

An in vitro study of the use of chelating agents in cleaning nickel-contaminated human skin: an alternative approach to preventing nickel allergic contact dermatitis

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The purpose of this study was to investigate the efficacy of organic ligands in cleaning human skin contaminated with nickel. 4 ligands were investigated, 5-chloro-7-iodoquinolin-8-ol (either as HL¹ or NaL¹), ethylenediaminetetraacetic acid (either as H₄L² or Na₂H₂L²), sodium diethyldithiocarbamate (NaL³) and L-histidine (HL⁴). The cytotoxicity of these ligands was assessed using HaCaT cells (a transformed human keratinocyte cell line). The cytotoxicity order of the ligands was NaL¹ > Na₂H₂L² > NaL³ > HL⁴. An in vitro methodology for examining nickel removal from viable human skin was developed. This methodology was then used to compare the efficiency of the ligands in removing nickel from skin, both alone and in combination with soap solutions. HL¹ and NaL³ were no more effective than control solutions in removing nickel over the pH range 2–11. In contrast, both H₄L² and HL⁴ removed between 74 and 87% (mean = 82 ± 3%) of nickel from human skin over the same pH range. Nickel removal from skin by sodium lauryl ethoxy sulfate (SLES, the active ingredient in most liquid skin cleansers) was independent of concentration and no more effective than phosphate-buffered saline (PBS). The amount of nickel removed by PBS solutions of Na₂H₂L² and HL⁴ was significantly greater than the amount removed by SLES and was concentration dependent. An evaluation of nickel removal from skin by commercial solid soap, liquid soap and PBS, both alone and with added Na₂H₂L² or HL⁴, was conducted. Commercial liquid soap with added HL⁴ was more effective than the untreated soap. PBS with either added Na₂H₂L² or HL⁴ was more effective than PBS alone.

Key words: ligand; skin; nickel; soap; complex; removal; cytotoxicity. © Munksgaard, 1998.

Accepted for publication 30 May 1998

It has been just over 45 years since Rostenberg & Perkins (1) first discovered that nickel chelation could prevent the symptoms of nickel allergic contact dermatitis. Since then, numerous investigators have demonstrated the effect in vivo using a variety of organic ligands (2). Nearly all of these studies employed patch testing, usually demonstrating that a positive reaction to nickel was avoided by prior application of a ligand-based preparation.

Using an alternative approach, we have investigated the potential of organic ligands in cleaning human skin contaminated with nickel. The ligands investigated (Fig. 1) were 5-chloro-7-iodoquinolin-8-ol (HL¹), ethylenediaminetetraacetic acid (H₄L²), sodium diethyldithiocarbamate (NaL³)

and L-histidine (HL⁴). Sodium salts of HL¹ and H₄L² were also used, depending on the solubility requirements of the experiment. With the exception of HL⁴, these ligands were selected because they have been used to treat nickel dermatitis prophylactically (2). HL⁴ was investigated because histidine motifs are potential nickel ligands for proteins with a function in nickel incorporation into hydrogenases and urease (3, 4). Consequently, HL⁴ has potential as an efficient, non-toxic, nickel chelator.

Materials and Methods

BDH AnalaR[®] grade ethanol (96% v/v), diethyl ether, H₄L², Na₂H₂L² · 2H₂O and Na₂CO₃ were

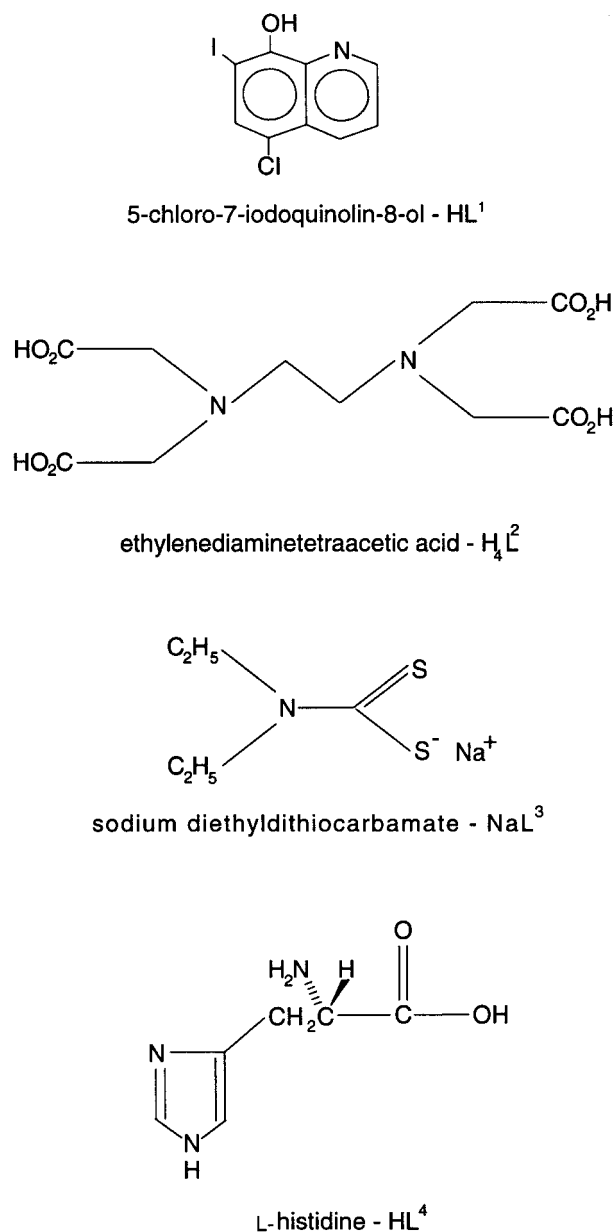


Fig. 1. Structures of the ligands.

obtained from Merck Ltd (Poole, Dorset, UK). $[\text{Ni}(\text{H}_2\text{O})_6]\text{Cl}_2$ (99.9999%), P_4O_{10} , methanol (99.9+%, HPLC grade), $\text{NaL}^3 \cdot 3\text{H}_2\text{O}$ (99+%), HL^4 (98%) and nickel atomic absorption standard solution (1000 $\mu\text{g}/\text{ml}$ nickel in 1% HNO_3) were obtained from Aldrich (Gillingham, Dorset, UK). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Poole, Dorset, UK). Dulbecco's modified Eagle's medium (DMEM), 1000 IU/ml penicillin, 1000 $\mu\text{g}/\text{ml}$ streptomycin and Fungizone were obtained from Gibco BRL, Life Technologies (Paisley, UK). Foetal calf serum (FCS) was obtained from GlobePharm Ltd

(Esher, Surrey, UK). Nitric and sulfuric acid (concentrated, "super pure grade") were obtained from Romil Ltd (Waterbeach, Cambridge, UK). HL^1 was obtained from Zyma Ltd (Alderley Edge, Cheshire, UK). EMPICOL[®] ESB 3 / M, sodium lauryl ethoxy sulfate (SLES) solution was a gift from Dr S Woodman (Albright and Wilson, Whitehaven, Cumbria, UK).

