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Less efficient skin penetration of the metal allergen Pd^{2+} compared to Ni^{2+} and Co^{2+} from patch test preparations

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Abstract

Background: Contrary to Ni^{2+}- and Co^{2+}-induced allergic contact dermatitis (ACD), reactions against Pd^{2+} are rare. However, Pd^{2+} activates a larger T cell fraction *in vitro*, suggesting an inefficient skin penetration.

Objectives: This study compares Ni^{2+}, Co^{2+} and Pd^{2+} skin penetration from commonly used diagnostic patch test preparations (PTPs) and aqueous metal salt solutions.

Methods: Using Franz diffusion cell assays, we applied the metals in PTPs (5% $NiSO_4$, 1% $CoCl_2$, 2% $PdCl_2$ and 3% Na_2PdCl_4) and in solution to pigskin for 48 h, thereby mirroring the time frame of a patch test. The different compartments were analysed individually by inductively coupled plasma mass spectrometry.

Results: Metal ions were mainly retained in the upper *stratum corneum* layers. After application of PTPs, concentrations in the viable skin were lower for Pd^{2+} (1 and 7 μM) compared to Ni^{2+} and Co^{2+} (54 and 17 μM).

Conclusions: Ni^{2+} and Co^{2+} penetrated the skin more efficiently than Pd^{2+} and thus may sensitize and elicit ACD more easily. This was observed for ions applied in petrolatum and aqueous solutions. We hypothesize that the differently charged metal complexes are responsible for the varying skin penetration behaviours.

KEYWORDS
Franz diffusion cell assay; metal allergy; Ni, Co, Pd; patch test preparation; skin penetration; T cell activation

1 | INTRODUCTION

Contact allergies are T cell-mediated diseases and are estimated to affect about 20% of the population.[1] Common contact allergens include metals such as nickel and cobalt which can be released from a variety of consumer products.[1] Therefore, nearly everyone is frequently exposed to metal allergens and thus at risk of allergic sensitization.

We recently quantified metal-specific CD4+ T cells by an *in vitro* activation-induced marker assay and identified the underlying interactions with conserved T cell receptor residues.[2,3] The particularly high frequencies we observed for Ni^{2+} and Co^{2+} were further exceeded by those of Pd^{2+}-specific T cells at optimal concentrations (ca. 400 μM

Abbreviations: ACD, allergic contact dermatitis; FDC, Franz diffusion cell; ICP-MS, inductively coupled plasma mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; PBS, phosphate-buffered saline; PTP, patch test preparation; s.c., *stratum corneum*; TLR4, Toll-like receptor 4.

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Pd^{2+}). However, palladium allergy is rarely diagnosed in the general population, which may be due to less efficient skin penetration of Pd^{2+}.

Patch testing—also known as epicutaneous testing—is used as a diagnostic tool to identify contact allergies.[4,5] Although it is the current diagnostic standard, the test has several limitations. Patch testing is supposed to induce the elicitation phase of allergic contact dermatitis (ACD) by delivering a critical amount of allergen into the skin to activate immune cells on a small test area. Antigen-specific tissue-resident memory T cells emerge during sensitization and become reactivated by renewed allergen exposure, for example, during patch testing.[6,7] Tissue-resident memory T cells predominantly reside in the epidermis near the dermal junction or in the dermis. However, it remains unclear whether metal ion concentrations on the skin eliciting ACD in real-life exposure scenarios and those used in patch testing are similar. As a result, the clinical relevance of a positive patch test remains unknown.

Patch test preparations (PTPs) of metals are undissolved metal salts dispersed in petrolatum, which are applied to the back of a patient. Various PTPs of different compositions used globally can potentially deliver varying amounts of metal ions into the skin thereby hindering the comparison of allergy prevalence.[8–13] Substance properties, including particle size and solubility of the metal salt, have a significant impact on the release and penetration of metal ions into the skin.[14,15] Therefore, using an inappropriate substance can lead to false positive or false negative patch test results, which can ultimately result in incorrect allergy diagnoses. For instance, the European Society of Contact Dermatitis guideline recommends the use of undissolved $PdCl_2$ in petrolatum as a PTP.[5] As $PdCl_2$ is poorly soluble in water, it is possible that not enough Pd^{2+} is migrating into the skin during patch testing. This can lead to false negative results when employing $PdCl_2$ as test substance. As an alternative for palladium-based PTPs, the water-soluble Na_2PdCl_4 has been suggested. Positive patch test results obtained with $PdCl_2$ were generally confirmed by Na_2PdCl_4. Furthermore, Na_2PdCl_4 allowed for the diagnosis of additional patients with palladium allergy. The results were obtained despite the fact that $PdCl_2$ was applied to the skin in dissolved form and Na_2PdCl_4 was applied dispersed in petrolatum.[16–18] In the literature, the permeation of metal ions through skin (human, pig and mouse) has been analysed mainly via Franz diffusion cell (FDC) assays. Most of these experiments were carried out with incubation times of 24 h, making it difficult to predict metal permeation rates after 48 h, which is the typical duration of the patch test.[19] Since most studies are designed to mimic real-life human exposure, mainly undissolved metal salts or metal nanoparticles in artificial sweat formulations have been investigated.[19–21] The analysis of skin penetration from artificial sweat is not representative of the metal ion release from PTPs into the skin because of the different physicochemical properties of the formulations. For instance, when investigating the penetration properties of 5% $NiSO_4$ PTPs, no Ni^{2+} was found in the receptor phase after 48 h.[22]

In the present study, the metal ion penetration of the patch test salts $NiSO_4$, $CoCl_2$, $PdCl_2$ and Na_2PdCl_4 into pigskin was determined using FDC assays combined with inductively coupled plasma mass spectrometry (ICP-MS) analysis. In a setting mimicking clinical use, the metal salts were applied as PTPs under occlusion for 48 h. In addition, metal salt solutions in phosphate-buffered saline (PBS) were tested. According to the *Registration, Evaluation, Authorisation and Restriction of Chemicals* legislation of the European Union, products that have direct and prolonged contact with the skin are restricted to release nickel to a maximum of 0.5 μg/(cm^2·week).[23] We used a salt solution in PBS to apply a similar concentration of 0.57 μg/cm^2 Ni^{2+} to the skin. Therefore, it can be assumed that the penetration of Ni^{2+} into the remaining skin provides an exposure scenario close to the regulatory limit. Co^{2+} and Pd^{2+} were applied in equal amounts for comparison.

