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Biological Sciences: Biophysics & Computational Biology

**Molecular diffusion in the human nail measured by stimulated Raman scattering microscopy**

Short title: Molecular diffusion in human nail

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## Abstract

The effective treatment of diseases of the nail remains an important unmet medical need, primarily because of poor drug delivery. To address this challenge, the diffusion, in real time, of topically applied chemicals into the human nail has been visualized and characterized using stimulated Raman scattering (SRS) microscopy. Deuterated water ( $D_2O$ ), propylene glycol ( $PG-d_8$ ) and dimethyl sulphoxide ( $DMSO-d_6$ ) were separately applied to the dorsal surface of human nail samples. SRS microscopy was used to image  $D_2O$ ,  $PG-d_8/DMSO-d_6$  and the nail through the O-D,  $-CD_2$  and  $-CH_2$  bond stretching Raman signals, respectively. Signal intensities obtained were measured as functions of time and of depth into the nail. It was observed that the diffusion of  $D_2O$  was more than an order of magnitude faster than that of  $PG-d_8$  and  $DMSO-d_6$ . Normalization of the Raman signals, to correct in part for scattering and absorption, permitted semi-quantitative analysis of the permeation profiles and strongly suggested that solvent diffusion diverged from classical behaviour and that derived diffusivities may be concentration-dependent. It appeared that the uptake of solvent progressively undermined the integrity of the nail. This novel application of SRS has permitted, therefore, direct visualization and semi-quantitation of solvent penetration into the human nail. The kinetics of uptake of the three chemicals studied demonstrated that each altered its own diffusion in the nail in an apparently concentration-dependent fashion. The scale of the unexpected behaviour observed may prove beneficial in the design and optimization of drug formulations to treat recalcitrant nail disease.

Keywords: nail plate | stimulated Raman scattering microscopy | chemical diffusion | imaging

## **Significance statement**

Diseases of the nail are particularly hard to treat because drug penetration to the target (which lies below the tightly woven keratin network) is extremely limited. To shed greater light on the problem, the diffusion of three pharmaceutically relevant solvents across the human nail has been imaged and characterized by stimulated Raman scattering microscopy. Remarkably, the kinetics of water transport were more than 10-fold faster than those of dimethyl sulphoxide and propylene glycol. Furthermore, the uptake of all three solvents, the diffusion of which appeared to be concentration-dependent, progressively undermined the integrity of the nail. These new insights may facilitate the improved formulation of drug products effective in the treatment of diseases such as fungal infections and nail psoriasis.

## Introduction

The effective treatment of nail disease requires efficient drug delivery into and through the barrier. However, the tightly woven keratin network of the nail plate means that poor drug uptake following topical administration is common. Despite considerable effort to improve formulations and to enhance drug delivery to the nail, progress has been slow at best. In general, the approaches adopted have failed to understand the complex interplay between drug, formulation components (including solvents) and the nail. For example, although it is quite clear that drug uptake from typical 'lacquer' formulations (comprising the active, a film-forming polymer and a volatile organic solvent) is intimately linked to the disposition of the solvent, and effectively stops once the solvent has gone, there has been little effort to characterize the transport of these key vehicle components into and across the nail. Only the diffusion of water has received attention, its overall time-dependent uptake having been measured by various techniques (1-3); otherwise, apart from some information on the concentration-depth profiles of water and dimethyl sulphoxide (DMSO) in the very superficial, outermost 20  $\mu\text{m}$  of the nail, there are essentially no time and position-dependent data on the movement of chemicals into the nail.

Stimulated Raman scattering (SRS) microscopy is a label-free imaging technique that offers a solution to this challenge. This method has been applied in a range of biomedical and pharmaceutical studies involving, for example, visualization in living cells (4), characterization of cortical vasculature morphology (5), imaging the constituents of solid, oral dosage forms (6), and tracking the pharmacokinetics of drugs and excipients in mammalian skin (7-9). In this paper, the first application of SRS microscopy to trace and visualize the diffusion of three pharmaceutically relevant solvents, water, propylene glycol (PG) and DMSO, as a function of depth and in real time, in human nail is presented. The use of deuterated solvents provides unique Raman-active molecular vibrations that are easily distinguished spectroscopically from those originating in the nail, resulting in excellent, and label-free, image contrast. Because of the linear relationship between the SRS signal and the concentration of the chemical, the spectroscopic signature of which is being monitored, a semi-quantitative analysis of solvent diffusion across the nail is possible and offers heretofore-unknown insight into the transport process.