The instrumentation used in this study included a Perkin-Elmer (Beaconsfield, Buckinghamshire, UK) Optima 3000 XL inductively coupled plasma atomic emission spectrometer and a Perkin-Elmer 2400 CHN elemental analyzer. A VG Prospec 3000 fast atom bombardment (FAB) mass spectrometer (Fisons Instruments Ltd, Crawley, Sussex, UK) and a MDS-81D microwave digestion system (CEM, High Wycombe, Buckinghamshire, UK) were also used.

Synthesis of metal-ligand compounds

Nickel complexes were prepared by heating appropriate quantities of $[\text{Ni}(\text{H}_2\text{O})_6]\text{Cl}_2$ and ligand in a suitable solvent. The synthesis of $[\text{Ni}(\text{L}^1)_2]$ required the addition of Na_2CO_3 . Yields of between 61 and 89% were achieved following isolation and purification of the complexes. $\text{NaL}^1 \cdot 0.5 \text{H}_2\text{O}$ was prepared by dissolving molar equivalents of HL^1 and NaOH in an appropriate solvent. After solvent evaporation, filtration and drying a yield of 72% was achieved. All products were characterized by elemental analysis and mass spectrometry.

Octanol-water distribution coefficient ($D_{o/w}$) estimations

$D_{o/w}$ values of the organic ligands were estimated using LogKOW, version 1.35a (Syracuse Research Corporation) for Microsoft Windows, running on an IBM compatible PC.

$D_{o/w}$ values of the nickel complexes were estimated using a modified literature procedure (5).

At least five different concentrations of each nickel complex were prepared in either octanol ($[\text{Ni}(\text{L}^1)_2]$ and $[\text{Ni}(\text{L}^3)_2]$) or the physiological incubation medium (5) ($[\text{Ni}(\text{H}_2\text{L}^2)] \cdot \text{H}_2\text{O}$ and $[\text{Ni}(\text{L}^4)_2] \cdot \text{H}_2\text{O}$). The octanol had been saturated with the physiological incubation medium and the physiological incubation medium had been saturated with octanol prior to use. The nickel complex solution (5.00 ml) was added to the same volume of either octanol (for $[\text{Ni}(\text{H}_2\text{L}^2)] \cdot \text{H}_2\text{O}$ and $[\text{Ni}(\text{L}^4)_2] \cdot \text{H}_2\text{O}$ solutions) or physiological incubation medium (for $[\text{Ni}(\text{L}^1)_2]$ and $[\text{Ni}(\text{L}^3)_2]$ solutions). The mixtures (contained in 25 ml Pyrex bottles with Teflon[®] lined screw caps) were allowed to equilibrate at 37.0°C in a shaking water bath.

Nickel concentrations in both phases were determined using inductively coupled plasma atomic emission spectrometry (ICP AES).

$D_{o/w}$ values of the complexes were obtained by measuring the gradient of a plot of nickel concentration in the octanol phase (y axis) versus nickel concentration in the aqueous phase.

$D_{o/w}$ values of $[\text{Ni}(\text{H}_2\text{L}^2)] \cdot \text{H}_2\text{O}$ and $[\text{Ni}(\text{L}^3)_2]$ were also determined in octanol/non-pH-buffered water systems, substituting water for the physiological incubation medium and using the method described above.

Cell culture

HaCaT cells (between passages 71 and 75) were a gift from Professor N. E. Fusenig and were cultured in DMEM, 10% FCS, 2 mmol/l L-glutamine, 100 IU/ml/100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin and 1.25 $\mu\text{g}/\text{ml}$ Fungizone. The cells were plated out (3×10^4 cells per well) in 96-well culture plates, as previously described (6).

MTT eluted stain assay

The viability of cultured HaCaT cells after 24 h in contact with serial dilutions of nickel complexes and ligands was determined using the MTT eluted stain assay, as previously described (6). Nickel complexes of HL^1 and NaL^3 could not be studied because they were virtually insoluble in water. HL^1 could not be studied for the same reason and $\text{NaL}^1 \cdot 0.5\text{H}_2\text{O}$ was substituted because it showed marginally better aqueous solubility.

Preparation of nickel-contaminated human skin

Skin was obtained from breast reductions and abdominoplasties and used within 24 h of the operation (patient consent for the use of tissue for research purposes is included in the consent form for surgical procedures for the Northern General Hospital NHS Trust). Circular shaped skin pieces were prepared using a 6-mm diameter skin biopsy punch (Stiefel Laboratories, High Wycombe, Buckinghamshire, UK) and placed in 96-well polystyrene culture plates (Costar, High Wycombe, UK) with the epidermal side facing upwards. To ensure a good seal between the dermal side of the skin and the culture plate, gentle pressure was applied to the skin piece using tweezer tips. An aqueous solution of NiCl_2 (5.0 μl , 25.0 mM) was then placed in the centre of the skin piece, ensuring that the solution did not come into contact with the edge of the cut skin. Following a 24-h incubation at 37°C (95% O_2 /5% CO_2), any nickel not bound in the epidermis was removed by 4 washes with

phosphate-buffered saline (PBS, 200 μl). Nickel-contaminated skin prepared in this manner was used immediately in all experiments.

