured after vigorous shaking as a function of the total surfactant concentration using a K12 Tensiometer (Krüss, Germany), employing the Du Noüy ring method. Briefly, this method measures the force needed to raise the ring from the liquid's surface (Du Noüy, 1925). The CMC is then graphically determined in a plot of surface tension and log concentration as the intercept of the two linear regions with different slopes. All measurements were carried out at 25 °C and at least three times.

2.8. Statistics

In this study, the difference in mean viability of epidermis models or keratinocytes is assessed after being treated either with a surfactant ‘A' alone at various concentrations or with surfactant ‘A' at the same concentrations but in combination with a second surfactant ‘B' at a fixed concentration. The statistical significance of a difference in mean viability was only tested between treatment groups containing the same concentration of surfactant ‘A'. For instance, if epidermis models were treated with different concentrations of SDS alone (surfactant ‘A') and with the same concentrations of SDS in combination with CAPB at a fixed concentration (surfactant ‘B'), respectively, statistical significance was evaluated for the difference in viability between epidermis models treated with a specific SDS concentration alone or in combination with CAPB. The difference in mean viability resulting from the different concentrations of SDS was not statistically evaluated.

In case of two treatment groups, Student's *t*-test was applied, whereas for three or more treatment groups, one-way ANOVA was used to assess differences of mean viability, with the latter informing about a significant difference between any of the treatment groups included in the test. *P*-values were adjusted for multiple comparison using the *false discovery rate* (FDR) method. All statistical analyses and graphical presentations were carried out using the R open-source statistical computing software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Viability of epidermis models after treatment with single surfactants

Different batches of epidermis models were treated with aqueous dilutions of SDS and CAPB at different concentrations in order to analyze the relationship between surfactant concentration and tissue viability for each surfactant (Fig. 1 A-B). The application of SDS resulted in a strong dose-dependent decrease of tissue viability. The dose-dependency shows a reverse-sigmoidal shape with a steep decline of viability at a concentration range of 0.15-0.25% SDS. In contrast, no clear dose-dependent decrease of tissue viability was observed for the application of CAPB, and even at a concentration of 15% as the highest test concentration the average viability remained at 93%. Taking the variabilities between different batches of epidermis models into account, an IC 50 (half maximal inhibitory concentration) in the range of 0.15% to 0.25% was estimated for SDS (Fig. 1A), whereas no IC 50 could be derived for CAPB.

3.2. Viability of epidermis models exposed to surfactant mixtures

Since treatment with SDS at concentrations of about 0.2% for 35 min reduced tissue viability below 50%, corresponding with a GHS Cat. 2 classification, different concentrations of SDS slightly below and above 0.2% were supplemented with CABP to evaluate the effect of mixtures on the epidermis models' viability.

SDS concentrations between 0.1 and 0.6% were chosen to cover the steep viability decrease observed when SDS is applied as such. CAPB was added to SDS at a concentration of 0.5% and 5% as mixtures with higher concentrations of CABP were difficult to rinse from the epidermis surface after the exposure period.

Even though the total surfactant concentration in the mixtures was higher compared with the SDS solution alone, mixtures of SDS with CAPB resulted in higher tissue viabilities (Fig. 2). In the case of mixtures with 5% CABP, viability of treated models did not decrease at all. These differences were found to be of high statistical significance for the applied mixtures of SDS and CAPB when compared to the treatment with the corresponding SDS solutions only.