After the incubation period, the FDCs were disassembled and the *stratum corneum* (s.c.) was separated from the remaining skin by tape stripping. The disassembly produced four compartments: donor, s.c., remaining skin and receptor fluid, with the remaining skin containing the dermal junction between the epidermis and dermis.

On the basis of our results, the accuracy of patch test-based diagnoses of metal contact allergies was evaluated. Furthermore, potential causes of the observed differences in the prevalence of metal contact allergies are discussed. The determined metal ion concentrations in the different FDC compartments can guide the selection of appropriate metal ion concentrations for *in vitro* studies, including diagnostic T cell assays.

2 | METHODS

For chemicals and materials, confer to the supporting information (SI) I, section A.1 Chemicals. The experimental strategy is summarized in Figure 1.

2.1 | Pigskin

Pigskin from two female pigs was received from Charité, Universitätsmedizin Berlin. Several earlier skin penetration investigations have substituted pigskin for human skin.[24–30] Pigskin and human skin do not significantly differ in terms of relevant penetration features for organic chemicals,[31] such as lag time and diffusion of drugs in the s.c.[26–28] Because pigskin shares key permeation characteristics with human skin, the Scientific Committee on Consumer Safety advises using it in tests of skin penetration.[32] Metal penetration was found to be qualitatively similar in pigskin as compared to human skin, indicating that pigskin is a suitable experimental model for human skin.[33] The skin was treated as published before.[34] Briefly, sows were sacrificed without impact on the skin's integrity after an unrelated surgical procedure. Flank skin was removed without delay and brought to our facility on ice. There, the skin was shaved and cut into pieces of about 10 × 20 cm^2 with a knife. These pieces were frozen in plastic bags and kept at a temperature of −20°C for up to 12 months.

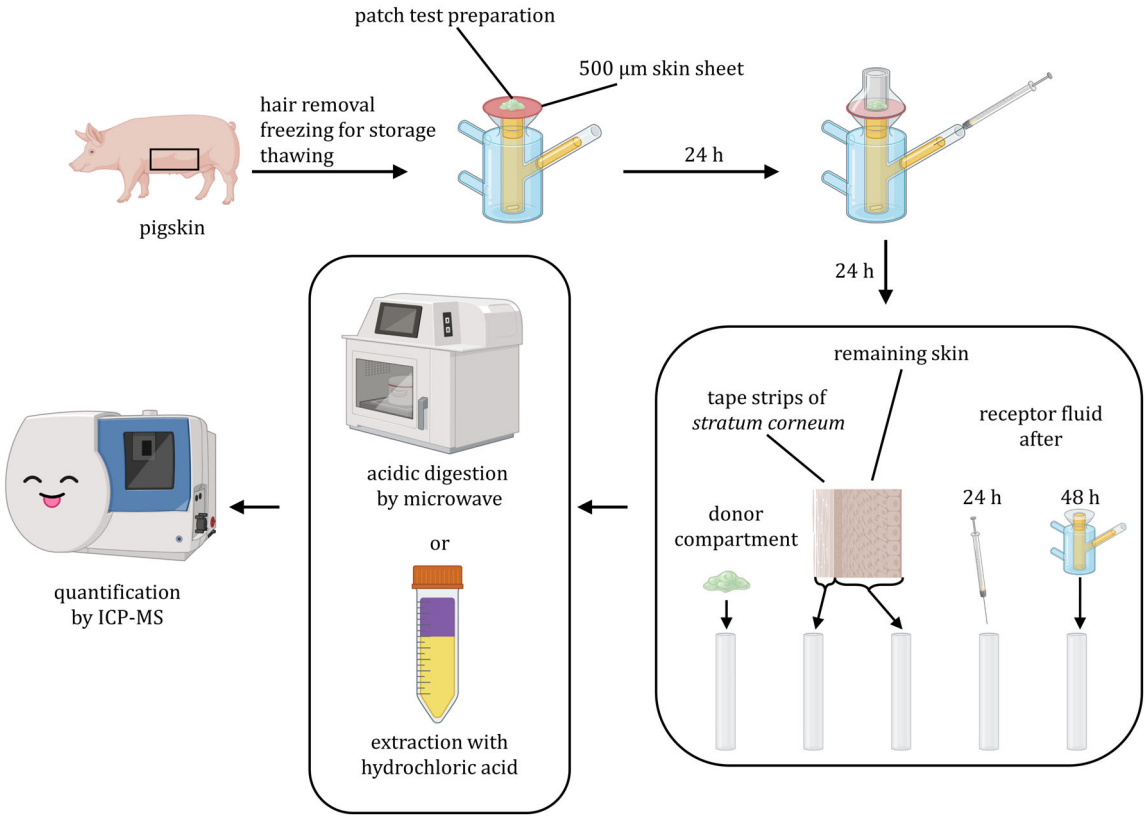

FIGURE 1 Experimental strategy. Donor substances were applied onto flank pigskin in Franz diffusion cell assays. After 24 h, a sample of the receptor fluid was taken. After 48 h, the compartments were separated including tape stripping of the *stratum corneum*. All compartments were digested with nitric acid in a microwave or extracted using hydrochloric acid. The metal contents of the resulting solutions were quantified by inductively coupled plasma mass spectrometry (ICP-MS). Created in part with BioRender.com.

TABLE 1 Donor substances used in Franz diffusion cell assays.

