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# Synergistic enhancement of skin permeability by *N*-lauroylsarcosine and ethanol

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**Abstract**

To develop formulations for transdermal drug delivery, this study tested the hypothesis that the anionic surfactant, *N*-lauroylsarcosine (NLS), and ethanol synergistically increase skin permeability by increasing the fluidity of *stratum corneum* lipid structure. Skin permeability experiments showed that transdermal delivery of fluorescein across human cadaver epidermis was increased by up to 47-fold using formulations containing NLS in aqueous ethanol solutions. Skin permeability was increased by increasing NLS concentration in combination with 25–50% ethanol solutions. Skin permeability was shown to correlate with skin electrical conductivity measurements, changes in differential scanning calorimetry lipid transition peak temperature, and Fourier transform infrared spectroscopy C–H stretching peak shifts indicative of *stratum corneum* lipid fluidization and changes in protein conformation. Evidence for lipid extraction was also evident, but did not appear to be responsible for the observed increases in skin permeability. We conclude that NLS in aqueous ethanol formulations can dramatically increase skin permeability by a mechanism involving synergistic lipid-fluidization activity in the *stratum corneum*.


*Keywords:* Differential scanning calorimetry; Fourier transform infrared spectroscopy; *N*-Lauroylsarcosine; Ethanol; Skin penetration enhancer; Skin resistance

## 1. Introduction

While most drugs are administered orally, there are numerous advantages to the transdermal route. These advantages include the potential for sustained release, controlled input kinetics, improved patient compliance, and avoidance of first-pass metabolism in the gastrointestinal tract. However, human skin is a very effective barrier and severely limits the transdermal delivery of drugs (Prausnitz et al., 2004). Corneocytes embedded in a lipid bilayer matrix comprise the unique hierarchical structure of the *stratum corneum* that provides the skin's barrier properties. Only a few drug molecules of optimal physicochemical properties can penetrate the skin sufficiently to be therapeutically efficient. The lipid barrier in the human *stratum corneum* principally consists of ceramides, fatty acids, cholesterol, and cholesteryl sulfate that are assembled into multi-lamellar bilayers (Elias, 1991).

In order to improve transdermal drug delivery, various penetration enhancers which disrupt the aforementioned structural hierarchy to decrease the barrier resistance of the *stratum corneum* have been investigated previously. Many compounds have been used as penetration enhancers, including Azone derivatives, fatty acids, fatty esters, sulphoxides, alcohols, pyrrolidones, glycols, surfactants, and terpenes (Williams and Barry, 2004). Among the various chemical enhancers, surfactants have been widely investigated as transdermal permeation enhancers, and also have a long history of use as emulsifiers, stabilizers, and suspending agents in many topical pharmaceutical formulations (Malmsten, 2002).

Many surfactants can interact with the *stratum corneum* to increase the penetration of drugs applied to skin (Zatz and Lee, 1997). Anionic surfactants have been shown to disrupt the intercellular lipid lamellae and to cause selective loss of intercellular lipids (Imokawa et al., 1989). In a mechanistic study, liposomes comprised of *stratum corneum* lipids were treated with an anionic surfactant, sodium lauryl sulfate (SLS), which was shown to increase the packing disorder of the lipid bilayer by partitioning of SLS into the bilayer (Friberg et al., 1988; Downing et al., 1993). In another study, insertion of SLS into a

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model of *stratum corneum* lipids resulted in fluidization of the intercellular lipid domain (Wilhelm et al., 1991).

Among the various anionic surfactants, *N*-lauroylsarcosine (NLS) has been used as a permeation enhancer and has shown a synergistic improvement of transdermal flux when used in combination with other enhancers, such as squalene and vitamin E (Aioi et al., 1993). When NLS salt was mixed with sorbitan monolaurate, the formulation exhibited not only synergistic transdermal flux enhancement, but also reduction of skin irritation (Karande et al., 2004).

Synergistic effects between chemical enhancers and various solvents have also been reported. A combination of surfactant and 50% propylene glycol showed strong synergistic effects on skin permeability (Nokhodchi et al., 2003). Polar solvents, such as ethanol, have been reported not only to have direct effects on skin permeability, but also to facilitate the solubilization of enhancers within the *stratum corneum*, thus further amplifying the lipid-modulating effect (Naik et al., 2000). Ethanol is a known enhancer for the transdermal delivery of lipophilic drugs and is currently used in commercialized transdermal systems for estradiol and fentanyl (Berner and Liu, 1995).

Given the observations that formulations combining lipid-disrupting penetration enhancers and appropriate solvents can be especially effective to increase skin permeability, we sought to study the mechanism by which this enhancement occurs using mixtures of NLS and ethanol as a model system. We hypothesize that NLS and ethanol synergistically increase skin permeability by increasing the fluidity of *stratum corneum* lipid structure. This hypothesis was tested by measuring the effect of NLS and ethanol concentrations on skin permeability to the model fluorescent probe fluorescein.

The mechanism of action was further investigated using skin resistance measurement, differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). Measurement of skin resistance can be used as a 'generic' measure of skin permeability that does not depend on the specific characteristics of target molecules such as hydrophobicity and charge. DSC and FTIR measurements allow us to probe the intercellular lipid domain of the *stratum corneum* to provide information especially on lipid fluidization. Using these tools, this is the first study to investigate the mechanism of skin permeability enhancement by NLS and the synergistic effect between an anionic surfactant and ethanol.

## 2. Materials and methods

### 2.1. Skin preparation

Human cadaver skin was obtained from the National Disease Research Interchange (Philadelphia, PA, USA) or Emory University School of Medicine (Atlanta, GA, USA) and stored at −75 °C until use for permeation studies with approval from the Georgia Tech Institutional Review Board. Immediately prior to a diffusion experiment, whole skin was thawed in deionized water at 30 °C for 1 h. Epidermis was isolated from dermis using a heat separation method (Scheuplein, 1965), in which the skin was immersed in deionized water for 2 min at 60 °C. The epidermis was then carefully peeled away from the dermis with a spatula and used for the diffusion study or other experiments.