Since SDS and CAPB belong to different classes of surfactants, with SDS being an anionic and CAPB a zwitterionic/amphoteric surfactant, it was evaluated whether a surfactant antagonism can also be observed if SDS is combined with a nonionic (Fig. 3A) or with another anionic surfactant (Fig. 3B). APG was chosen as the nonionic surfactant and LAS as an anionic surfactant as both are widely used in consumer products. Treatment of epidermis models with mixtures of SDS and APG overall resulted in significantly higher viabilities than treatment with SDS alone, although the effect was not as pronounced as for CAPB (Fig. 3A). Such a maintenance of viability was not observed when the two anionic surfactants LAS and SDS were combined (Fig. 3B). Treatment of epidermis models with various concentrations of LAS in combination with 0.2% SDS led to viabilities that were both lower than those after treatment with only LAS at the corresponding concentrations as well as lower than the viability of a treatment with 0.2% SDS only. This strongly contrasts the observation that adding APG or CAPB at different concentrations to 0.2% SDS always resulted in higher viabilities than the treatment with 0.2% SDS alone (Fig. 2 and Fig. 3A).

3.3. Critical micelle concentration (CMC) of surfactant mixtures

The lower CMC of surfactant mixtures in comparison to single surfactants solutions has been discussed as a possible explanation for the increased mildness of surfactant mixtures compared to single surfactants

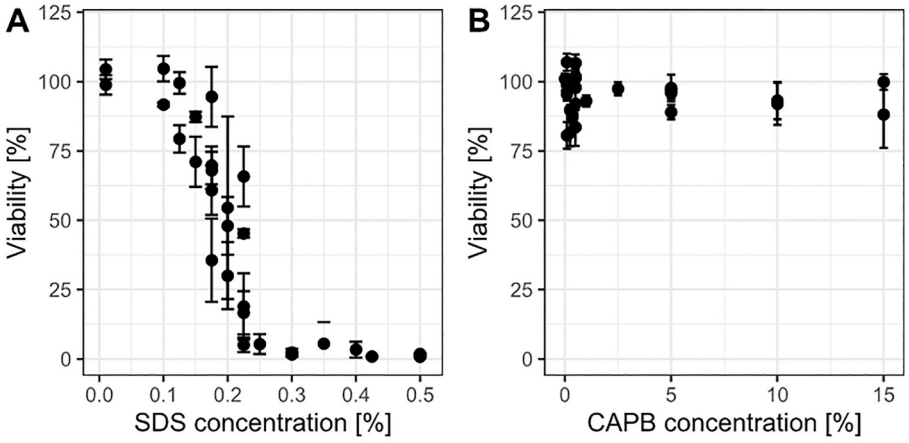

Fig. 1. MTT-viability assay of epidermis models exposed to (**A**) SDS and (**B**) CAPB for 35 min. The graphs represent an overlay of several experiments with different batches of epidermis models. While exposure to SDS results in a decrease of viability of epidermis models below 5% at a concentration of 0.25%, application of 15% CAPB decreases the average viability to 93%. The average IC 50 for SDS is determined as about 0.2%. Viability is expressed as the percentage of water treated control. Values represent the mean ± SD ($n = 3$).

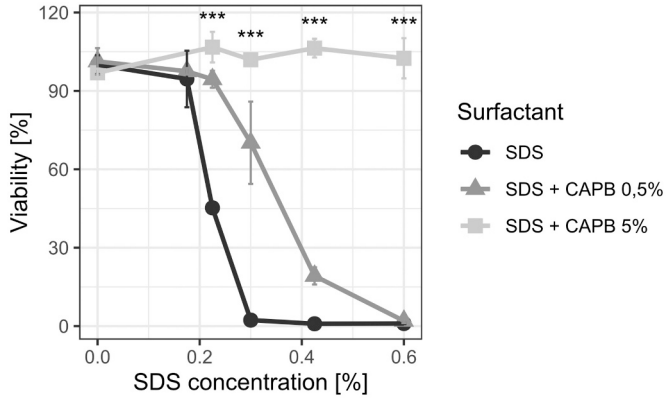

Fig. 2. MTT-viability assay of epidermis models exposed for 35 min to SDS alone or to mixtures of SDS with CAPB. Exposure to mixtures of SDS with CAPB resulted in higher viabilities compared to the application of SDS alone, with 5% CAPB being most effective in retaining viability of epidermis models. Viability is expressed as the percentage of water treated control. The values represent the mean ± SD ($n = 3$). (***) $P < 0.001$ (one-way ANOVA is applied for comparing the treatment with SDS alone and the treatments with the mixtures with CAPB containing the same concentration of SDS).