Donor substance	ζ	β (mg/mL)	c_n (mM)	β_A (µg/cm^2)
$NiSO_4$ PTP (5% $NiSO_4 \cdot 6H_2O$)	1.1%	9	160	127
$CoCl_2$ PTP (1% $CoCl_2 \cdot 6H_2O$)	0.2%	2	35	28
$PdCl_2$ PTP (2% $PdCl_2$)	1.2%	10	95	136
Na_2PdCl_4 PTP (3% $Na_2PdCl_4 \cdot H_2O$)	1.0%	9	81	116
Ni^{2+} solution ($NiSO_4 \cdot 6H_2O$ in PBS)	5 ppm	$5 \cdot 10^{-3}$	$8.5 \cdot 10^{-3}$	0.57
Co^{2+} solution ($CoCl_2 \cdot 6H_2O$ in PBS)	5 ppm	$5 \cdot 10^{-3}$	$8.5 \cdot 10^{-3}$	0.57
Pd^{2+} solution ($Na_2PdCl_4 \cdot H_2O$ in PBS)	5 ppm	$5 \cdot 10^{-3}$	$4.7 \cdot 10^{-3}$	0.57

Note: PTPs are declared as mass percentages (m/m). Concentration metrics for each metal ion: mass percentages of the metal ζ, mass concentrations β, molar concentrations c_n and applied surface concentrations β_A on the skin in the Franz diffusion cell assay with a skin surface area of 1.76 cm^2. Molar masses: SI II sheet 8.
Abbreviations: PBS, phosphate-buffered saline; PTP, patch test preparation.

2.2 | Donor substances

For this study, commercially available and clinically applied PTPs (ChemotechniqueMB Diagnostics AB, Vellinge, Sweden) were used (concentrations: Table 1). The amount of PTP applied to the skin was 20 mg as recommended by the European Society of Contact Dermatitis.[5] Furthermore, a solution of the three metals in PBS was used. The solution was prepared by solving the respective metal salts in hydrochloric acid (6.7%, salt concentration: $NiSO_4 \cdot 6H_2O$: 3.36 g/mL, $CoCl_2 \cdot 6H_2O$: 3.03 g/mL and $Na_2PdCl_4 \cdot H_2O$: 2.21 g/mL) and subsequent dilution in PBS to a concentration of 5 µg/mL (pH ≈ 7). About 200 µL of this solution was applied to the skin. The resulting surface concentrations on the skin after the application of PTPs or PBS solutions are summarized in Table 1.

2.3 | FDC assay

The FDC assay is commonly applied in skin penetration studies. Here, we illustrate an overview of the used method (Figure 1). The assays were carried out as six (per individual metal PTP) or seven (metal salt solution in PBS) independent experiments. For details on the experimental design and microwave digestion of samples, see the SI I, section A.2 Protocol of Franz diffusion cell assay.

For the assay, the receptor chamber (12 mL) of the FDC was filled with fetal bovine serum and a piece of dermatomed pigskin (1.5 × 1.5 cm^2, 500 µm thick) was placed onto the cell and fixed with the donor chamber cap, resulting in an application area of 1.76 cm^2. The skin integrity was assured monitoring the trans-epidermal water loss, using the AquaFlux device AF200 (Biox Systems Ltd, London, UK) in accordance with Guideline 428 of the Organisation for Economic Co-operation and Development.[35] Skin pieces were excluded and replaced if the trans-epidermal water loss exceeded 13 $g \cdot m^{-2} \cdot h^{-1}$.

The PTPs or the metal salt solution in PBS were applied to the skin and the cell was incubated at 32°C. After 24 h, a 100 µL sample was taken from the receptor chamber using a syringe. After a total incubation time of 48 h, the FDC was disassembled and the s.c. was separated from the remaining skin by tape stripping (20 strips, four consecutive strips were pooled to obtain five groups). All compartments were processed by microwave digestion with nitric acid or an extraction with hydrochloric acid (conditions: Tables S1–S3 in SI I). The samples were then quantified using ICP-MS.

2.4 | Tape stripping and skin layer volume calculation

The s.c. was separated from the skin by tape stripping.[34,36,37] Each tape strip removes about one layer of the s.c., corresponding to ca. 0.38 µm of s.c. removed per tape strip, as determined in our recent study.[34] In this study, four consecutive tape strips were pooled. Thus, every pooled sample corresponds to approximately 1.52 µm of s.c. removed. Given the 1.76 cm^2 permeation area in the FDC, this amounts to a volume of 268 nL. After 20 tape strips, 7.60 µm of the 500 µm thick skin sheet is removed, leaving a volume of 86.7 µL for the remaining skin. These values were used for the calculation of the concentrations of metal ions in the different skin layers. Importantly, 20 tape strips were found to not completely remove the s.c. but leave behind about seven layers in pigskin.[34]

2.5 | Establishment of sample preparation for quantification

Sample treatments were optimized to ensure an efficient digestion, especially for palladium, and metal ion recoveries for each compartment of the FDC assays were determined from spiked matrix samples applying optimized conditions. Recoveries were within the range of 100 ± 15%, except for the recovery of palladium from the tape strip matrix (42.1 ± 2.2%, Table S4). For quantification, the determined recovery values were used to correct for losses during sample preparation. Limits of detection (LODs) and limits of quantification (LOQs) were determined for each of the metals from noise levels of blank solutions of 3% nitric acid (Table S5). A detailed description can be found in SI I, section A.3 Establishment of sample preparation for ICP-MS.

2.6 | Solubility assessment of palladium salts in water

To determine the water solubility of the palladium salts used in the PTPs ($PdCl_2$ and Na_2PdCl_4), aqueous saturated solutions of both salts were prepared. The Pd^{2+} concentrations of the supernatants were determined by ICP-MS yielding the solubility of each salt. For details on the experimental set-up, see SI I, section A.5 Solubility of palladium salts.

2.7 | Inductively coupled plasma mass spectrometry

A quadrupole ICP-MS (iCAP Q, Thermo Fisher Scientific GmbH, Dreieich, Germany) with a perfluoroalkoxy alkane ST-Nebulizer, a quartz cyclonic spray chamber and a 2.5 mm quartz O-ring-free injector (all from Elemental Service and Instruments GmbH, Mainz, Germany) was used. The sample solutions were injected at a flow rate of 0.4 mL/min. A performance report was evaluated on each measurement day and the instrument was tuned if necessary. All isotopes were measured with collision gas (5 mL/min, H_2: 7 vol%; He: 93 vol%) in kinetic energy discrimination mode. Calibration solutions were prepared in nitric acid (3.5%) ranging from 0.2 to 120 ng/mL. Blank solutions (3.5% nitric acid, 1% hydrochloric acid) were measured before and after the calibration solutions as well as after the analysis of every five samples. Solutions were injected using a prepFast dilution system (Elemental Scientific, Omaha, Nebraska). The system was tuned using a diluted solution of Tune B (Thermo Fisher Scientific Co. LLC Waltham, Massachusetts) at a concentration of 1 ppb in 2% isopropanol. For more details, confer to the SI I, section A.5 ICP-MS quantification.