For experiments on isolated *stratum corneum*, a *stratum corneum* sheet was isolated from human epidermis by trypsin digestion. The epidermis was incubated in phosphate-buffered saline (PBS, Sigma–Aldrich, St. Louis, MO, USA) containing 0.25% trypsin (Mediatech, Herndon, VA, USA) and 0.01% gentamicin (Clonetics, Walkersville, MD, USA) at 32 °C for 24 h. The isolated *stratum corneum* then was rinsed with distilled water three times and stored on polymer-coated paper (Fisher Scientific, Waltham, MA, USA) under vacuum (KNF Neuberger, Trenton, NJ, USA) overnight at room temperature, 23–24 °C (Kuriharabergstrom et al., 1990).

### 2.2. Transdermal flux measurements

Transdermal flux experiments consisted of three steps. The first step was the pretreatment of skin with a chemical enhancer formulation in a vertical Franz diffusion glass cell apparatus (PermeGear, Bethlehem, PA, USA). This Franz cell was used to hold epidermis samples (0.7 $cm^2$ exposed skin surface area) between the donor (upper) and receiver (lower) chambers during an *in vitro* permeation experiment. The *stratum corneum* side was exposed to 0.3 ml of the enhancer formulations in the donor chamber. Solutions of varying *N*-lauroylsarcosine (98%, Fluka, Buchs, Switzerland) concentrations in solvents of various ethanol (Sigma–Aldrich) concentrations was placed in the donor chamber. PBS was placed in the receiver chamber. In some cases, the sodium salt of *N*-lauroylsarcosine (Sigma–Aldrich) was used, when indicated. The Franz cell was kept in the refrigerator at 4 °C for 12 h for pretreatment. This pretreatment was carried out at 4 °C to minimize skin degradation, although preliminary skin permeability experiments carried out at 25 °C showed similar behavior (data not shown). The concentration of NLS solution used in the penetration experiment was 2% (w/v) and the concentration of ethanol was 50% (v/v) in water, unless otherwise mentioned.

The second step was an equilibration step. The Franz cell was placed in a heater/stirrer block (PermeGear) maintained at 32 °C and stirred at 455 rpm for 3 h.

For the final step, the chemical enhancer formulation was removed from the donor chamber and 0.3 ml of 1 mM fluorescein (Sigma–Aldrich) in PBS buffer solution was placed in the donor chamber. The amount of fluorescein in the receiver chamber (4.5–4.7 ml) was measured by calibrated fluorescence spectroscopy (Photon Technology International, Birmingham, NJ, USA) every hour for 5 h to determine the transdermal fluorescein flux. At each sample time, all of the solution in the receiver chamber was removed and replaced with fresh PBS.

### 2.3. Skin resistance measurements

The electrical resistance across the epidermis was measured by a Keithley 3322 LCZ meter (Keithley Instruments, Cleveland, OH, USA). A Ag/AgCl disk electrode (In Vivo Metric, Healdsburg, CA, USA) was inserted into the receiver chamber through the sampling outlet of a vertical Franz diffusion cell apparatus

and another Ag/AgCl electrode was placed in the donor chamber. In these experiments, there was no pretreatment of the skin. Electrical measurements were initiated at the same time that the chemical enhancer formulation was applied to the skin, which was maintained at 32 °C. Electrical conductivity ($\sigma$) was calculated from electrical resistance measurements ($R$) as $\sigma = L/RA$, where $L$ is *stratum corneum* thickness (20 μm) and $A$ is skin cross-sectional area (0.7 $cm^2$).

### 2.4. Differential scanning calorimetry

DSC was used to characterize the thermal transitions in *stratum corneum* samples that were either untreated or treated with a chemical enhancer formulation. Thermal analysis employed a DSCQ100 differential scanning calorimeter fitted with a refrigerated cooling system (TA Instruments, New Castle, DE, USA). The *stratum corneum* samples were desiccated under vacuum for 12 h. Desiccation has been shown to sharpen DSC thermal transitions without altering the location of *stratum corneum* lipid peaks (Goodman and Barry, 1986). *Stratum corneum* samples were then each soaked in various chemical enhancer formulations for 24 h at 4 °C and then washed with PBS solution. Samples were then hermetically sealed within an aluminum holder (Perkin-Elmer, Wellesley, MA, USA) and heated from 0 to 120 °C at a heating rate of 10 °C/min.

### 2.5. Fourier transform infrared spectroscopy

Prior to spectral analysis by FTIR, *stratum corneum* samples were treated as described for DSC samples, but were not desiccated and were instead left in a fully hydrated state. Using a Magma-IR 560 FTIR spectrometer (Nicolet, Thermo Electron, Waltham, MA, USA), all spectra (4 $cm^{-1}$ resolution, representing the average of 64 scans) were obtained in the frequency range 4000–1000 $cm^{-1}$. OMNIC professional software (Thermo Electron) was used to determine the peak position and area under each peak. Although the FTIR had a data collection resolution of 4 $cm^{-1}$, interpolation between points is reliable because the noise level is so low and the reproducibility of FTIR spectra is so high. This permits one to determine the location of a peak, even if it exists between data points that were actually collected. This is well established in the spectroscopy literature (Cameron et al., 1982) and is consistent with many previous studies involving FTIR analysis of skin, where peak shifts much smaller than the data collection spacing are reported (Anigbogu et al., 1995; Naik et al., 1995).