(Hall-Manning et al., 1998). The CMC describes the concentration at which surfactants start forming micelles in a solution, and it was proposed that micelle formation results in less surfactant-skin lipid interaction. Hence, if micelles are already formed at quite low surfactant concentrations, the number of free surfactant molecules markedly decreases, resulting in a lower irritation potential compared to hypothetical solutions of non-micellar surfactant molecules. To investigate the relationship between CMC and damage caused by surfactants, the CMC of SDS as well as of the combination of CAPB and SDS was determined (Fig. 4A). In parallel, the viability of epidermis models exposed to the same mixtures of CAPB and SDS was measured for comparison (Fig. 4B). The CMC of SDS and CAPB alone were measured in individual experiments and determined as 0.1% and 0.009%, respectively. Fig. 4A illustrates the decrease of the CMC of 0.225% SDS which is combined with an increasing amount of CAPB. A concentration of 0.025% of CAPB mixed with 0.225% SDS already lowers the CMC considerably in comparison to SDS only. However, ten times as much CAPB (0.25%) must be added to a solution of 0.225% SDS to reduce tissue viability loss in a range observed after exposure to the pure surfactants (Fig. 4B).

3.4. *Exposure of keratinocyte in monolayer cultures to mixtures of SDS and CAPB*

In the previous experiments, we have demonstrated that single surfactants decrease the viability of epidermis models more than certain

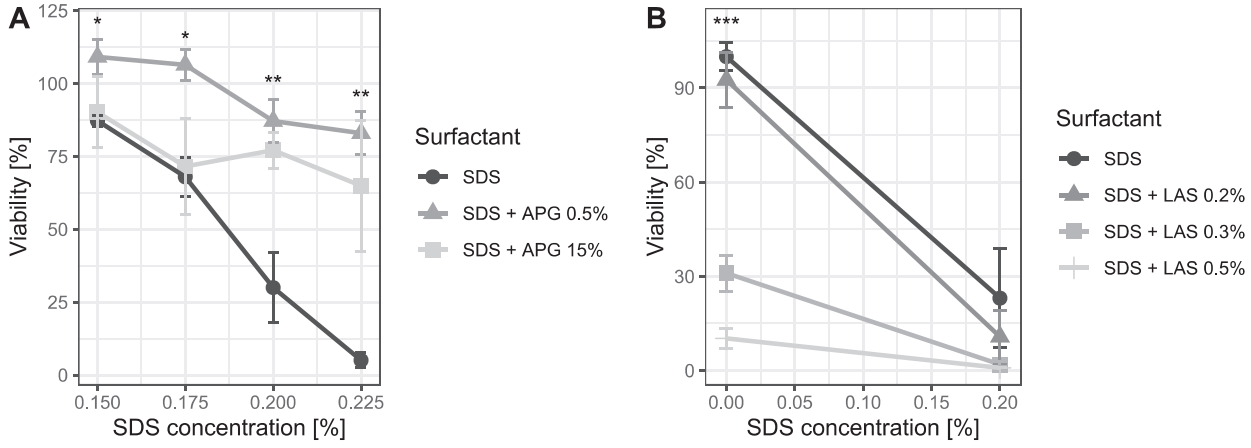

Fig. 3. MTT-viability assay of epidermis models exposed to mixtures of SDS with either the nonionic surfactant APG (**A**) or anionic LAS (**B**). (A) The combination of the anionic surfactant SDS with the nonionic surfactant APG led to a retention of viability. (B) Exposure to the combination of the two anionic surfactants LAS and SDS did not result in higher viabilities as for the treatment with each individual surfactant. The values represent the mean ± SD ($n = 3$). ((*) $P < 0.05$, (**) $P < 0.01$; statistical tests were performed for each experimental condition with the same concentration of SDS using one-way ANOVA.