2.8 | Data analysis and statistics

Exported raw data (comma-separated values files) were analysed in R (version 4.2.2) and data points corresponding to metal concentrations below the LODs (Table S5) were marked as below LOD (<LOD). In the next step, the means of the concentrations of the negative control FDC assay results (pure petrolatum applied) were calculated and these values were subtracted (negative values are documented in SI II sheet 1) from the values obtained from the FDC cells loaded with metal-containing donor substances. From these background-corrected

values, the total metal ion masses in each of the compartments were calculated. The data were then subjected to a Shapiro–Wilk normality test.[38] If the Shapiro–Wilk normality test failed, the dataset was checked for outliers using boxplots (Figures S1–S3 in SI I). Outliers were excluded from the data set if the data were not normally distributed and if the data point was outside 1.5 times the interquartile range of the boxplot (outside of whiskers). Differences were considered significant when a *t*-test resulted in $p < 0.05$. Data given in the text are reported as means ± standard deviations.

3 | RESULTS

The aim of this study was to quantitatively compare skin penetration of Ni^{2+}, Co^{2+} and Pd^{2+} ions from both common diagnostic PTPs and an aqueous salt solution. Following incubation of pigskin in FDC assays for 48 h, the metal contents of all compartments including different s.c. layers and the remaining skin were analysed by ICP-MS. Total recoveries are in the range of 80%–104% (Table 2) illustrating that the metal ion contents were reliably quantified.

3.1 | Skin penetration of metal ions from patch test preparations

When applied in PTPs with metal ion concentrations used in clinical patch testing, Ni^{2+} and Co^{2+} more effectively penetrate into the skin than Pd^{2+} from $PdCl_2$ PTP (Figure 2A,B, SI II sheets 2 and 3). A greater share of Ni^{2+} and Co^{2+} than Pd^{2+} from either PTP was absorbed into the skin, corresponding to the recovery of all compartments but the donor compartment, even though the difference between $NiSO_4$ and Na_2PdCl_4 was not significant ($NiSO_4$: 3.98 ± 2.09%; $CoCl_2$: 5.40 ± 2.64%; $PdCl_2$: 1.47 ± 0.45%; Na_2PdCl_4: 2.00 ± 0.99%, SI II sheet 4 for *p* values). Furthermore, a greater share of Ni^{2+} and Co^{2+} was found in the remaining skin after tape stripping ($NiSO_4$: 0.120 ± 0.045%; $CoCl_2$: 0.166 ± 0.105%) compared with Pd^{2+} ($PdCl_2$: 0.004 ± 0.004%; Na_2PdCl_4: 0.032 ± 0.031%, SI II sheet 5 for *p* values). No Pd^{2+} was found in the receptor fluid after 48 h incubation, while recoveries of Ni^{2+} and Co^{2+} above the LOQ were determined (Ni^{2+}: 0.182 ± 0.186%; Co^{2+}: 0.124 ± 0.045%). No recoveries above the LOD of Ni^{2+}, Co^{2+} or Pd^{2+} were found in the receptor fluid after 24 h incubation time.

The first four tape strips contain the highest amount of any metal ion regardless of the PTP (Figure 2B). Ni^{2+} and Co^{2+} show very similar results, however, the decrease after the first four tape strips is more pronounced for Ni^{2+} than for Co^{2+}. After incubation with $PdCl_2$ PTP, Pd^{2+} was only found in the first eight tape strips in amounts above the LOD. After incubation with Na_2PdCl_4 PTP, the amount of Pd^{2+} decreased less rapidly and Pd^{2+} was detected up to 16 tape strips deep.

The concentration of Ni^{2+} in the first s.c. layers is significantly ($p = 0.025$) higher than the other metals probably due to the higher concentration in the PTP (Figure 2C, Table 3 for values of s.c. layers 17–20 and the remaining skin, SI II sheet 6 for all values). Molar concentrations in deeper s.c. layers do not differ significantly with few exceptions (SI II sheet 7). Pd^{2+} from $PdCl_2$ was not detected in tape strips nine through 16 and then again in tape strips 17–20.

Pd^{2+} from $PdCl_2$ PTP was found in a significantly lower concentration in the remaining skin than Ni^{2+} and Co^{2+} from their respective PTPs ($p = 0.02$). Contrarily, the Pd^{2+} concentration in the remaining skin from Na_2PdCl_4 PTP is only significantly different from Ni^{2+} ($p = 0.03$, $NiSO_4$: 53 ± 37 µM; $CoCl_2$: 17 ± 10 µM; $PdCl_2$: 1.1 ± 1.0 µM; Na_2PdCl_4: 11.5 ± 5.8 µM).

3.2 | Skin penetration of metal ions from metal salt solutions in PBS

To investigate the different skin penetration capabilities of the metal ions independent from salt solubility in water, FDCs applying an aqueous solution of the salts in PBS were performed (Figure 3). Metal salts were dissolved in PBS to maintain a stable, neutral pH. Since we found no reliable values in the literature, the solubilities of $PdCl_2$ and Na_2PdCl_4 in water were determined. The values obtained are 7.74

TABLE 2 Total recovery and absolute masses of metal ions after application of patch test preparations (PTPs) and aqueous salt solutions in Franz diffusion cell assays.

Application	Applied metal ion mass (µg)	Recovered metal ion mass (µg)	Total recovery (%)
$NiSO_4$ PTP	229 ± 5.5	219 ± 1.5	96 ± 0.5
$CoCl_2$ PTP	50.6 ± 1.1	48.4 ± 0.3	96 ± 0.7
$PdCl_2$ PTP	246 ± 5.1	195 ± 1.2	80 ± 0.6
Na_2PdCl_4 PTP	210 ± 2.3	177 ± 1.3	84 ± 0.6
Ni^{2+} solution in PBS	1.0	1.04 ± 0.06	104 ± 6
Co^{2+} solution in PBS	1.0	0.90 ± 0.05	90 ± 5
Pd^{2+} solution in PBS	1.0	0.85 ± 0.03	85 ± 3

Note: Mean recoveries/masses ± standard deviations with $n = 6$ (PTP) or $n = 7$ (solution) independent experiments. Total recoveries/masses represent the sum of the quantified metal contents in all analysed compartments.
Abbreviation: PBS, phosphate-buffered saline.