### 2.6. Statistical analysis

The transdermal flux of fluorescein, skin resistance, DSC spectra, and FTIR spectra were measured using at least three skin specimens each, from which the mean and standard error of the mean were calculated. A two-tailed Student's $t$-test ($\alpha = 0.05$) was performed when comparing two different conditions. When comparing three or more conditions, a one-way analysis of variance (ANOVA, $\alpha = 0.05$) was performed. In all cases, a value $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effect of NLS and ethanol concentration on transdermal flux

To better understand the mechanism by which NLS increases skin permeability, we first measured the effect of NLS concentration on transdermal fluorescein delivery across human cadaver skin. Fluorescein is a moderately large and hydrophilic molecule that serves as a model drug that is normally difficult to deliver across the skin. As shown in Fig. 1A, accumulated fluorescein delivered across the skin increased with time (ANOVA, $p < 0.05$). The accumulated amount delivered over the 5 h experimental period is shown in Fig. 1B. These data demonstrate that skin permeability increased with increasing NLS concentration (ANOVA, $p < 0.05$) and did not show saturation over the range of conditions studied. At the highest NLS concentration studied, the presence of NLS increased transdermal fluorescein flux 47-fold relative to the control sample without NLS (Student's $t$-test, $p < 0.01$).

Because we were unable to fully dissolve NLS in water, the above experiments were carried out in an aqueous solution containing 50% ethanol. In addition to increasing NLS solubility, the presence of ethanol is also known to increase skin permeability itself. We therefore examined the effect of ethanol concentration on transdermal fluorescein delivery. As shown in Fig. 1C and D, skin permeability exhibited a complex dependence on ethanol concentration. In the absence of ethanol, the addition of NLS did not significantly enhance transdermal flux (0% ethanol without NLS vs. 0% ethanol with NLS, Student's $t$-test, $p > 0.05$). This data point was based on addition of NLS to PBS, even though the NLS was generally insoluble in the PBS. As an additional experiment, we instead added the sodium salt of NLS to PBS. This salt form of NLS was completely dissolved, but only resulted in a very small increase in transdermal flux (i.e., $0.061 \pm 0.013$ μg fluorescein delivered, Student's $t$-test, $p < 0.05$).

Transdermal flux in the presence of NLS with 25 and 50% ethanol increased by a factor of 20 and 24, respectively, relative to the control sample with NLS in 0% ethanol (Student's $t$-test, $p < 0.01$). There was no statistical difference between the flux measured at 25 and 50% ethanol (Student's $t$-test, $p > 0.05$). Transdermal flux at 75 and 100% ethanol were progressively lower, but were still six and three times greater, respectively, than the control sample with NLS in 0% ethanol (Student's $t$-test, $p < 0.05$). Altogether, we conclude that NLS in the absence of ethanol does not enhance skin permeability, NLS with high concentration of ethanol (i.e., 75 or 100% ethanol) is a weak enhancer, and NLS with moderate concentration of ethanol (i.e., 25 or 50% ethanol) is a strong enhancer.

To facilitate better interpretation, we ran an additional control experiment using 50% ethanol in the absence of NLS, which can decouple the direct effects of ethanol on enhancement from the synergistic effects of ethanol and NLS together. In this case, ethanol increased transdermal flux by a factor of 3 relative to the control sample without ethanol or NLS, but

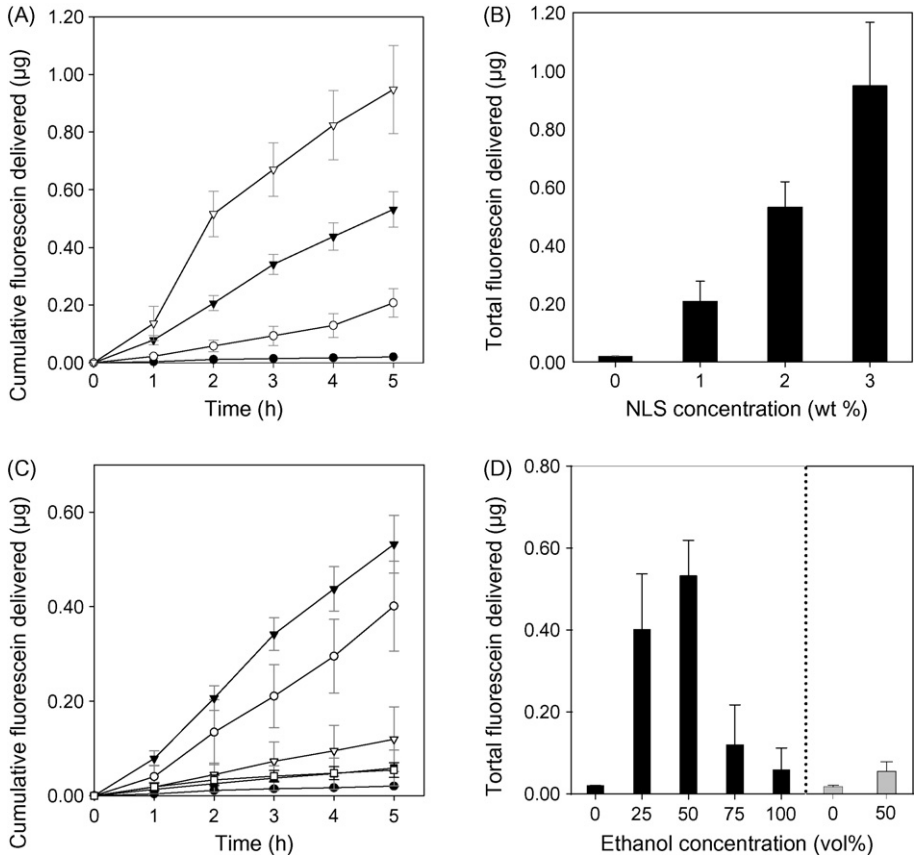


Fig. 1. Transdermal delivery of fluorescein across human cadaver epidermis as a function of NLS and ethanol concentration. (A) Cumulative fluorescein delivered across the skin as a function of time from a formulation containing (●) 0%, (◯) 1%, (▼) 2%, and (▽) 3% NLS in 50% ethanol. (B) Total fluorescein delivered after 5 h as a function of NLS concentration. Data were taken from part (A). (C) Cumulative fluorescein delivered as a function of time from a formulation containing 2% NLS in (●) 0%, (◯) 25%, (▼) 50%, (▽) 75%, and (■) 100% ethanol in PBS or a formulation containing (□) 50% ethanol without NLS. (D) Total fluorescein delivered after 5 h as a function of ethanol concentration either (■) with or (▩) without 2% NLS. Data were taken from part (C). All ethanol concentrations are reported as % (v/v) and all NLS concentrations are reported as % (w/v). Data points show the average of $n \geq 3$ replicates and error bars correspond to the standard error of the mean.