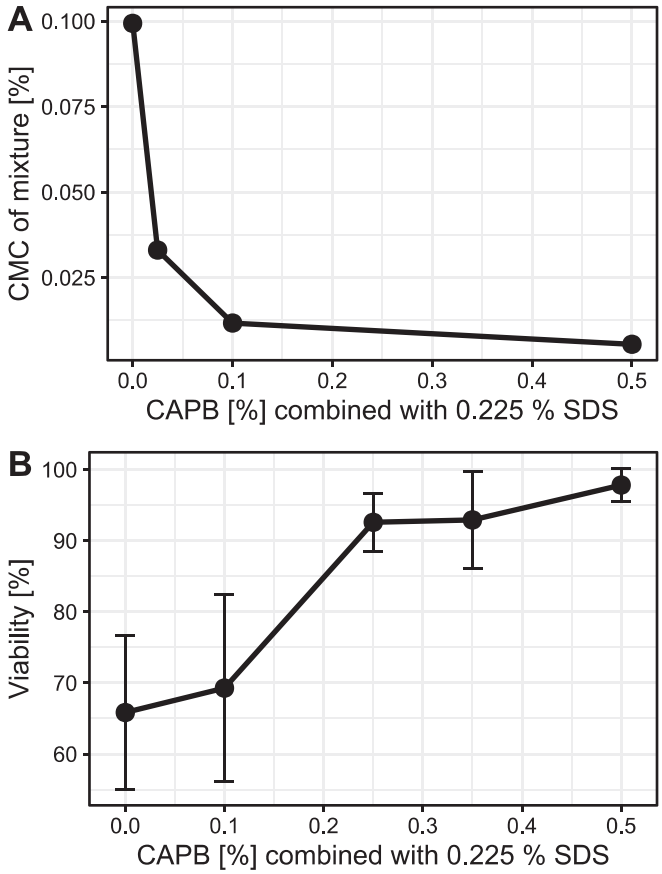

Fig. 4. CMC of mixtures of 0.225% SDS and different concentrations of CAPB and the effect of these mixtures on the viability of epidermis models was determined. **(A)** The CMC of the mixtures were determined using a Du Noüy tensiometer at 25 °C, demonstrating a considerable decrease at a combination of 0.225% SDS with 0.025% CAPB. **(B)** Models were treated with surfactants for 35 min and the viability was determined using the MTT assay. Retention of viability was observed for mixtures of 0.225% SDS with CAPB at and above 0.25%. Viability is expressed as the percentage of water-treated control; values represent mean ± SD ($n = 3$).

mixtures of the same surfactant in combination with a second, milder surfactant (Fig. 2). In order to analyze whether this effect depends on the existence of a fully differentiated epidermis or whether it also can be observed with single cells not integrated in a complex tissue architecture, a comparative experiment was conducted with keratinocytes in a 2D monolayer culture. This test was also intended to dissect possible mechanisms behind the effect on a cellular basis. Analogous to the epidermis models, we exposed proliferating, non-differentiated human keratinocytes to surfactants alone and in combination with a second, milder surfactant.

Specifically, the effects of mixtures of SDS and CAPB on the keratinocyte viability in monolayer culture were evaluated. Primary human keratinocytes from the same donors that were used for the generation of the epidermis models were cultured as subconfluent monolayers and exposed to several concentrations of SDS alone or in combination with various concentrations of CAPB, respectively.

The addition of CAPB to SDS did not prevent the decrease in cell viability observed for the application of SDS alone (Fig. 5). The incubation with surfactant mixtures generally resulted in lower or similar viability values than the exposure to the individual surfactants, with the mixtures of SDS with 0.005% and 0.0075% CAPB, respectively, resulting in a significant decrease of viabilities compared to those observed for the treatment with the corresponding SDS solutions.