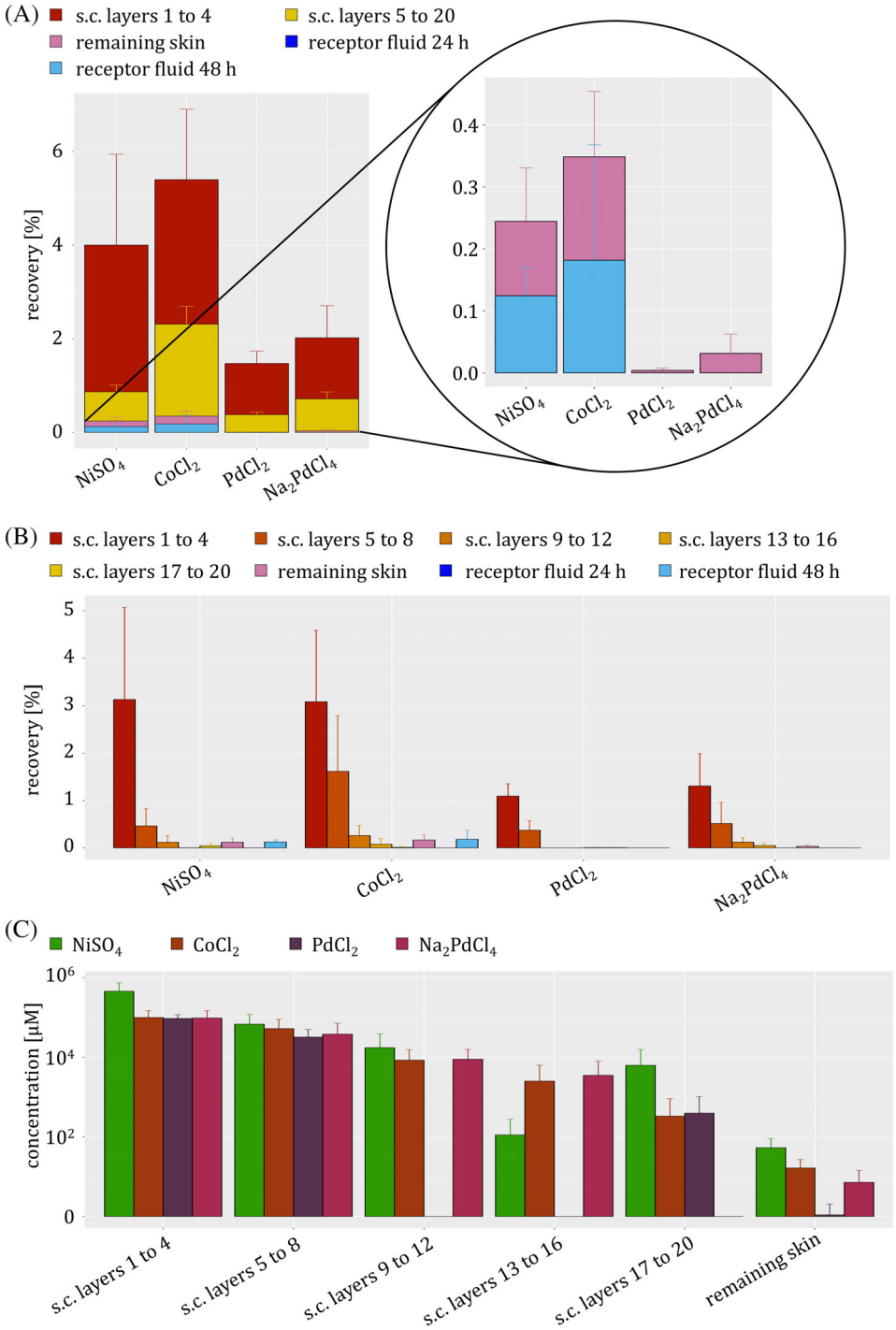

FIGURE 2 Recoveries of Ni^{2+}, Co^{2+} and Pd^{2+} after the application of different patch test preparations (PTPs, see Table 1 for metal concentration) in a Franz diffusion cell assay after 48 h of incubation. (A) Stacked diagram. Magnification: data from the *stratum corneum* (s.c.) were omitted for clarity. (B) Bar diagram of recoveries to compare different compartments and layers of the s.c. (C) Bar diagram showing micromolar concentrations in the respective skin layers to compare different PTPs; logarithmic scale. No ions were recovered after 24 h in the receptor fluid. Means ± standard deviation with $n = 6$ independent experiments.

± 1.17 mM of Pd^{2+} for $PdCl_2$ and 241 ± 49 mM of Pd^{2+} for Na_2PdCl_4. The values determined here are used as guidance to show that Na_2PdCl_4 is indeed better soluble in water than $PdCl_2$. For comparison, the water solubilities of $NiSO_4$ and $CoCl_2$ are 1.9 M of Ni^{2+} and 4.08 M of Co^{2+}, respectively.[39,40]

Notably, the proportion of the applied ions that was detected in the entire skin after 48 h did not significantly differ between the metals (Ni^{2+}: 64.5 ± 16.4%; Co^{2+}: 69.4 ± 16.2%; Pd^{2+}: 68.5 ± 7.3%). However, the distribution in individual skin compartments is different: the recoveries in the uppermost s.c. layers (tape strips one to four) significantly vary for all three ions with Pd^{2+} being retained the most, which is followed by Co^{2+} and Ni^{2+} ($p < 0.02$, Ni^{2+}: 13.8 ± 3.8%; Co^{2+}: 22.7 ± 4.0%; Pd^{2+}: 39.9 ± 5.7%). In s.c. layers 5–20, the three metals are statistically equally abundant (Ni^{2+}: 11.0 ± 2.2%; Co^{2+}: 14.7 ± 2.5%; Pd^{2+}: 17.5 ± 2.5%). A higher recovery of Ni^{2+} compared to Pd^{2+} is found in the remaining skin ($p = 0.006$); Co^{2+} recovery does not significantly differ from both (Ni^{2+}: 23.6 ± 8.5%; Co^{2+}: 17.5 ± 8.1%; Pd^{2+}: 10.6 ± 5.4%). In the receptor fluid after 24 h, the only metal that was detected above LOD was Co^{2+} (5.18 ± 1.96%). After 48 h, only a small recovery of Pd^{2+} was detected in the receptor fluid