this was not significant (Student's *t*-test, $p > 0.05$). This increase in flux due to ethanol alone was also not statistically different from that caused by NLS in the absence of ethanol or NLS in the presence of 75% ethanol or NLS in the presence of 100% ethanol (Student's *t*-test, $p > 0.1$). In contrast, transdermal flux in the presence of NLS and 25 or 50% ethanol was significantly greater than for 50% ethanol alone (Student's *t*-test, $p < 0.01$). This indicates a synergistic interaction between ethanol and NLS, because the sum of the permeability enhancement from NLS alone (non-significant) and ethanol alone (3-fold) is much less than the enhancement caused by their combination (24-fold).

The reasons for this mechanistic enhancement at 25–50% ethanol concentrations will be further investigated below. At 75–100% ethanol concentrations, the reduced enhancement may be explained in part by dehydration of the *stratum corneum*. Previous results have shown that exposure to high ethanol concentrations can substantially dehydrate the outer layer of the *stratum corneum* and thereby decrease skin permeability (Berner et al., 1989). Dehydration has also been shown to shrink the keratins of the *stratum corneum*, which similarly decreases skin permeability (Levang et al., 1999).

### 3.2. Skin resistance measurement

To further validate the synergistic effects of NLS and ethanol of skin barrier properties, we examined the effect of ethanol concentration on the electrical resistance of skin. Skin's electrical resistance is generally considered a marker of skin permeability and changes in skin resistance due to exposure to different chemical enhancers has been shown to correlate with increased skin permeability to model drug compounds (Lackermeier et al., 1999; Karande et al., 2006). Moreover, because skin resistance can be easily measured on a continuous basis, electrical measurements permit better characterization of the kinetics of skin permeability changes.

Fig. 2A shows skin resistance over time for different formulations. Skin samples exposed to 50% ethanol, without NLS, showed a small and insignificant (Student's *t*-test, $p > 0.1$) drop in resistance over the 24-h experimental period. However, the combination of NLS and ethanol decreased skin resistance more rapidly and to a greater extent, where NLS in 50% ethanol had the greatest effect (Student's *t*-test, $p < 0.01$); NLS in 25 and 75% ethanol had similar, but lesser effects (Student's *t*-test, $p < 0.01$); and NLS in 100% ethanol had much less effect, but still more

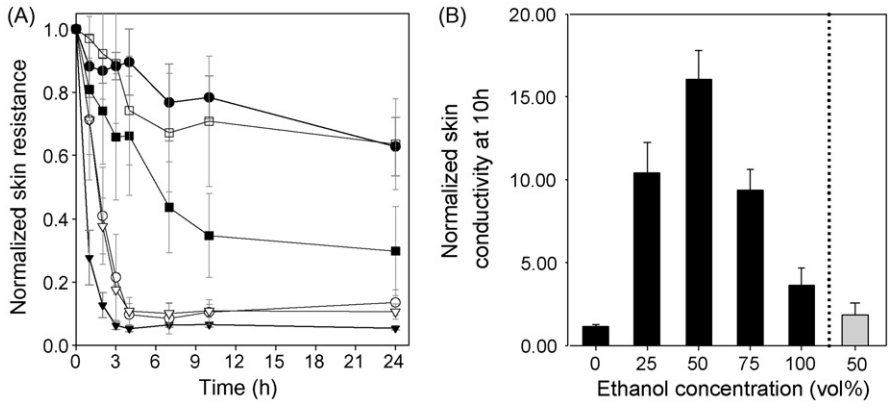


Fig. 2. Skin electrical resistance and conductivity as a function of ethanol concentration. (A) Normalized electrical resistance of human cadaver epidermis treated with a formulation containing 2% NLS in (●) 0%, (◯) 25%, (▼) 50%, (▽) 75%, and (■) 100% ethanol in PBS and a formulation containing (□) 50% ethanol without NLS. Resistance values were normalized relative to their pretreatment levels. (B) Normalized electrical conductivity (i.e., the inverse of resistance) of human epidermis after 10 h of treatment (■) with and (▩) without NLS as a function of ethanol concentration. Data were taken from part (A). Resistance and conductivity values were normalized to value at time = 0. Data points show the average of $n \geq 3$ replicates and error bars correspond to the standard error of the mean.

than the NLS-only or ethanol-only controls (Student's *t*-test, $p < 0.05$).

Fig. 2B summarizes these data and replots them as electrical conductivity, which is the inverse of electrical resistance and is known to correlate directly with skin permeability (Karande et al., 2006). The dependence of changes in skin resistance and conductivity on ethanol concentration is strongly consistent with the dependence found in measurements of transdermal flux of fluorescein (Fig. 1), which further validates the findings and suggests that these decreases in skin barrier properties may be broadly applicable to many drugs.

From a mechanistic viewpoint, skin's electrical resistance is known to be governed primarily to the highly ordered, lipophilic barrier of the *stratum corneum* lipid bilayers (Kontturi et al., 1993). Therefore, changes in skin resistance are a sensitive measure of changes in *stratum corneum* lipid bilayer integrity. These measurements suggest that the combination of NLS and ethanol act by disrupting *stratum corneum* lipid bilayer structure.

A final observation of the skin resistance data concerns the kinetics of action. Changes in skin resistance are seen to occur with a lag time of one or more hours (Fig. 2A), which suggests a kinetic barrier that may be a diffusive transport limitation. Lag times for transdermal diffusion of small drugs can similarly be a few hours (Prausnitz et al., 2004), which suggests that the lag time observed for NLS-based enhancement may be limited by the time it takes for NLS to diffuse into and throughout the *stratum corneum*.