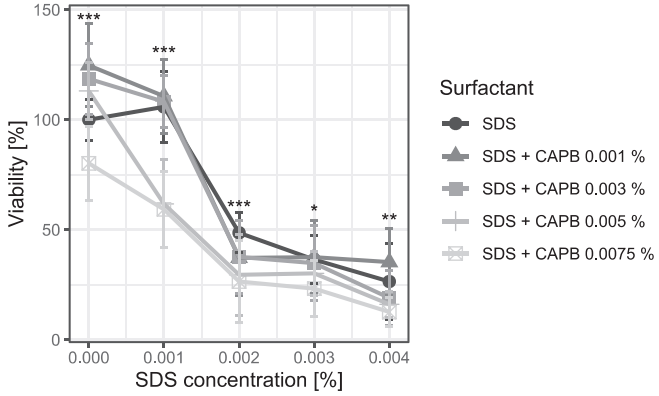

Fig. 5. MTT viability assay of human primary keratinocytes after 4 h exposure to mixtures of SDS and CAPB. Subconfluent keratinocytes were exposed to SDS and CAPB alone or as mixtures. In general, mixtures of the surfactants result in a lower viability compared to the individual surfactants. Viability is expressed as the percentage of medium treated control; values represent the mean ± SD ($n = 8$). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, one-way ANOVA for each experimental condition with the same concentration of SDS; statistical test is applied for comparing the treatment with SDS alone and the treatments with the mixtures with CAPB containing the same concentration of SDS.

3.5. *Immunofluorescence staining of aquaporins in surfactant-treated epidermis models*

To investigate whether the model's barrier plays a role in the differences observed in viability of skin models treated with surfactant mixtures or individual surfactants, the effect of surfactant treatment on the epidermis model's structural integrity was studied. Immunohistochemistry was applied to stain aquaporins in surfactant-treated epidermis models (Fig. 6). Aquaporins were chosen because they are pore-forming cell membrane proteins whose staining can visualize the integrity of cell membranes and hence can be used as a marker for cell damage (Bollag et al., 2020).

Epidermis models were treated with 0.5% SDS alone or in combination with a mixture of 5% CAPB and compared to a water-treated control. In the control, staining of aquaporins revealed a clear and well-organized morphology of the cell membranes (Fig. 6). Epidermis models exposed to 0.5% of SDS lack this clear labelling of the membranes, and only a faint fluorescence signal is visible in the cell periphery. However, following an exposure to SDS supplemented with CAPB, the epidermis model's cell membranes display the same distinct structure as the water treated control. Epidermis models treated with 5% CAPB alone displayed the same clear membrane morphology as the control (data not shown).

3.6. *Fluorescence permeability of surfactant- treated epidermis models*

Skin barrier integrity after exposure to surfactants was further evaluated by qualitatively investigating the permeation of sodium fluorescein through epidermis models. It is assumed that measuring higher fluorescence due to increased permeability corresponds with greater surfactant-mediated damage of the barrier. Epidermis models were exposed to SDS solutions of various concentrations alone or in combination with 5% CAPB, respectively. For one set of epidermis models, viability was determined (Fig. 2), while the amount of fluorescein permeated into the medium was measured in a second set, comprising two epidermis models (Fig. 7). The exposure of epidermis models to SDS alone resulted in a dose-dependent increase of fluorescein permeation. An increase in permeability was observed at concentration levels that also impacted the models' viability, whereas at low concentrations neither a decrease in tissue viability nor in permeability was observed (Fig. 2). Adding 5% of CAPB to SDS prevented both fluorescein