Investigating the seal formed between skin and polystyrene

The integrity of the seal formed between the dermal side of the skin piece and the base of the culture plate well was investigated by estimating the amount of a PBS solution of trypan blue due (100 μl) incorporated into the seal over a 24h period at 37°C.

pH dependence of nickel removal from skin by ligands

Buffer solutions with pH values between 2 and 11 were prepared using published compositions (7). Phthalate buffers were used for pH 2–6, phosphate buffers were used for pH 7–8 and borax buffers were used for pH 9–11. These buffer solutions were used to prepare solutions (6.25 mM) of HL^1 , H_4L^2 , NaL^3 and HL^4 . Suspensions were formed in all HL^1 preparations and the low-pH H_4L^2 preparations. These were shaken immediately prior to use. Nickel-contaminated skin was treated with buffered solutions (100 μl) of the various ligands. Following 24 h incubation at 37°C (95% O_2 /5% CO_2), the buffer solution was removed from the skin and the skin washed 4 $\times$ with water (200 μl). The buffer solution and washings were combined and nickel determined in both the resulting solution and in the skin.

Nickel removal from skin by SLES, $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4

Solutions of SLES, $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 were prepared in PBS and used to remove nickel from contaminated skin. A range of concentrations were investigated. The procedure was the same as that employed in the pH dependence study, with the exception that incubation time was 4 h.

Rate of nickel removal from skin by $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4

Solutions (50.0 mM) of $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 in PBS were used to remove nickel from contaminated skin. The procedure was the same as that employed in the pH dependence study, with the exception that incubation length was varied.

Nickel removal from skin by soaps and PBS containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4

Solutions of commercial soap preparations in water were investigated for their ability to remove nickel from contaminated skin. One liquid and one

solid soap were used and 5% aqueous dilutions of the soap were examined. For each soap, 3 formulations were prepared; 1 containing 15% (m/v) $\text{Na}_2\text{H}_2\text{L}^2$, 1 containing 15% (m/v) HL^4 and 1 not containing any added ligand. In addition, PBS solutions of the ligands and PBS not containing any added ligand were also investigated. The ligand concentrations in the PBS solutions were the same as the ligand concentrations in the aqueous dilutions of the soap/ligand formulations. Again, the procedure was the same as that employed in the pH dependence study, with the exception that the incubation times were 1 h.

Sample digestions and nickel determinations

All glassware was cleaned with detergent solution and rinsed with dilute aqueous acetic acid solution (using a laboratory washing machine). Further cleaning was carried out by leaving the equipment to soak for at least 24 h in 4 M nitric acid and rinsing with water.

Nickel complex concentrations in octanol/aqueous phases were determined by carefully withdrawing an aliquot from each phase, transferring the aliquot to a tall-form 50 ml Pyrex[®] beaker and removing the solvent by evaporation on a hot plate set at 250°C. The resulting solid was treated with concentrated nitric acid (5 ml), evaporated to near dryness, treated again with concentrated nitric acid (5 ml) and evaporated to 1 ml. This was diluted to a total volume of 10.0 ml and the nickel concentration of the resulting solution determined using ICP AES.

Skin pieces were digested using a microwave digestion system (20 min at 50% power) with concentrated nitric acid (1 ml) in 3 ml screw-top Teflon[®] digestion vessels (CEM, High Wycombe). The resulting solutions were diluted with water to 10.0 ml. Buffered ligand solutions were evaporated to dryness in tall-form 50 ml Pyrex[®] beakers

on a hot plate set at 180°C. The resulting solid was treated with concentrated nitric acid (5 ml) and evaporated to 1 ml. This solution was diluted to 10.0 ml.

SLES, soap and liquid soap solutions were evaporated to dryness and treated with concentrated sulfuric acid (2 ml). The solution was evaporated to 0.1 ml on a hot plate set at 280°C. During the evaporation, the hot solution was treated with hydrogen peroxide (less than 0.1 ml, slowly, dropwise). The setting of the hotplate was changed to 180°C and concentrated nitric acid (5 ml) added. After evaporation to 1 ml, the solution was diluted to 10.0 ml. Nickel concentrations were determined using ICP AES.

Statistical analysis

Differences in the data were tested for significance using a paired Student *t*-test.

Results

$D_{o/w}$ estimations

The results of the log $D_{o/w}$ estimations and literature values for other selected physicochemical parameters are shown in Table 1. The plots (nickel concentration in octanol versus nickel concentration in the aqueous phase) for $[\text{Ni}(\text{H}_2\text{L}^2)] \cdot \text{H}_2\text{O}$ and $[\text{Ni}(\text{L}^3)_2]$ log $D_{o/w}$ estimations were non-linear, possibly due to interactions between the complexes and the buffer solutes (12). Consequently, log $D_{o/w}$ estimations for these complexes were repeated in an octanol/non-buffered water system. This system gave improved linearity and these are the results reported. The pH ranges of the aqueous phases of the $[\text{Ni}(\text{H}_2\text{L}^2)] \cdot \text{H}_2\text{O}$ and $[\text{Ni}(\text{L}^3)_2]$ log $D_{o/w}$ estimation experiments were 2.5–3.5 and 4.4–4.7 respectively. This is a potentially unsatisfactory situation since the other estimations were deter-

Table 1. Physicochemical data for nickel complexes and ligands

Ligand	Nickel complex	Stability		Lipophilicity		Ionisation			
		log β_1 of complex (8)	log β_2 of complex (8)	estimated log $D_{o/w}$ of ligand ^a	estimated log $D_{o/w}$ of complex	pK _a of ligand			
						step 1	step 2	step 3	step 4
HL ¹	$[\text{Ni}(\text{L}^1)_2]$		17.8–21.8 ^b	3.47	3.2	3.17 ^c	8.05 ^c		
H ₄ L ²	$[\text{Ni}(\text{H}_2\text{L}^2) \cdot \text{H}_2\text{O}]$	18.52 ^d		−3.86	−3.4		5.8 ^{e,f}	6.47 ^f	10.1 ^f
NaL ³	$[\text{Ni}(\text{L}^3)_2]$		12.1 ^g	0.27	1.3	NF			
HL ⁴	$[\text{Ni}(\text{L}^4)_2] \cdot \text{H}_2\text{O}$		15.94 ^h	−3.22	−3.4	1.82 ⁱ	6.04 ⁱ	9.12 ⁱ	

NF: not found in the literature. ^a estimated using LogKOW, version 1.35a for windows. ^b log β_2 values of nickel complexes with mono halo substituted, di halo substituted and unsubstituted quinolin-8-ol ligands are in the range 17.8–21.8 (2). Therefore, log β_2 of $[\text{Ni}(\text{L}^1)_2]$ is likely to fall within this range. ^c Ref.(9). ^d Determined at 25°C in 0.1 M KNO_3 using a glass electrode. ^e Only the product of the first 2 dissociation constants ($K_{1,2}$) was quoted because it was more reliable than either of the first 2 constants taken separately. ^f Determined at 25°C (10). ^g Determined by voltammetry at 25°C in 1.0 M KCl. ^h Determined at 25°C in 0.2 M Cl^- using a glass electrode. ⁱ Determined at 25°C (11).

mined in systems where the aqueous phase pH was 7.4 and log $D_{o/w}$ can vary significantly with pH (13).