### 3.3. Differential scanning calorimetry

To further explore the effects of NLS and ethanol on the skin barrier, we are guided by the hypothesis that NLS and ethanol increase skin permeability by increasing the fluidity of *stratum corneum* lipid structure. To test this hypothesis, DSC was employed to investigate the potential fluidizing effects of NLS in ethanol solution on the thermal properties of human *stratum corneum* (Fig. 3). DSC analysis of negative control samples of human *stratum corneum* exposed to PBS without NLS or ethanol showed two major endothermic transition peaks, $T_{m1}$ and $T_{m2}$, that occur near 75 and 90 °C, respectively. $T_{m1}$ is known to correspond to lipid structure transformation from a lamellar to disordered state and $T_{m2}$ corresponds to protein-associated lipid transition from gel to liquid form (Tanojo et al., 1999). It should be mentioned that a commonly observed, lower temperature transition peak (at 35–40 °C) is not easily observed (Potts et al., 1991) and an additional transition peak (at 105–120 °C), representing changes in *stratum corneum* protein conformation, is also not seen, because it requires a skin water content of at least 15% (Leopold and Lippold, 1995) and our *stratum corneum* samples were desiccated before DSC analysis.

The effect of NLS concentration on the thermal profiles of *stratum corneum* is shown in Fig. 3A and B. These data show that increasing NLS concentration (in the presence of 50% ethanol) significantly decreased both of the transition temperatures, $T_{m1}$ and $T_{m2}$, by as much as 9 and 11 °C, respectively (Student's *t*-test, $p < 0.01$). These lipid transitions involve decreased packing order relative to the initial state of the *stratum corneum,* such that the observed decreases in $T_m$ reflect thermal transitions starting from less ordered and more fluidized states of *stratum corneum* (Golden et al., 1987). These changes in transition temperatures therefore suggest that incorporation of NLS into the *stratum corneum*, with the aid of the ethanol solution, leads to increased lipid disorder or fluidization. Moreover, the changes in $T_{m1}$ and $T_{m2}$ as a function of NLS concentration scale with the corresponding changes in skin permeability (Fig. 1A and B), which suggests that increased skin permeability correlates increased *stratum corneum* lipid fluidization, consistent with previous observations (Golden et al., 1987; Francoeur et al., 1990).

To further investigate the fluidizing effect of NLS–ethanol mixtures on *stratum corneum* lipids, DSC analysis was performed as a function of ethanol concentration. Fig. 3C and D indicates that the lipid transition temperatures are reduced by the addition of ethanol and that the largest reduction is found at 25 and 50% ethanol (Student's *t*-test, $p < 0.01$), although reductions at 75 and 100% ethanol were also statistically different from the

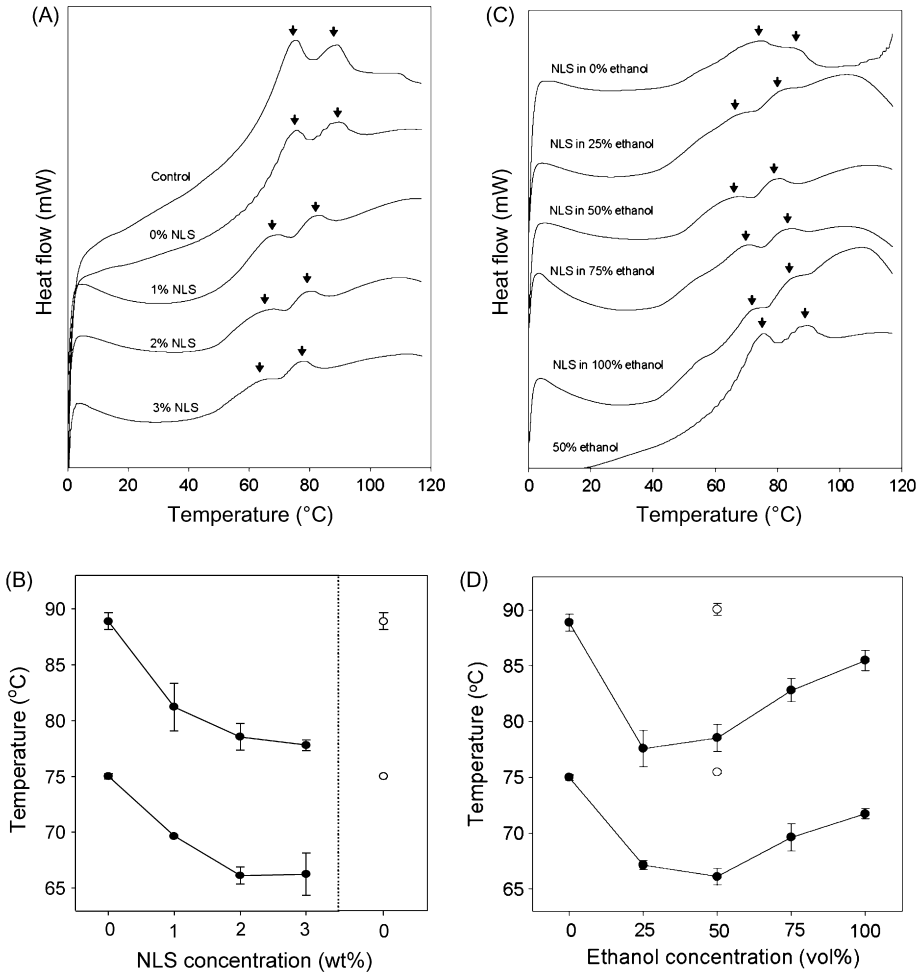


Fig. 3. Differential scanning calorimetry analysis of human *stratum corneum* as a function of NLS and ethanol concentration. (A) DSC thermograms of *stratum corneum* treated with various concentrations of NLS in 50% ethanol and negative control thermogram for untreated skin (i.e., no NLS and no ethanol). (B) Peak temperature of two characteristic order–disorder transitions associated with *stratum corneum* lipids as a function of NLS concentration (●) and for the negative control skin (◯). Data were taken from part (A). (C) DSC thermograms of *stratum corneum* treated with 2% NLS in various concentrations of ethanol and control thermogram for 50% ethanol without NLS. (D) Peak temperature of the two characteristic transitions as a function of ethanol concentration (●) with NLS and (◯) without NLS. Data points show the average of $n \geq 3$ replicates and error bars correspond to the standard error of the mean.