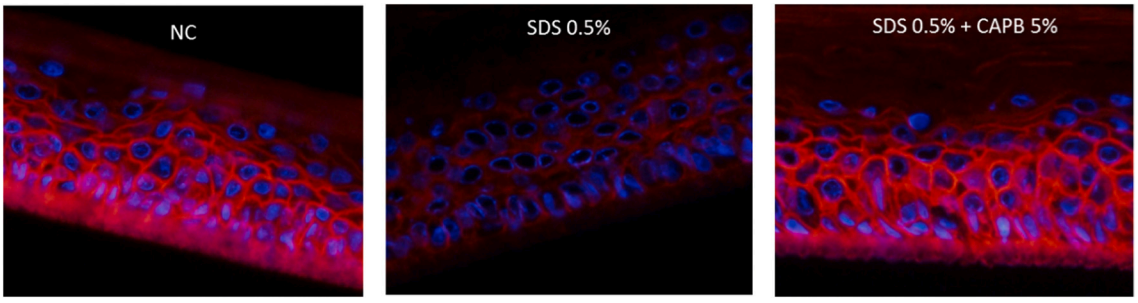

Fig. 6. Immunofluorescent labelling of Aquaporins in epidermis models treated with 0.5% SDS alone or in combination with 5% CAPB. Aquaporins were detected with an anti-aquaporin antibody (red fluorescence), nuclei were stained by DAPI (blue fluorescence). Pictures are representative of two independent experiments with two epidermis models per exposure condition. Staining is weak and diffuse in epidermis models treated with SDS alone. Models treated with the mixture of 0.5% SDS and 5% CAPB show a similar clear staining of the cell membranes as the water-treated control (NC). Epidermis models exposed to 5% CAPB alone show no difference to the water-treated control (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

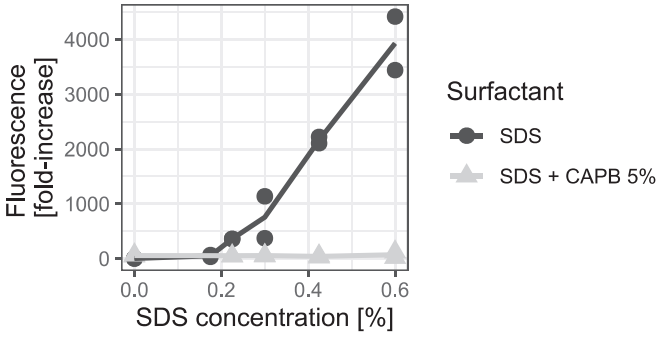

Fig. 7. Permeation of fluorescein through epidermis models after exposure to SDS alone and SDS in combination CAPB. Epidermis models were exposed for 35 min to increasing concentration of SDS, either alone or in combination with 5% CAPB. After a post-incubation phase of 42 h, fluorescein was applied to the stratum corneum of the epidermis models and the fluorescence intensity in the medium under the models was determined ($n = 2$, dots represent measurements of single epidermis models and the line represents the average of the two measurements of each concentration). The viability of epidermis models treated with the same surfactants are shown in Fig. 2.

permeation and viability loss up to the highest tested SDS concentration (0.6%).

3.7. LDH release of treated epidermis models

LDH release is considered a marker for permeabilization of cell membranes which is typically linked to necrotic processes (Ka-Ming Chan et al., 2013) but can be associated with general cell lysis processes as well. The epidermis model's release of LDH was measured after exposure for 35 min to various concentrations of SDS alone or in combination with 5% CABP. Starting at a concentration level at which tissue viability begins to decrease (Fig. 2), treatment with SDS alone results in release of LDH compared to water-treated controls (Fig. 8), even though no clear concentration dependent increase of LDH release is found at higher concentrations of SDS, and high variability being observed at the highest tested concentration. However, in combination with 5% CAPB, at which retention of viability is observed, essentially no release of LDH is measured even at the highest SDS concentration.