Cytotoxicity of ligands and nickel complexes in HaCaT cells

The concentration dependencies of the cytotoxicity of ligands and nickel complexes towards HaCaT cells over 24 h are shown in Fig. 2. Over the concentration range studied (up to 10 mM), $[\text{Ni}(\text{H}_2\text{L}^2)]$, HL^4 and $[\text{Ni}(\text{L}^4)_2]$ did not display any significant cytotoxicity. The cytotoxicity order of the other compounds studied was $\text{NaL}^1 > \text{NiCl}_2 > \text{Na}_2\text{H}_2\text{L}^2 > \text{NaL}^3$. The IC_{50} values of these compounds were $549 \pm 212 \mu\text{M}$, $1315 \pm 148 \mu\text{M}$, $3400 \pm 169 \mu\text{M}$ and $5147 \pm 487 \mu\text{M}$, respectively, based on results from 5 different experiments.

Nickel absorption by skin

In preparing nickel-contaminated skin, the procedure was the same in all experiments and the amount of nickel spiked onto skin pieces was $7.33 \mu\text{g}$. The amount of nickel absorbed by the skin after 24 h incubation at 37°C followed by washing with PBS was determined using the results of the nickel determinations in the skin and ligand/soap solution digests. A frequency histogram of the results obtained from over 600 determinations is shown in Fig. 3. The mean, median and mode of the data are $5.38 \mu\text{g}$, $6.10 \mu\text{g}$ and $6.20 \mu\text{g}$, respectively. Thus, the mean absorption of nickel by the skin over 24 h in this model was 73%. However, the wide and apparently bimodal variation in nickel uptake in normal adult skin, studied under identical experimental conditions, was wider than expected.

Seal formed between skin and polystyrene

Experiments to investigate the integrity of the seal between the dermal side of skin pieces and the well bottoms, using trypan blue dye solution, revealed that in all cases the seal was intact for at least 1 h. After a 24-h time period, dye had been incorporated into approximately 50% of the seals. The extent of the incorporation (based on visual surface area estimations) was 10–90%.

Nickel removal from skin by ligands as a function of pH

The influence of pH on the removal of nickel from skin by ligands is shown in Fig. 4. The results clearly show that the amount of nickel removed from skin by buffered aqueous solutions which do

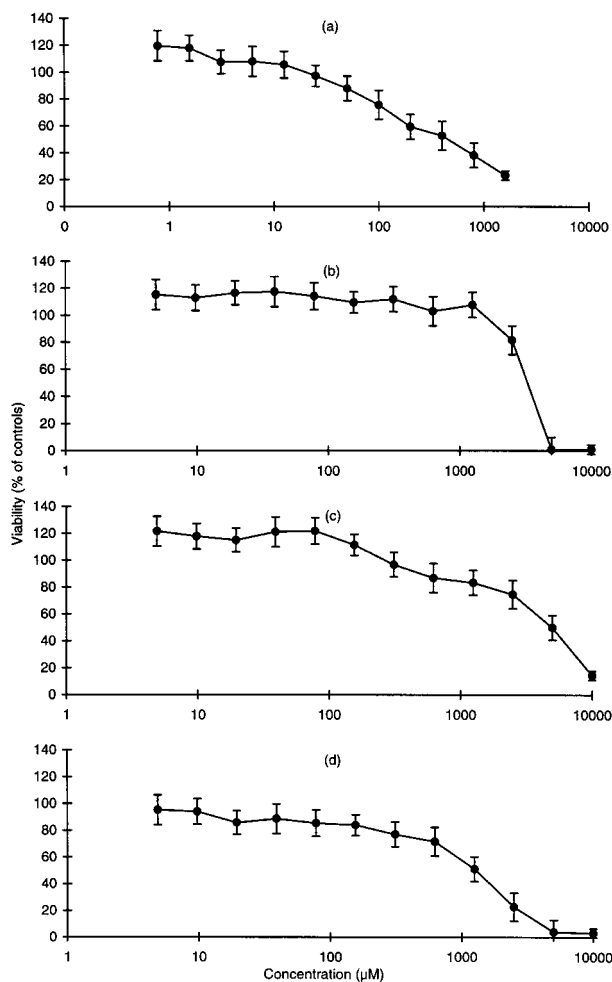


Fig. 2. Concentration dependence of cytotoxicity in HaCaT cells. (a) = NaL^1 , (b) = $\text{Na}_2\text{H}_2\text{L}^2$, (c) = NaL^3 and (d) = $[\text{Ni}(\text{H}_2\text{O})_6]\text{Cl}_2$. HaCaT cells (between passages 71 and 75) were cultured in DMEM and treated with serial dilutions of ligands and nickel complexes in DMEM for 24 h at 37°C . $[\text{Ni}(\text{H}_2\text{L}^2)]$, HL^4 and $[\text{Ni}(\text{L}^4)_2]$ were also studied and displayed no significant cytotoxicity up to 10 mM. Values are expressed as means $\pm$ SEM of 5 experiments. 5 replicates of each dilution were performed in each experiment.

not contain any added ligand decreases with increasing pH. At pH 2, $88 \pm 4\%$ is removed, while at pH 11, only $38 \pm 8\%$ is removed. In addition, it is apparent that both HL^1 and NaL^3 are no more effective than the control solutions in removing nickel from the skin. By contrast, both H_4L^2 and HL^4 remove between 74 and 87% (mean = $82 \pm 3\%$) between pH 2–11.