0% ethanol control (Student's *t*-test, $p < 0.05$). In contrast, when *stratum corneum* was treated with a 50% ethanol solution without NLS, neither transition temperatures was shifted (Student's *t*-test, $p > 0.1$). Once again, these increases in lipid fluidization indicated by lower lipid transition temperatures correlate closely with increases in skin permeability (Fig. 1C and D) and increases in skin conductivity (Fig. 2).

### 3.4. Fourier transform infrared spectroscopy

To further assess changes in molecular conformations within *stratum corneum* after exposure to NLS and ethanol, we employed FTIR spectroscopy. Characteristic peaks are found in FTIR spectra of *stratum corneum* near 2920 $cm^{-1}$, which corresponds to asymmetric C–H stretching; near 2850 $cm^{-1}$, which corresponds to symmetric C–H stretching; and near 1650 $cm^{-1}$, which is the amide I band corresponding to C=O stretching (Garidel, 2002). The frequencies of the two C–H stretching bands are related to lipid order in *stratum corneum* and are significantly influenced by the degree of conformational order, the freedom of alkyl chain motion and possible incorporation of chemical enhancers, such as NLS. The frequency of the C=O stretching band is related to *stratum corneum* protein conformation (Goates and Knutson, 1994).

Representative IR absorbance spectra from 3000 to 2750 and 1850 to 1300 $cm^{-1}$ of human *stratum corneum* samples are displayed in Figs. 4 and 5. In Figs. 4A and 5A, changes in the C–H stretching region and the amide I band, respectively, are shown after treatment of *stratum corneum* with different NLS concentrations. The wavenumber position of the three characteristic spectral peaks is shown in Figs. 4B and 5B as a function of NLS concentration. Negative control samples exposed to PBS or to 50% ethanol in the absence of NLS both have C–H asymmetric, C–H symmetric, and C=O stretching peaks at 2918, 2850 and 1648 $cm^{-1}$, respectively. In contrast, addition of NLS in 50% ethanol increased the wavenumber of each of these three peaks by 1 to 4 $cm^{-1}$ in a dose-dependent manner (Student's *t*-test, $p < 0.01$). Although these wavenumber shifts are relatively small, they are statistically significant and are consistent in mag-