4. Discussion

Testing irritation properties of substances or mixtures with *in vitro* methods based on human 3D skin models became today's standard for decisions on classification for skin irritation or corrosion according to UN GHS. Apart from deriving a GHS classification, these models

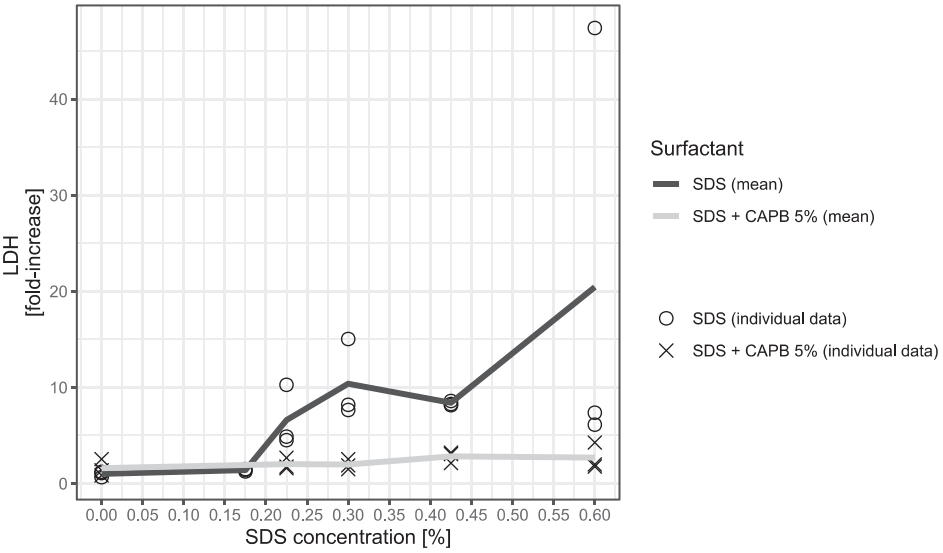

Fig. 8. LDH release from epidermis models after exposure to SDS alone and SDS in combination with CAPB. Epidermis models were exposed for 35 min to increasing concentration of SDS, either alone or in combination with 5% CAPB. After a post-incubation phase of 42 h, the epidermis model's release of LDH was measured ($n = 3$, circles/crosses represent measurements of single epidermis models and the line represents the average of the three measurements of each concentration). The viability of epidermis models treated with the same concentrations of surfactants are shown in Fig. 2.

additionally offer a means for evaluating the mechanistic basis of the irritation effect exerted by a specific substance or mixture.

In the present study, the open-source reconstructed epidermis (OS-REp) is used to investigate the so-called surfactant antagonism, which describes the observation that the skin irritation potential of surfactant mixtures is often lower than expected from the irritation potential of each individual surfactant of the mixture. Hitherto, the surfactant antagonism has only been described in a few studies involving human patch tests (Hall-Manning et al., 1998; Lee et al., 1994; Dillarstone and Paye, 1993; Rhein et al., 1990).

In line with the OECD Test Guideline 439 (OECD, 2021), the skin irritation potential of surfactants and their mixtures were assessed by determining the change in viability of the epidermis models after application of the surfactants based on the protocol developed by Mewes et al. (2016) for small organic molecules. In contrast to the protocol described by Mewes et al. (2016), the surfactants were not tested neat but in dilution and the irritation potential was assessed in a benchmark approach, considering that a larger decrease in viability corresponds to a stronger irritation potential of the surfactant dilution when comparing to other dilutions.

The anionic surfactant SDS resulted in a significant decrease in the model's viability, which is well in line with its known irritation potential (Hall-Manning et al., 1998). However, this decrease of viability is prevented when SDS is combined with the nonionic surfactant APG or the zwitterionic surfactant CAPB, both of which are only mild irritants. Due to the surfactant antagonism, viability loss is prevented even when the total concentration of surfactants in the solution is increased by adding the second surfactant. On the contrary, no surfactant antagonism was observed when SDS is combined with LAS, another anionic surfactant with skin corrosive properties.

Immunofluorescence staining of the transmembrane protein aquaporin revealed that exposure to SDS leads to cell membrane damages which correspond with the decrease of viability of the tissue.