TABLE 3 Metal concentrations in the lower *stratum corneum* (s.c.) layers and the remaining skin.

	s.c. layers 17–20		Remaining skin	
Donor substance	**(µM)**	**(µg/ml)**	**(µM)**	**(µg/ml)**
$NiSO_4$ PTP	6263 ± 9349	368 ± 549	53.5 ± 37.4	3.14 ± 2.20
$CoCl_2$ PTP	333 ± 576	19.6 ± 33.9	16.5 ± 10.5	0.97 ± 0.62
$PdCl_2$ PTP	394 ± 622	41.9 ± 66.2	1.1 ± 1.0	0.12 ± 0.10
Na_2PdCl_4 PTP	<LOD	<LOD	7.2 ± 7.1	0.77 ± 0.76
Ni^{2+} solution in PBS	718 ± 1110	42.2 ± 65.1	46.3 ± 16.7	2.72 ± 0.98
Co^{2+} solution in PBS	753 ± 923	44.4 ± 54.4	34.2 ± 15.9	2.02 ± 0.94
Pd^{2+} solution in PBS	454 ± 645	48.3 ± 68.6	11.5 ± 5.8	1.22 ± 0.62

Note: Mean concentrations ± standard deviations with $n = 6$ (PTP) or $n = 7$ (solution) independent experiments. Molar masses: SI II sheet 8.
Abbreviations: PBS, phosphate-buffered saline; PTP, patch test preparation.

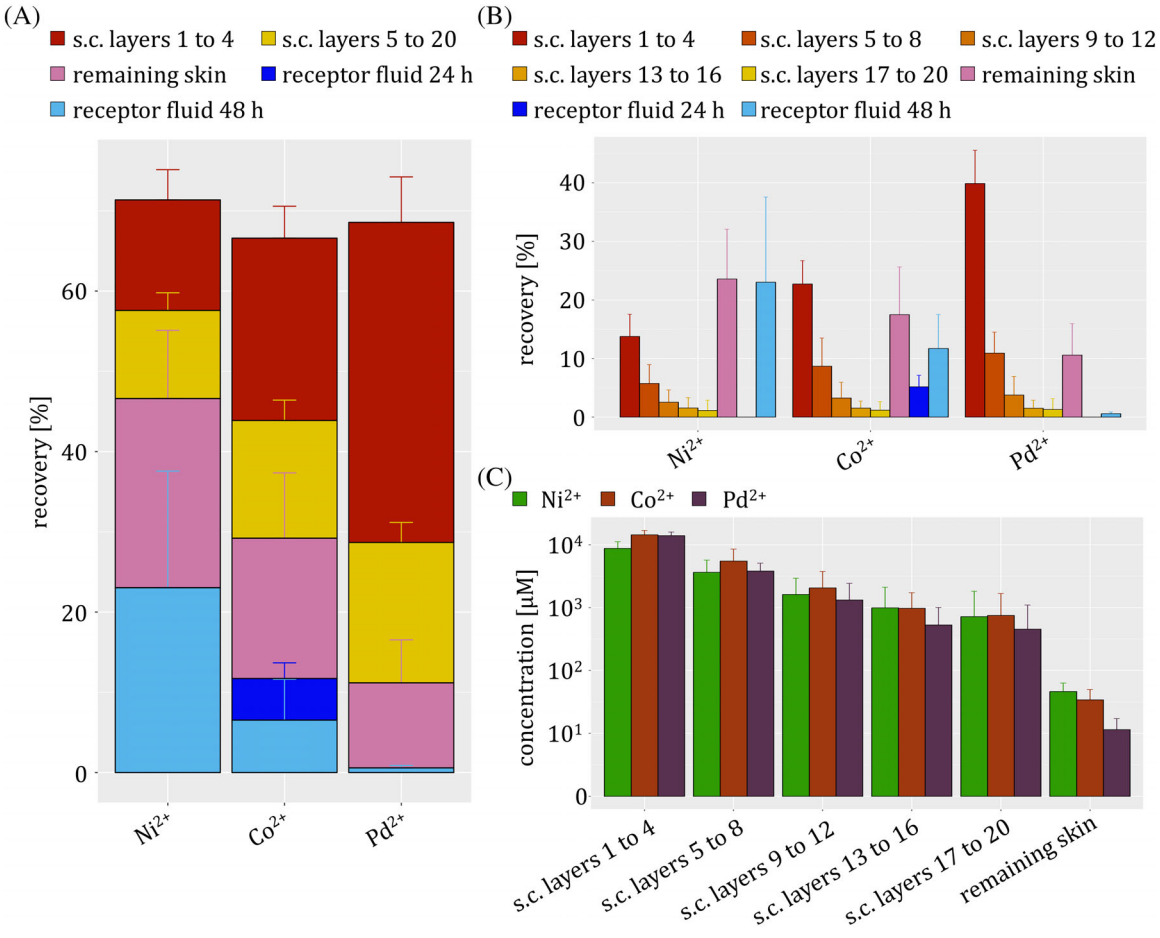

FIGURE 3 Recovery of Ni^{2+}, Co^{2+} and Pd^{2+} after application of a solution of the three metals (see Table 1 for metal concentration) in a Franz diffusion cell assay at 48 h incubation time. (A) Stacked diagram. Recovery after 24 h was subtracted from recovery after 48 h. (B) Bar diagram of recoveries to compare different compartments and layers of the *stratum corneum* (s.c.). (C) Bar diagram of micromolar concentrations in the respective skin layers to compare different metals; logarithmic scale. Means ± standard deviation with $n = 7$ independent experiments.

compared to Ni^{2+} and Co^{2+} ($p < 0.013$; Ni^{2+}: 23.0 ± 14.5%; Co^{2+}: 11.7 ± 5.8%; Pd^{2+}: 0.59 ± 0.29%).