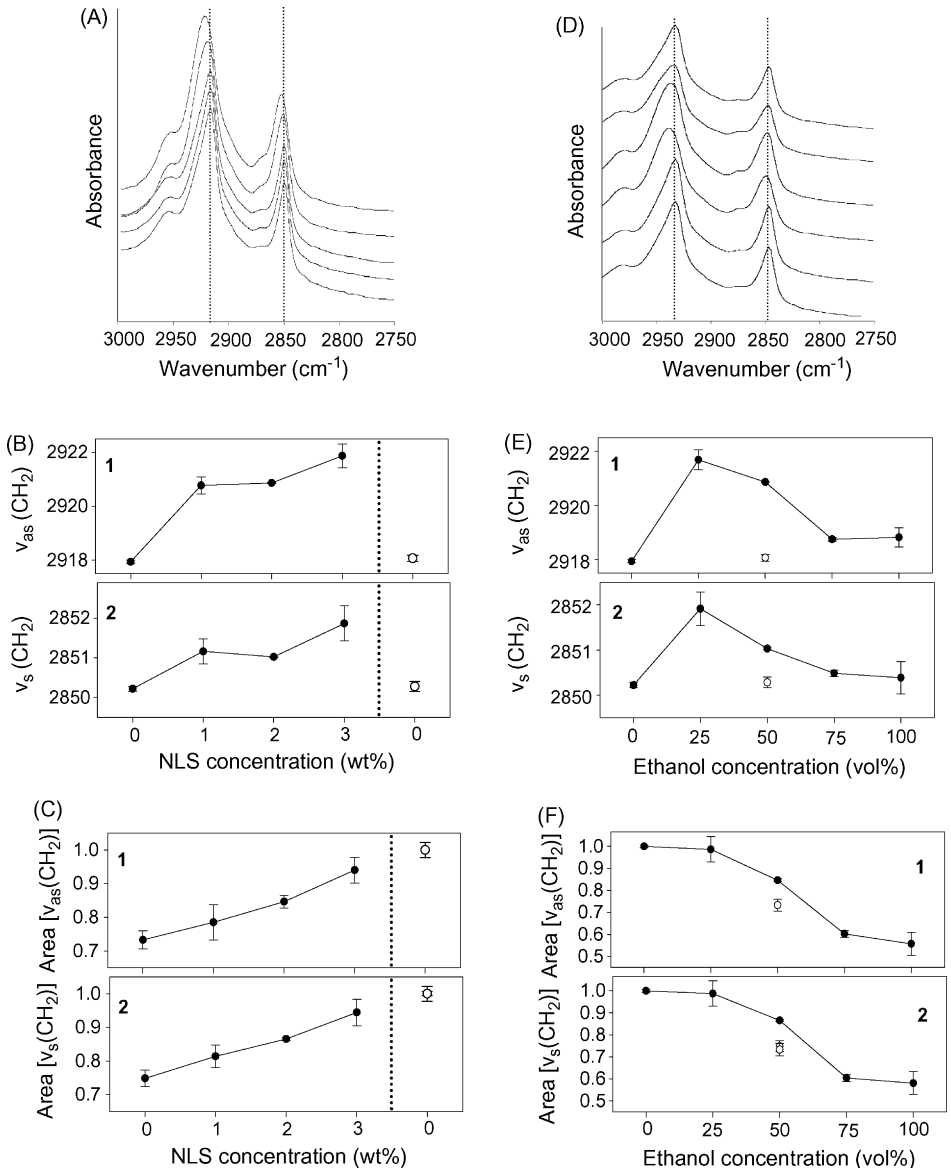


Fig. 4. Fourier transform infrared spectral analysis of human *stratum corneum* lipids as a function of NLS and ethanol concentration. (A) FTIR spectra of *stratum corneum* treated with various concentrations of NLS in 50% ethanol. Curves correspond, from top to bottom, to 3, 2, 1, and 0% NLS in 50% ethanol, and untreated skin. (B) Peak wavenumber and (C) area of characteristic spectral peaks corresponding to (1) asymmetric C—H stretching and (2) symmetric C—H stretching as a function of NLS concentration in (●) 50% ethanol and (◯) in PBS. (D) FTIR spectra of *stratum corneum* treated with 2% NLS in various concentrations of ethanol. Curves correspond, from top to bottom, to 100, 75, 50, 25 and 0% ethanol with 2% NLS, and 50% ethanol without NLS. (E) Peak wavenumber and (C) area of characteristic spectral peaks corresponding to (1) asymmetric C—H stretching, (2) symmetric C—H stretching as function of ethanol concentration (●) with NLS and (◯) without NLS. Data points show the average of $n \geq 3$ replicates and error bars correspond to the standard error of the mean. In (C and F), area is normalized to 0% NLS in 0% ethanol.

nitude with previous studies of chemical enhancers (Potts et al., 1991; Bhatia et al., 1997).

### 3.4.1. Lipid fluidization

The increased wavenumber of the two C–H stretching peaks (Fig. 4B) indicates that increasing NLS concentration increased the fluidization of *stratum corneum* lipids, according to established interpretations of FTIR spectra (Casal and Mantsch, 1984). This fluidization could be from dispersed incorporation of NLS among the *stratum corneum* lipids and, possibly, from pooling of NLS in discrete domains among the lipids. Comparison with the ethanol-only negative control shows that this was clearly the effect of NLS (in the presence of 50% ethanol) and not the effect of ethanol alone. This is consistent with previous observations that short-chain alcohols have little effect on *stratum corneum* lipid order below 40 °C (Knutson et al., 1990) and, despite forming localized regions of greater free volume within the lipid alkyl chain regions, exposure to ethanol does not cause overall fluidization of *stratum corneum* lipids (Goates and Knutson, 1994).

### 3.4.2. Protein conformation

The increased wavenumber of the C=O stretching peak (Fig. 5B) indicates that increasing NLS concentration also

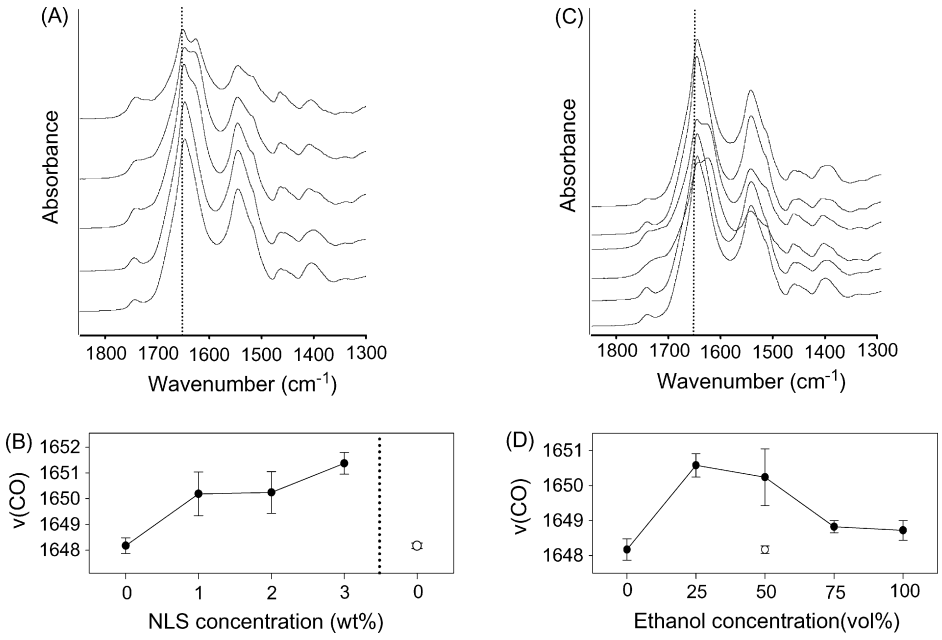


Fig. 5. Fourier transform infrared spectral analysis of human *stratum corneum* proteins as a function of NLS and ethanol concentration. (A) FTIR spectra of *stratum corneum* treated with various concentrations of NLS in 50% ethanol. Curves correspond, from top to bottom, to 3, 2, 1, and 0% NLS in 50% ethanol, and untreated skin. (B) Wavenumber of spectral peak corresponding to C═O stretching as a function of NLS concentration in (●) 50% ethanol and (◯) in PBS. (C) FTIR spectra of *stratum corneum* treated with 2% NLS in various concentrations of ethanol. Curves correspond, from top to bottom, to 100, 75, 50, 25 and 0% ethanol with 2% NLS, and 50% ethanol without NLS. (D) Wavenumber of spectral peak corresponding to C═O stretching as function of ethanol concentration (●) with NLS and (◯) without NLS. Data points show the average of $n \geq 3$ replicates and error bars correspond to the standard error of the mean.

altered *stratum corneum* protein conformation. Closer analysis of Fig. 5A also shows the appearance of an intense new band at 1628 $cm^{-1}$ and increased absorption at 1519 $cm^{-1}$. Altogether, these changes indicate that increasing NLS concentration caused a shift from α-helix protein conformation to β-sheet structure (Anigbogu et al., 1995). When considered in the context of skin permeability data (Fig. 1A and B), this analysis shows that increased transdermal flux correlates with increased *stratum corneum* lipid fluidization and altered *stratum corneum* protein conformation caused by exposure to increasing concentration of NLS in 50% ethanol.