The measurements of LDH release from the models, even though considered rather indicative due to the high degree of variability at the highest SDS concentrations when tested alone, overall support the hypothesis that the cell membranes of the model's keratinocytes are disintegrated once the surfactants have penetrated the skin barrier and have reached the living cells. This process is prevented when a second milder surfactant is added to the SDS solution, which can be seen by higher tissue viability, intact tissue architecture and prevented LDH release.

While surfactant antagonism can be demonstrated in epidermis models, this effect is not observed in monolayer cultures of keratinocytes. This indicates that the effect is linked to a component of the epidermis that is not present in the viable keratinocytes. More specifically, a preliminary analysis of the fluorescein permeability suggests that the skin barrier of the epidermis plays an important role in the surfactant antagonism. Although requiring confirmation by an increased number of repetitions, the initial experiment shows the clear trend that the dose-dependent increase of the model's permeability to fluorescein after treatment with SDS is reduced by the addition of a milder surfactant. Based on these results, the surfactant antagonism is primarily related to the surfactant interaction with the skin barrier, in which adding a surfactant of low irritation potential to a strongly irritating surfactant prevents the latter from destroying the skin barrier. However, once the surfactants have passed the barrier and reached the living layers of the epidermis, the cells are lysed. The relevance of the skin barrier in this process is also reflected by the reverse-sigmoidal shape of the dose-dependency curve found for the model's viability when treated with different dilutions of SDS alone. As long as the barrier remains intact with increasing concentrations of SDS, no relevant change of viability can be observed. However, at SDS concentrations at which the barrier function is destroyed, an immediate and steep decrease of viability is observed.

In the past, the lower CMC of surfactant mixtures has been proposed as a possible explanation of the lower skin irritation potential (Dillarstone and Paye, 1993; Rhein et al., 1990). In our study, surfactant solutions with a lower CMC also reduced the epidermis models' viability to a lesser extent than surfactant solutions with a higher CMC. However, the ratio of the two surfactants at which either the change of CMC or a change of cytotoxic effects occurs are not identical, which is in line with the findings of Rhein et al. obtained by patch tests (Rhein et al., 1990).

Therefore, the data presented in this study do not support the hypothesis that the antagonism is directly caused by a lowering of the CMC. It is possible that some other physico-chemical effects related to the CMC causes the surfactant antagonism. Moore et al. (2003), for instance, suggested that the bigger size of mixed micelles might be the reason for the surfactant antagonism, as they are too big to enter the skin's barrier and to interact with its components. The exact mechanism of how the addition of a milder surfactant decreases the skin barrier disintegration caused by the more irritating surfactant remains unclear. In addition, this study is limited to binary surfactant mixtures and does not address how more complex mixtures with additional surfactants and non-surfactant substances may influence the degree of surfactant antagonism. Further studies are needed to shed light on the underlying mechanisms of the epidermis barrier's damage by surfactants and their mixtures.

When applying the 50% viability cut-off value according to the prediction model defined in OECD TG 439 (OECD, 2021), a solution of about 0.2% SDS already requires classification as skin irritating category 2 (H315); in combination with 5% CAPB, the mixture does not require classification for skin irritation, as the viability remains at about 100%. This contrasts the strict additive nature considered in the UN GHS (United Nations, 2019) when deriving the classification of the mixture from the concentration and classification of each individual ingredient of the mixture. Even though it is appreciated that the summation approach as stipulated in UN GHS aims at being sufficiently conservative to avoid under-classification of mixtures, the irritation potential of surfactant mixtures may be overestimated by this method because it neglects surfactant antagonism effects.

In conclusion, the use of a 3D reconstructed epidermis model that mimics the epidermal architecture of the human skin allowed to investigate the mechanism of surfactant antagonism and to identify the interaction of surfactants with the skin barrier as the main driver for surfactant antagonism. Furthermore, it could be demonstrated that the surfactant antagonism does not occur in all types of surfactant mixtures but seems to be linked to the combination of surfactants of high and comparably low irritation potential. The use of an MTT-based method in line with the OECD Test Guideline 439 shows that the results might affect the classification of surfactant mixtures for skin effects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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