Nickel removal from skin by ligands as a function of ligand concentration

The results of this study (Fig. 5) show that nickel removal from the skin over 4 h by SLES is independent of SLES concentration and constant at

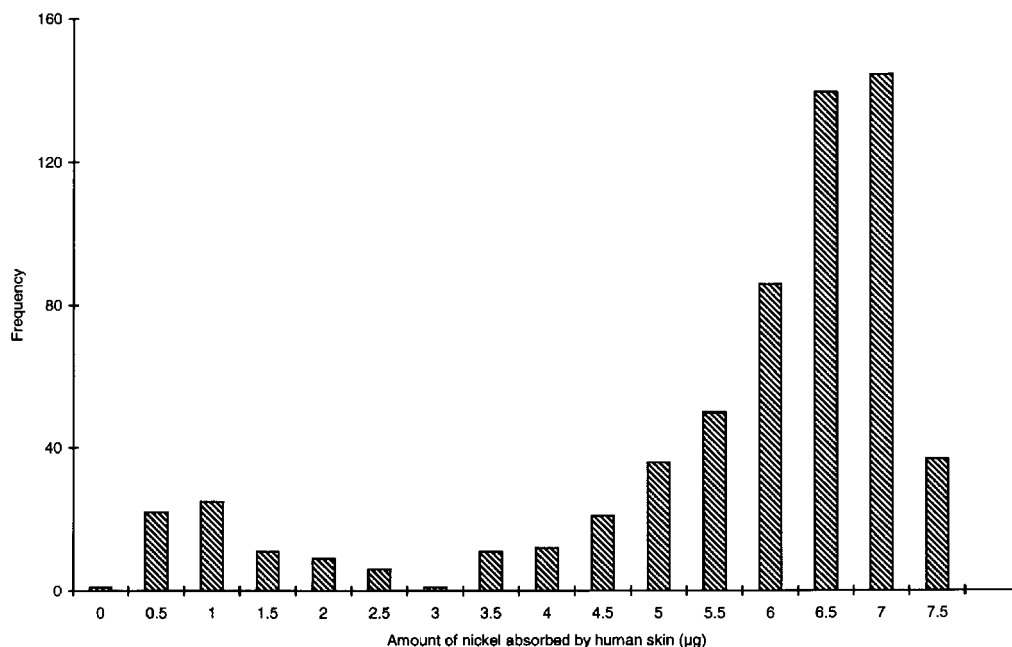


Fig. 3. Frequency distribution of the amount of nickel absorbed by human skin. Epidermal nickel dose = $7.33 \mu\text{g}$, incubation = 24 h at 37°C . After incubation, unbound nickel was removed by washing with PBS. Following nickel removal experiments, the amount of nickel originally bound by the skin was calculated from the sum of the amount of nickel remaining in the skin and the amount of nickel removed by the solution under study. Nickel concentrations were determined using ICP AES. The results of over 600 separate determinations, using skin from 20 donors, are shown.

around 35% across the concentration range studied. There was no significant difference between the amount of nickel removed by aqueous SLES and PBS. In contrast, the amount of nickel removed by $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 was concentration dependent. The maximum amount of nickel removed by solutions (81.0 mM) of $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 was $79 \pm 2\%$ and $70 \pm 4\%$ respectively.

Rate of nickel removal from skin by ligands

The relative rates of nickel removal from skin by solutions (50.0 mM) of $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 are shown in Fig. 6. Based on 5 experiments and assuming a linear relationship over the 1 h studied, the average rates of nickel removal for $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 were $0.82\%/ \text{min}$ and $0.59\%/ \text{min}$, respectively. However, it appears that the greatest rate of nickel removal occurred within the first 10 min, with a slower rate of removal occurring between 10 and 60 min.

Nickel removal from skin by soaps and PBS containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4

The amount of nickel removed from skin over 1 h by soap formulations containing 15% (m/v) $\text{Na}_2\text{H}_2\text{L}^2$, 15% (m/v) HL^4 and no added ligand are shown in Fig. 7. Liquid soap and solid soap with

no added ligand removed $25 \pm 5\%$ and $17 \pm 2\%$ nickel, respectively. For liquid soap, the amount of nickel removed by formulations containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 was $41 \pm 9\%$ and $48 \pm 6\%$, respectively. For solid soap, the amount of nickel removed by formulations containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 was $21 \pm 3\%$ and $28 \pm 7\%$, respectively. For PBS, the amount of nickel removed by formulations containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 was $44 \pm 7\%$ and $41 \pm 7\%$, respectively. PBS alone removed $15 \pm 2\%$.

Considering these results in combination with the results of other nickel removal experiments, it is apparent that both liquid soap and PBS supplemented with either $\text{Na}_2\text{H}_2\text{L}^2$ or HL^4 are superior to preparations with no added ligand in their ability to clean human skin contaminated with nickel.

Discussion

The purpose of this study was to develop a practical approach to dealing with nickel contamination of skin (14, 15), which might be developed for use in situations of unavoidable nickel exposure (16). Such situations often result from occupational exposure, which can lead to the development of a dermatitis and the possibility of time off work or inability to work at all.

The cytotoxicity results (Fig. 2) reflect literature

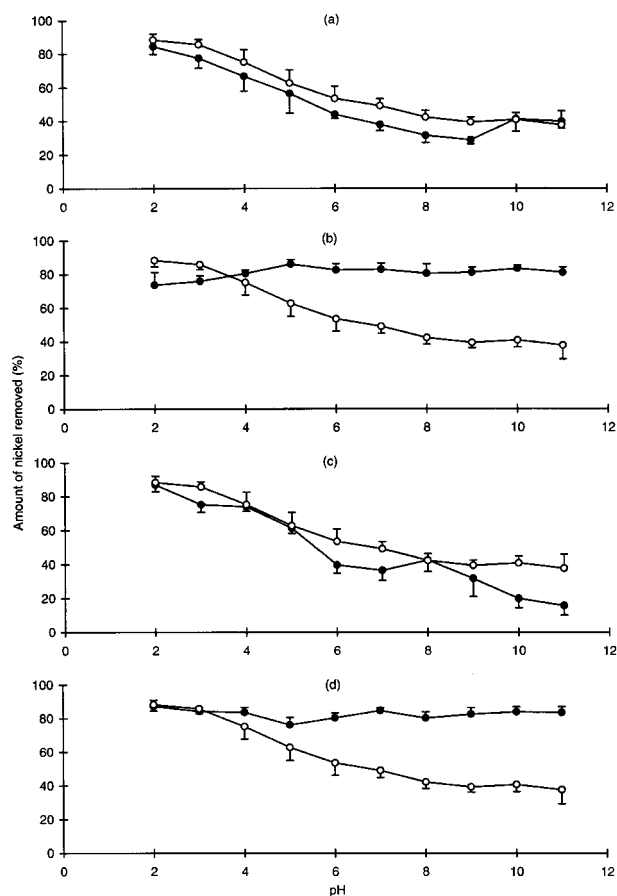


Fig. 4. pH dependence of nickel removal from human skin. (a)= HL^1 , (b)= H_4L^2 , (c)= NaL^3 , (d)= HL^4 and $\circ$ =control. Incubation=24 h at $37^\circ C$. 100 μl of a 6.25 mM ligand solution was used at each pH value, which gave a ligand:nickel ratio of 5:1. Values are expressed as means $\pm$ SEM of 5 experiments. Nickel removal by ligand/control solutions at each pH value studied was not replicated in individual experiments.