### 3.4.3. Effect of ethanol concentration

We carried out a similar analysis of changes in FTIR spectra for *stratum corneum* samples treated with NLS in different concentrations of ethanol. Changes in the C–H stretching region and the amide I band frequencies are shown in Figs. 4D and 5C, respectively, and summarized in Figs. 4E and 5D. Treatment of *stratum corneum* with NLS in 25 and 50% ethanol significantly increased the wavenumber of all three characteristic spectral peaks relative to the NLS-only and ethanol-only control samples (Student's *t*-test, $p < 0.05$), whereas treatment with NLS in 75 and 100% ethanol had much less effect (Student's *t*-test, $p > 0.05$).

### 3.4.4. Lipid extraction

Ethanol and surfactants have been reported to extract lipids from the *stratum corneum* and in that way increase skin permeability (Imokawa et al., 1989; Bommannan et al., 1991). To assess the possible role of extraction in this study, the area under the two C–H stretching peaks was measured as a function of NLS and ethanol concentration. Exposure of skin to 50% ethanol significantly decreased the area under both of these peaks (Fig. 4C, Student's *t*-test, $p < 0.01$), which indicated lipid extraction by ethanol, in agreement with previous findings (Bhatia and Singh, 1998). Addition of NLS in 50% ethanol at progressively larger NLS concentration diminished the effect on peak area (Fig. 4C, ANOVA, $p < 0.05$), which suggests that addition of NLS either reduced the degree to which 50% ethanol extracted lipids or that NLS possibly replaced the extracted lipids and thereby increased the peak area. At constant NLS concentration, increasing ethanol concentration progressively decreased peak area for both lipid peaks (Fig. 4F, ANOVA, $p < 0.01$), which indicated that higher ethanol concentrations increased lipid extraction.

Notably, the dependence of peak area on NLS and ethanol concentration is different from that of skin permeability. For example, the addition of 50% without NLS caused a large drop in peak area, but had no effect on transdermal flux. Moreover, increasing ethanol concentration (in combination with NLS) progressively decreased peak area, but caused transdermal flux to go through a maximum at 25–50% ethanol. These different functionalities of lipid extraction measured by peak area and skin permeability indicated that although lipid extraction may have occurred, it was not responsible for the observed increases in skin permeability.

Altogether, these observations indicate that NLS in the presence of 25–50% ethanol increases lipid fluidization and alters protein conformation (Fig. 4), which correlates with dramatically increased skin permeability (Fig. 1). NLS in the presence of 75–100% ethanol had little effect on lipid fluidity and protein

conformation in *stratum corneum*, which similarly correlates with the modest increases in skin permeability seen under those conditions.

## 4. Discussion

The results of this study show a remarkably consistent correlation between four different analyses of skin properties as a function of NLS and ethanol concentration. Consistently, treatment using NLS without ethanol or ethanol without NLS had little to no significant effect on skin permeability to fluorescein, skin electrical resistance, DSC thermograms, and FTIR spectra. In contrast, the combination of NLS and ethanol at an optimized concentration of 25–50% uniformly increased skin permeability, decreased skin resistance, decreased DSC transition temperatures, and increased FTIR peak wavenumbers.

Interpretation of these findings demonstrates a correlation, and suggests a causative relationship, between increased skin permeability and increased *stratum corneum* lipid fluidization, as well as changes in protein conformation. We hypothesize that NLS inserts into the *stratum corneum* lipid structures with the aid of ethanol, which disrupts the lipid packing. NLS alone may have difficulty partitioning into the *stratum corneum* lipid domain and ethanol alone is known not to affect lipid fluidity. However, the combination of NLS and ethanol has a synergistic effect, such that ethanol may facilitate penetration of NLS into the *stratum corneum* lipids, where NLS is then able to fluidize lipid structure.

Although we observed changes in protein conformation as well, we do not expect that these changes are mechanistically responsible for the skin's increased permeability. Skin's barrier properties have been widely established to reside predominantly in the *stratum corneum* lipids, where *stratum corneum* proteins play a secondary role (Grubauer et al., 1989; Imokawa et al., 1991).

## 5. Conclusion

This study sought to determine the mechanism by which mixtures of NLS and ethanol increase transdermal transport by testing the hypothesis that NLS and ethanol synergistically increase skin permeability by increasing the fluidity of *stratum corneum* lipid structure. When NLS in 50% ethanol was applied to human cadaver skin over a range of NLS concentrations, transdermal flux of fluorescein was increased by up to 47-fold in a manner that depended strongly on NLS concentration. Ethanol concentration also strongly affected skin permeability, where formulations containing NLS in 25 and 50% ethanol dramatically enhanced transdermal delivery, whereas NLS in 0, 75, and 100% ethanol had modest or no effect. Ethanol in the absence of NLS had only a small effect on skin permeability.

The degree of skin permeability enhancement correlated with companion measurements of skin electrical resistance, which is a general measurement of skin barrier function associated with an intact *stratum corneum* lipid domain. Increased skin permeability also correlated with altered DSC transition temperatures and FTIR peak shifts that indicate increased fluidity of the *stratum corneum* lipids and changes in protein conformation from α-helix to β-sheet structure. These correlations suggest a causative relationship in which increased lipid fluidity is the mechanism by which combinations of NLS and an aqueous ethanol solution increase skin permeability. This formulation also appeared to cause extraction of *stratum corneum* lipids, but the level of extraction did not correlate with skin permeability changes, which suggested it was a side effect. Finally, the synergistic effect of this formulation may result from the ability of the ethanol solution to improve the permeation of NLS in the lipid bilayer matrix of the *stratum corneum*, thereby improving the ability of NLS to disrupt the lipid order.

Overall, we conclude that a mixture of NLS in 25–50% ethanol acts synergistically to increase skin permeability. This formulation may be useful for transdermal drug delivery applications.

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