The distribution profile in the s.c. after incubation of the metals dissolved in PBS is similar for Ni^{2+} and Co^{2+} (Figure 3B). Molar concentrations (Figure 3C; Table 3 for values of s.c. layers 17–20 and remaining skin) on the other hand do not differ significantly in the different s.c. layers, except for lower Ni^{2+} concentration in the uppermost s.c. layers ($p = 0.002$). The molar concentration of Pd^{2+} in the remaining skin is significantly lower than that of the other two metals ($p < 0.01$). Even though the applied molar concentration of Pd^{2+} is

about half of that of Ni^{2+} and Co^{2+}, the molar concentration of Pd^{2+} in the s.c. is similar. The drop in Pd^{2+} concentration in the remaining skin is thus not due to a lower applied concentration but shows less efficient Pd^{2+} penetration through the skin.

4 | DISCUSSION

Here, we present an investigation of the skin penetration of Ni^{2+}, Co^{2+} and Pd^{2+} from commonly applied PTPs. Using FDC assays, we incubated pigskin with the PTPs for 48 h, mimicking the settings of diagnostic patch testing as recommended by the European Society of Contact Dermatitis guidelines.[5] Pigskin served as an often used alternative to human skin, as it is similar in relevant penetration features.[31] The skin penetration of metal ions from a solution in PBS was analysed to investigate penetration independent from water solubility. Based on the results, we attempt to understand the link between metal ion penetration and epicutaneous patch testing results for metal-mediated contact allergies.

4.1 | Chemical factors influencing metal penetration rates

Ni^{2+} and Co^{2+} from $NiSO_4$ and $CoCl_2$ PTPs penetrated the s.c. and the viable skin much more efficiently than Pd^{2+} from $PdCl_2$ or Na_2PdCl_4 PTPs (Figure 2A). This can be explained by the different metal salt water solubilities, which are reported to be in the molar range for $NiSO_4$ and $CoCl_2$ but measured to be in the mid to low millimolar range for the two palladium salts.[39,40] In PTPs, the metal salts are dispersed in petrolatum, while for skin penetration, it is feasible to assume a dissolution in an aqueous medium such as sweat prior to being taken up into the skin. In FDC assays, the necessary water is provided by the transepidermal water loss (measured at about 10 g/(h·m^2)). Thus, the higher water solubilities of Ni^{2+} and Co^{2+} salts compared to Pd^{2+} salts contribute to higher recoveries of Ni^{2+} and Co^{2+} in the different s.c. layers and the remaining skin.

The water solubility of metal salts was not the only factor leading to a lower skin penetration of Pd^{2+} into the skin. Previous research on the skin penetration of dissolved metal salts indicates that metal ions are retained in the s.c.[14,41–43] The effect, where Pd^{2+} was not detected in tape strips 9–16 of the s.c., but in tape strips 17–20, could be due to the s.c./viable epidermis boundary layer. Here, the diffusion from the s.c. into the viable epidermis is slow. A similar trend was observed for Ni^{2+}, whose concentration in the s.c. also increased when approaching this boundary layer. In the literature, the middle and lower s.c. layers have been shown to provide a barrier to metals, for example chromium.[44] Histidine-rich filaggrin proteins strongly chelate nickel, which is hypothesized to slow down skin penetration. The effective retention of metal ions may therefore be attributed to the barrier effect of filaggrin.[44–46]

Infinite dose conditions,[35] which occur due to the excessive abundance of metal salts in PTPs, could compensate for the lower solubility. We observed that the majority (>90%) of the metal ions remain in the donor compartment and do not penetrate the skin. The metal salt concentration of the PTPs is therefore likely not the determining factor for the efficient delivery of Pd^{2+} into the remaining skin. Hence, the difference between the penetration of Ni^{2+} and Co^{2+} on the one hand and Pd^{2+} on the other hand must at least partially be due to physico-chemical differences in the ions—such as radius or complexation behaviour—and biochemical properties determining their interactions with the skin.

The skin is a permselective membrane preferring cation transport.[47,48] The chloride concentration in the skin is around 79.9 ± 4.8 mM,[49] approximately corresponding to the molar Pd^{2+} concentration in the PTPs. Since Pd^{2+} prefers complexation with chloride, it is plausible to assume that dermally applied Pd^{2+} will form complexes with the general structure of $\left[PdCl_x(H_2O)_{4-x}\right]^{2-x}$ ($x = 2, 3, 4$).[50,51] These chloropalladate complexes are negatively charged or neutral. Ni^{2+} and Co^{2+} on the other hand form complexes with the general structure of $[M(H_2O)_{6-x}]^{2+}$ ($M = Ni^{2+}$, Co^{2+}, $x = 0, 2$).[52] These complexes are always positively charged. We thus hypothesize that the difference in charge of the metal ion complexes contributes to the observed differences in penetration rates through the skin.

This hypothesis is supported by the skin penetration data for metal salts in buffered solutions. While the molar concentrations in the s.c. are similar for all three metal salts ($NiSO_4$, $CoCl_2$ and Na_2PdCl_4), the concentration of Pd^{2+} in the remaining skin is significantly lower than that of Ni^{2+} and Co^{2+}. This difference can be explained with the pH gradient of the skin: the s.c. surface pH is acidic, ranging from 4.1 to 5.8.[53] In deeper cell layers, the pH rises to about 7.[54] A higher pH corresponds to more deprotonated carboxylic acid groups and the membrane becomes more permselective for cations. This effect is intensified by the application medium (PBS), with a pH of about 7. At this point, the negatively charged Pd^{2+} complexes are retained stronger while the positively charged Ni^{2+} and Co^{2+} complexes are able to penetrate more readily through the skin into the receptor fluid, which was held at a physiological pH of 7.4.