toxicology reports on the compounds studied. Toxicity of HL^1 has been found in dogs treated daily with 5 g of a 3% preparation for a month (17) and there may be a risk of toxicity to infants and children from its topical use (18). In addition, HL^1 is absorbed through the skin (19, 20) and may be a neurotoxin (21). In a study of the cytotoxic effects of chromium and nickel (6) in normal and transformed human keratinocytes, the relative cytotoxicity order was $Cr(VI) \gg Ni(II) > Cr(III)$. The IC_{50} value of $NiCl_2$, after 24 h incubation with normal human keratinocytes ranged from 1800–>5000 μM . The variation was attributed to donor variation. The IC_{50} value of $NiCl_2$, after 24 h incubation with HaCaT cells was around 1000 μM . HaCaT cells were more sensitive than normal human keratinocytes to the cytotoxic effects of the metal compounds studies. It has been demonstrated that H_4L^2 displays a low mutagenic and chromosome-breaking activity in mammalian cell

cultures at elevated concentrations and/or prolonged incubation (22). From these results, it was concluded that H_4L^2 was unlikely to be a harmful drug with respect to genotoxicity, although the mechanism by which it produces genotoxic effects is poorly understood (22). The use of NaL^3 and the related compound tetraethylthiuram disulphide by mouth for treating nickel dermatitis (2) is restricted by their limited efficacy and side-effects. The side-effects include hepatotoxicity (23), lassitude (24) and the risk of $[Ni(L^3)_2]$ accumulation in brain cells (25).

The variation in nickel uptake by human skin (Fig. 3) is not directly relevant to the purpose of this investigation, but is interesting nevertheless. The variation is possibly due to the non-homogeneous nature of human skin and the path taken by nickel in percutaneous absorption. There are 3 possible absorption pathways across the stratum corneum (26): transcellular, intercellular and appendageal. Nickel penetrates the stratum corneum via the intercellular lipids (27) and the appendages (28) and maybe found preferentially by carboxyl groups in the stratum corneum (29). Thus, differing appendageal surface density in the skin pieces studied and different nickel take-up rates by the 2 routes may be responsible for the variation in nickel binding. Alternatively, the variation may be due to differences in the chemical (e.g., lipid) composition of the skin.

The trypan blue due penetration tests indicated that the initially sound seal formed between the dermal side of the skin pieces and the plastic of the 96-well plate was compromised after 24-h incubation at $37^\circ C$ in around 50% of cases. However, this should not present a problem in this work. Previous work (14) has demonstrated that the distribution of nickel in human skin following exposure to 5% $NiCl_2$ for 96 h is highest on the skin surface. In addition, in this latter study, most of the nickel was bound in the stratum corneum and the ratio of epidermal nickel/dermal nickel was around 40. Therefore, the amount of nickel removed by ligand solutions in contact with the dermal side of the skin pieces was negligible.

We suggest that for a ligand to be effective in cleaning nickel contaminated skin safely it should satisfy a number of criteria. Firstly, the nickel-ligand complex should be significantly more stable than the nickel-“skin” complex (i.e., nickel chelated in both the appendages and the intercellular lipids). Secondly, both the ligand and its nickel complex should not be absorbed by the skin. Finally, the ligand should be in the correct ionization state for nickel complexation. If these conditions are met, then the ligand will compete more effectively for nickel than the skin components respon-

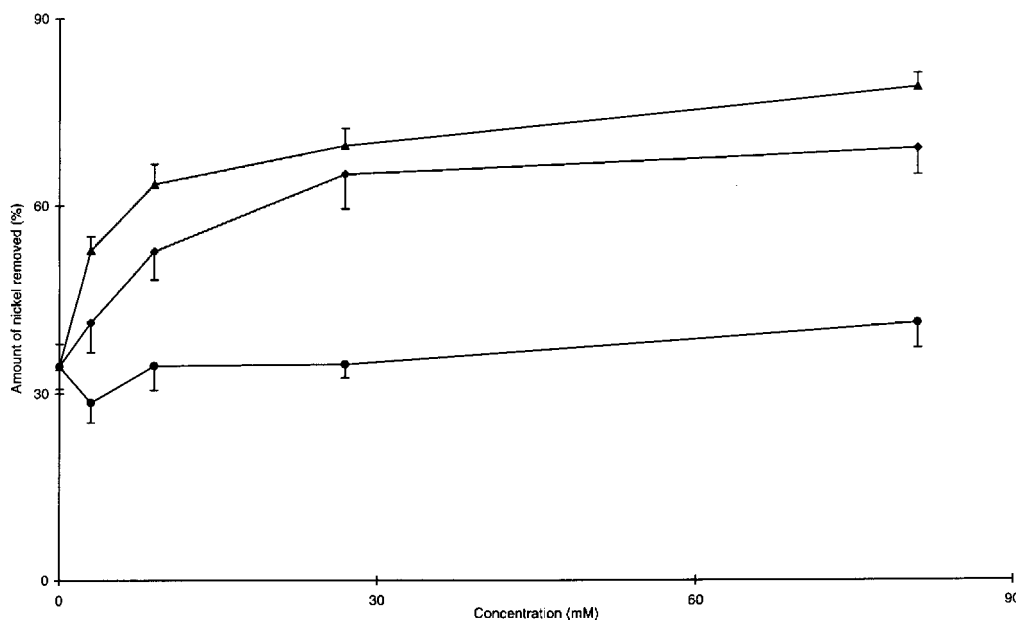


Fig. 5. Comparison of the effect of ligand and SLES concentration on nickel removal from human skin. $\blacktriangle$ =Na₂H₂L₂, $\blacklozenge$ =HL⁴ and $\bullet$ =SLES. The volume of ligand or SLES solution was 100 μ l and the amount of nickel removed was determined after 4 h at 37°C. 3 replicates of each dilution were performed in each experiment and values are expressed as means $\pm$ SEM of 5 experiments. A paired *t*-test was used to determine significant differences between nickel removal by Na₂H₂L₂ and HL⁴. Significant differences were found at 3 and 9 mM ($p<0.001$) and 81 mM ($p<0.01$). The difference was not significant at 27 mM.

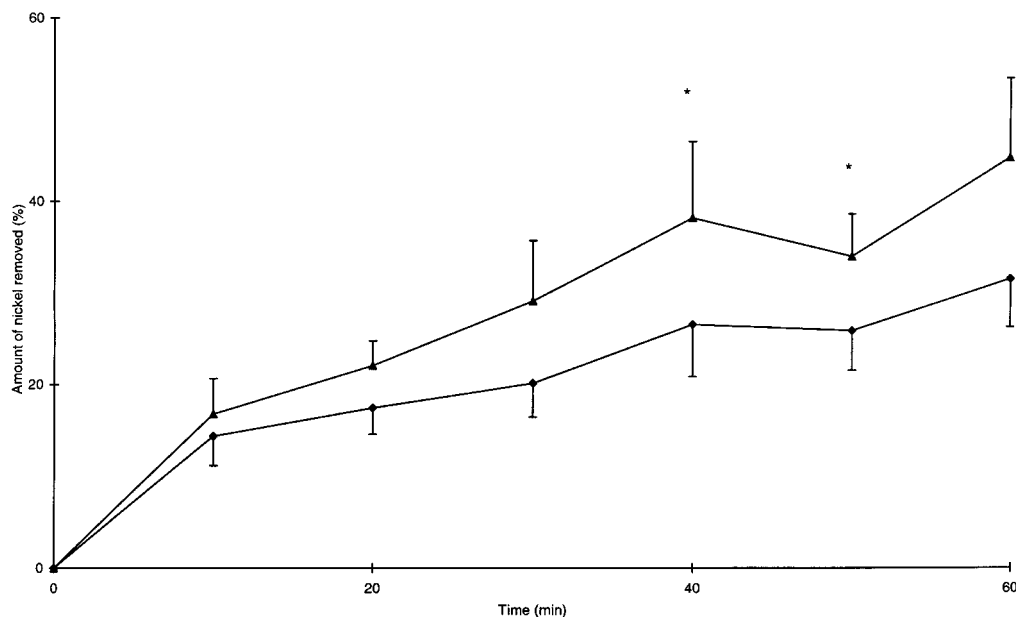


Fig. 6. Rate of nickel removal from human skin. $\blacktriangle$ =Na₂H₂L₂ and $\blacklozenge$ =HL⁴. The volume of ligand solution was 100 μ l and the amount of nickel removed was determined at 37°C. The ligand:nickel ratio was 40:1 and values are expressed as means $\pm$ SEM of 5 experiments. 2 replicates of individual time points were performed in each experiment. Significant differences, determined by a paired *t*-test, are indicated by * ($p<0.05$).

sible for nickel complexation and the nickel-ligand complex will not be reabsorbed. The criteria of stability, percutaneous absorption and ionisation are quantified by constants of complex stability (β_n), octanol-water distribution ($D_{o/w}$) and acid dis-

sociation (K_a), respectively. These are summarized in Table 1 for the ligands we have investigated.

The influence of pH on nickel removal from skin by ligands (Fig. 4) can mostly be explained by considering the physicochemical parameters listed in

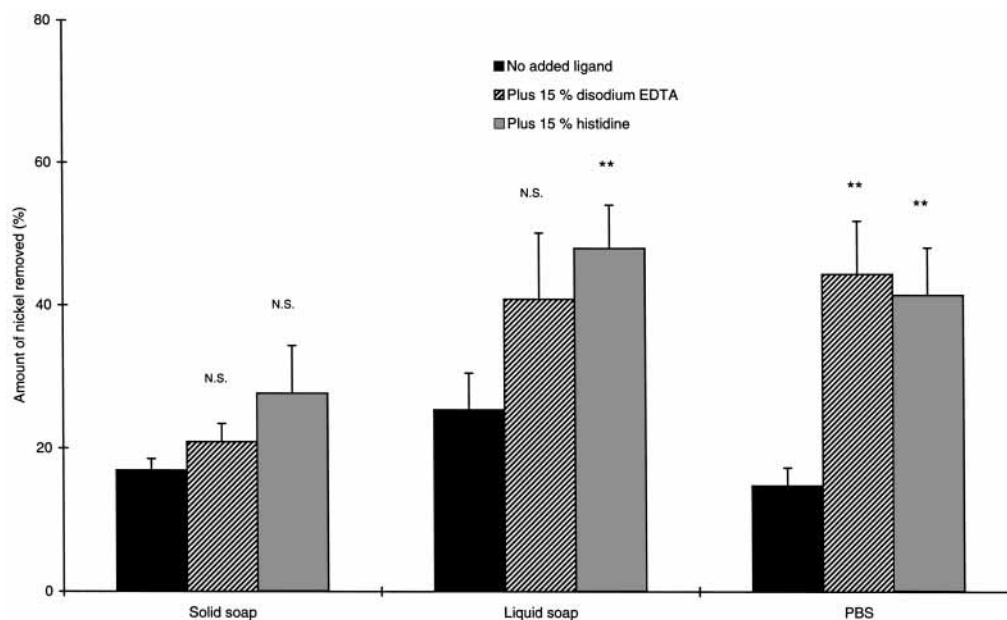


Fig. 7. Nickel removal from human skin by preparations containing $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 . 100 μl of each preparation was used and the amount of nickel removed was determined after 1 h at 37°C. Values are expressed at means $\pm$ SEM of 5 experiments. 2 replicates of individual preparations were performed in each experiment. Significant differences, determined by a paired *t*-test, are indicated by ** ($p < 0.01$). NS: no significance.