4.2 | Potential immunological interactions

Determining the concentration of metal ions in skin layers is crucial because immune system interactions that trigger metal allergies are concentration-dependent. During sensitization, the innate immune system becomes activated, resulting in the maturation of dendritic cells such as epidermal Langerhans cells. The dendrites of Langerhans cells can extend outwards into the lower layers of the s.c.[53] In this study, concentrations of about 450–750 µM of Ni^{2+}, Co^{2+} and Pd^{2+} were determined in the lower layers of the s.c. (strips 17–20). Such concentrations were shown to activate a plethora of pathways in dendritic cells *in vitro*, for example, due to reactive oxygen species formation.[55] *In vitro* studies demonstrated that THP-1 cells were activated by 500 µM Ni^{2+} and Co^{2+}, while human Toll-like receptor 4 (TLR4) activation occurred at 250 µM Ni^{2+} and 750 µM Co^{2+} in HEK293 cells.[56] TLR4 is not expressed on freshly isolated Langerhans cells or keratinocytes,[57] but

TLR4-expressing cells in the dermis may be in contact with similarly high metal ion concentrations in case of skin injury. Our results thus demonstrate that exposure to a Ni^{2+} solution in PBS at concentrations comparable to the regulatory limit may lead to Ni^{2+} concentrations in the skin relevant for dendritic cell activation.

Using the novel activation-induced marker assay, our previous research has shown that T cell activation is highly concentration-dependent, peaking at about 400 μM for all three metal ions.[3] Notably, at this concentration, Pd^{2+} activates a large fraction of the T cell pool. To ensure an accurate allergy diagnosis, PTPs must release metals to yield concentrations in the viable epidermis comparable to those that elicit ACD to re-activate tissue-resident memory T cells. In healthy skin of nickel allergic donors, as little as 7.7 μg/cm^2 Ni^{2+} was sufficient to elicit ACD.[58,59] However, these concentrations may not be the same as those for optimal *in vitro* T cell activation.

In our FDC assays using the $NiSO_4$ PTP, a Ni^{2+} concentration of about 54 μM was found in the remaining skin. A concentration within the same range could therefore be optimal for replicating patch test results *in vitro*. Consequently, this concentration could be utilized for an alternative *in vitro* assay for diagnosing contact allergies. In accordance with our results, Cavani et al. identified approximately 40 μM Ni^{2+} as the most suitable concentration for conducting a nickel allergy diagnosis using a quantitative limited dilution assay.[60] Such a quantitative approach may reveal an increased blood frequency of Ni^{2+}-specific T cells in allergic individuals and thereby enable allergy diagnosis.

Conventional lymphocyte transformation test protocols use lower metal salt concentrations. Higher concentrations may lead to ambiguous results due to an allergy non-related T cell proliferation caused by the presence of Ni^{2+} reactive cells in each individual.[61,62] In FDC assays using $CoCl_2$ PTP, we found considerably less Co^{2+} in the remaining skin compared to Ni^{2+}. Hence, lower concentrations than those used for Ni^{2+} may be necessary in *in vitro* experiments to replicate the results of patch tests.

Of the three metals analysed, Pd^{2+} had the lowest concentration in the remaining skin, regardless of whether PTPs or a metal salt solution were applied. This suggests that even though Pd^{2+} has the ability to activate a large portion of the T cell pool, only a very small fraction of Pd^{2+}-specific T cells become engaged at the low Pd^{2+} concentrations in the skin.[3] This may explain why palladium allergies are less common than nickel allergies, in addition to a generally lower dermal exposure. On the other hand, individuals may become sensitized to higher Pd^{2+} concentrations (for example, in case of skin injury), but $PdCl_2$ PTP may not deliver enough ions to enable diagnoses. This may lead to false negative results when employing $PdCl_2$ in a PTP. Previous research by Muris et al. suggested higher reliability in patch tests conducted with Na_2PdCl_4 compared to $PdCl_2$,[18] proposing the use of the water-soluble Na_2PdCl_4 for patch testing.[16,17]

4.3 | Limitations

Due to the experimental set-up, only 100 μL of the receptor solution was sampled for analysis after 24 h. As a result of this small volume, it is possible that the metal ions in this aliquot were below the LODs of the ICP-MS method and therefore the migrated amount of metal ions could not be determined. This limitation does not apply to the receptor solution after 48 h.

FDC assays were carried out using either a solution of equal mass of Ni^{2+}, Co^{2+} and Pd^{2+} in PBS or diagnostic PTPs. An alternative approach would be the use of equimolar solutions and PTPs. If the penetration of the metal ions is concentration-dependent, an evaluation of higher concentrated solutions could lead to diverging concentrations in the skin. However, we do not believe that the overall trend (Pd^{2+} penetrates the skin less efficiently than Ni^{2+} and Co^{2+}) would change. We base this on the similar trend in penetration after the application of the PBS solution and the PTPs.

The obtained results could further be verified with human skin. However, the literature indicates that pigskin is a good surrogate for human skin, yielding qualitatively comparable results.[33]

An additional limitation is the use of skin from only two pigs. Data from a higher number of specimens could better account for variability between different individuals, even though we found no variance between the two analysed individuals.

5 | CONCLUSION

In conclusion, we elucidate a lower skin penetration capacity of Pd^{2+} compared to Ni^{2+} and Co^{2+}. The approach allows the comparison of metal ion penetration into healthy pigskin from aqueous solutions to that of metal ion penetration from diagnostic PTPs. Our results provide orientation values on local metal ion concentration in different layers of the skin, which are important for an understanding of metal allergies and the patch testing procedure. The less efficient skin penetration of Pd^{2+} could potentially result in lower sensitization rates or underdiagnoses of contact allergies through patch testing on intact skin, which could partially be addressed by employing Na_2PdCl_4-based PTPs. Our findings can guide the further development of *in vitro* testing strategies and the choice of relevant concentrations for cross-reactivity assessment, given that metal ion interactions with the immune system are highly concentration-dependent.

AUTHOR CONTRIBUTIONS

Konstantin Simon: Conceptualization; methodology; formal analysis; investigation; resources; writing – original draft; writing – review and editing; visualization; project administration. **Philipp Reichardt:** Methodology; investigation. **Andreas Luch:** Conceptualization; writing – review and editing; resources; funding acquisition. **Alexander Roloff:** Conceptualization; methodology; writing – review and editing; supervision; project administration; funding acquisition. **Katherina Siewert:** Conceptualization; writing – review and editing; supervision; project administration; funding acquisition. **Franziska Riedel:** Conceptualization; methodology; formal analysis; investigation; resources; writing – original draft; writing – review and editing; visualization; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All data are made available in the tables provided in the Supporting Information.

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