Table 1. HL^1 is no more effective than control solutions in removing nickel at all pH values. To a large extent, this is probably due to its aqueous insolubility. However, at pH values greater than around 8, where HL^1 displayed a degree of solubility, the hydroxyl group will be deprotonated and nickel complexation can occur. At pH 8.05, 50% of HL^1 exists as $[\text{L}^1]$, at pH 9.05 about 90% exists as the monoanion and at pH 10.05 about 99% exists as the monoanion. The site of nickel complexation will either be at the stratum corneum-buffer solution interface or within the stratum corneum. Nickel complexed at the stratum corneum-buffer solution interface is likely to be reabsorbed because the resulting complex is lipophilic. Nickel complexation within the stratum corneum is possible because HL^1 is percutaneously absorbed (19, 20), although its lipophilicity decreases sharply at pH values greater than 5.6 (9). Thus, if nickel complexation occurs, then, regardless of the site, the amount of nickel removed from the skin will be no greater than that removed by the control solutions. NaL^3 is soluble across the pH range studied and is also no more effective than control solutions in removing nickel at all pH values. Because NaL^3 and $[\text{Ni}(\text{L}^3)_2]$ are both relatively lipophilic, similar arguments can be used to explain the ineffectiveness of NaL^3 in removing nickel from the skin. Both H_4L^2 and HL^4 remove an average of $82 \pm 3\%$ nickel between pH 2–11. These ligands and their nickel complexes are hydrophilic and the complex-

es have relatively large β_n values (Table 1). Consequently, the ligands satisfy 2 of the 3 criteria for cleaning nickel contaminated skin effectively. The pK_a data listed in Table 1 are more difficult to reconcile with the observed results for H_4L^2 and HL^4 .

The results for H_4L^2 and HL^4 partially agree with an earlier study, where it was found that HL^4 was equivalent to $\text{Na}_2\text{H}_2\text{L}^2$ in removing nickel from human epidermis (15) at an initial nickel concentration of 2 $\mu\text{g}/\text{ml}$. At lower nickel concentrations, HL^4 removed significantly less than $\text{Na}_2\text{H}_2\text{L}^2$. However, a comparison of the 2 studies is not fully justified because the authors used powdered epidermis which had been prepared by ball milling human epidermis at -196°C (15). In addition, powdered epidermis was incubated in a medium containing both the ligand and nickel. In this study, ligand solutions were added after a 24-h incubation with nickel and intact viable skin was used.

For aqueous buffer solutions not containing added ligand, it was apparent that the amount of nickel removed from human skin decreased with increasing pH. Carboxylic acid functionality within the stratum corneum has been suggested (29) as a possible mode of nickel chelation and the observed profile is consistent with this hypothesis. The carboxylic acid anion has a greater affinity for protons compared with nickel and so, at low pH, carboxylic-acid-bound nickel is displaced by protons.

The dependence of concentration on the extent of nickel removal by SLES, $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 (Fig. 5) clearly demonstrated that SLES does not play an active role in nickel removal. In contrast, the amount of nickel removed by $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 is concentration dependent. This demonstrates that these ligands do play an active role in nickel removal and is consistent with the ligands successfully competing for nickel chelated within the stratum corneum. Furthermore, in 3 out of 4 ligand concentrations investigated, there were significant differences between the nickel removal data. It was apparent that PBS solutions of $\text{Na}_2\text{H}_2\text{L}^2$ were more effective than similar solutions of HL^4 . However, when combined with liquid soap, HL^4 is more effective than $\text{Na}_2\text{H}_2\text{L}^2$ (Fig. 7). Also, the difference between the amount of nickel removed by the liquid soap/ $\text{Na}_2\text{H}_2\text{L}^2$ mixture and liquid soap was not significant ($p > 0.05$). These apparent anomalies can be explained by considering the probable composition of commercial liquid soap and the chemical properties of $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 . Commercial liquid soap probably contains metal ions (eg calcium and magnesium) which will compete with nickel for $\text{Na}_2\text{H}_2\text{L}^2$ and reduce its effectiveness. HL^4 , on the other hand, forms much less stable complexes with group 2 metal ions and is therefore more effective in their presence.

The data shown in Fig. 6 reveal that the average rates of nickel removal for $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 were 0.82 %/min and 0.59 %/min, respectively. Although the amount of nickel removed by $\text{Na}_2\text{H}_2\text{L}^2$ was slightly greater than that removed by HL^4 , the difference was only significant ($p < 0.05$) at $t = 40$ and $t = 50$ min. The relatively large variation in the nickel removal data may have been due to the amount of time taken to separate the ligand solution from the skin. This was relatively large compared to the times at which nickel removal was determined.

Our results indicate that $\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 can be used safely in vivo to clean human skin contaminated with nickel. When mixed with commercial liquid soap, HL^4 will be more efficient than $\text{Na}_2\text{H}_2\text{L}^2$, possibly because of the formation of group 2 metal complexes with $\text{Na}_2\text{H}_2\text{L}^2$. Our study also supports the view that nickel complex stability and nickel complex/ligand lipophilicity are the most important factors determining the effectiveness of a ligand in cleaning human skin contaminated with nickel. We tentatively suggest that ligand ionization state is a less important determinant.

$\text{Na}_2\text{H}_2\text{L}^2$ and HL^4 might best be used to prevent the symptoms of nickel dermatitis in individuals who are occupationally exposed to nickel and who cannot prevent their exposure (e.g., hairdressers,

jewellers, car mechanics, etc). 3 possible modes of use are envisaged. At work, a liquid skin cleanser comprising SLES and either of the 2 ligands could be used at the end of each shift to clean the hands in the normal manner. This method offers the advantage that it does not involve changing the work culture of the employee. In addition, if used by employees who are not sensitized to nickel, it has the potential to reduce the risk of becoming sensitized. At home, a ligand-containing hand cream could be applied or a bath additive containing SLES and a ligand could be used. The efficacy of these preparations and methods of use remain to be investigated in vivo and in workplace situations.

Acknowledgements

We gratefully acknowledge the Royal Society (London, UK) for providing one of us (JH) with an Industry Fellowship. We thank Professor N. E. Fusenig (Institute of Biochemistry, German Cancer Research Centre, Heidelberg) for the gift of HaCaT cells. We thank Dr S. Woodman (Albright and Wilson, Whitehaven, Cumbria) for the gift of sodium lauryl ethoxy sulfate. We are grateful to the plastic surgeons of the Northern General Hospital NHS Trust for provision of skin, especially Mr D. R. Ralston. We also thank Mr D. Towers (Sheffield University, UK) for FAB MS analyses and Mr A. Jones (Sheffield University, UK) for elemental analyses.

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