## Results & Discussion

### Raman spectroscopy and imaging

SRS involves an energy transfer between a pump pulse and a Stokes pulse, the wavelengths of which are tuned so that the energy difference between them matches a specific Raman-active vibration of the sample, generating a coherent, chemically-specific image contrast. In this study, unique vibrational modes of the human nail samples and of the solvents were targeted for imaging, specifically  $\text{-CH}_2$  bond stretching ( $2855\text{ cm}^{-1}$ ) from the nail,  $\text{-CD}_2$  stretching ( $2120\text{ cm}^{-1}$ ) from PG- $\text{d}_8$  and DMSO- $\text{d}_6$ , and O-D stretching ( $2500\text{ cm}^{-1}$ ) from  $\text{D}_2\text{O}$  (Fig. S1A). An off-resonance signal at  $1802\text{ cm}^{-1}$  provided a suitable background where neither the nail nor the solvents are Raman-active (Fig. S1B). Control images (Fig. S1B) from untreated nails were acquired using the  $\text{-CH}_2$  stretching vibration; the nail surface was clearly observed. For the skin, this vibrational mode originates primarily from the lipids in the outermost layer, the stratum corneum (SC) (8). However, the lipid content of the nail is reported to be very low (0.1-1%) (10) (i.e., an order of magnitude or two less than that in the SC) and the  $\text{-CH}_2$  signal more likely originates from keratin, the principal protein component present. The signal from untreated nails, either at off-resonance or at the  $\text{-CD}_2$  stretching frequency, was negligible; the infrequent and small punctate ‘spots’ on the otherwise uniformly black images are due to two-photon absorption (TPA) (11) from residual dirt particles that were not removed when the nails were cleaned.

To enable a more quantitative analysis of the chemical diffusion results from SRS imaging, there remains the unresolved issue, ubiquitous to confocal imaging, of signal attenuation with increasing depth into the sample (in this case, the nail) due to light absorption and scattering that reduce optical excitation and collection efficiencies. Therefore, the signals emanating from deeper into the nail likely reflect underestimates of the actual amount of chemical present. Although it has been suggested that the non-resonant contribution may be used to correct for the signal loss along the sample depth in coherent anti-Stokes Raman scattering (CARS) imaging (12), such a method may not be reliable for SRS, in which the background noise generally arises from other quasi-instantaneous nonlinear optical processes of ubiquitous sources (13).

Instead, an approach to account for the loss of the target chemical signal via normalization to the nail  $\text{-CH}_2$  resonance has been adopted. This method took advantage of mapping the  $\text{-CH}_2$  signal intensity (Fig. 1A) of the nail cross-section. Data were obtained from nail samples provided by three different donors (Fig. 1B). The  $\text{-CH}_2$  signal profile across the nail from the outer surface towards the inner was similar for the three samples examined (Fig. 1C), and followed a consistent trend. No statistical difference was found in the keratin Raman signature, at least over the outermost  $60\text{ }\mu\text{m}$  of the nails, meaning that attenuation of this resonance during optical sectioning in SRS imaging may be confidently attributed to light scattering and absorption (Fig. 1D).

## Nail penetration of D<sub>2</sub>O

To measure D<sub>2</sub>O uptake into the nail plate, the SRS microscope was tuned to the O-D stretching vibration at 2500 cm<sup>-1</sup>. After application of the solvent to the nail and positioning the sample on the microscope stage, an x-z line scan (x = 353 μm) was performed at t = 10 minutes capturing every 1 μm in the z-direction into the nail. This scan (150 lines) was repeated on 9 further occasions, every 2.7 minutes (i.e., until t = 34.3 minutes post-application of D<sub>2</sub>O). Acquisition of each line scan required 1.07 seconds. At the end of the experiment, the nail was imaged by re-tuning the SRS microscope to 2855 cm<sup>-1</sup>, and an off-resonance signal was then recorded at 1802 cm<sup>-1</sup>.

The results are presented in Fig. 2 (and in Movie S1), which shows superimposed x-z orthogonal views of the O-D signals obtained from each scan as a function of time from 5 different regions (35 x 150 μm<sup>2</sup>) of the D<sub>2</sub>O-treated nail. The SRS image from the -CH<sub>2</sub> contrast of the nail permits the surface to be clearly delineated; the off-resonance ‘image’ only reveals (as before) a very few bright points of light scattering due to residual particulate matter not removed by the cleaning process prior to starting the experiment.

The O-D signal recorded at each 2.7-minute interval is represented by a separate color on the visible spectrum scale shown on Fig. 2; that is, red corresponds to the measurement at t = 10 minutes, yellow to that at t = 12.7 minutes, and so on. These scans have then been superimposed (prepared using the ImageJ ‘transparent zero’ function), one upon the other, to generate (at each of the 5 different regions visualized) an image of D<sub>2</sub>O diffusion into the nail. Within 35 minutes, it can be seen that the deuterated water had diffused approximately 100 μm into the sample. This relatively rapid uptake of water into the nail has been inferred from previous investigations (14, 15) but the transport process has never been visualized before in such a direct fashion. The results are consistent with the nail being characterized as a dense hydrogel containing overlapping keratin fibres, which create small, tortuous, pore pathways that favour the permeability of small, hydrophilic molecules, such as water (16).

## Nail penetration PG-d<sub>8</sub> and DMSO-d<sub>6</sub>

To follow the penetration of PG-d<sub>8</sub> and DMSO-d<sub>6</sub> into the nail, SRS imaging at 2120 cm<sup>-1</sup> (the -CD<sub>2</sub> stretching vibration) was performed. As the diffusion of these solvents was much slower than that of D<sub>2</sub>O, images were also recorded at each time point for the nail (-CH<sub>2</sub> at 2855 cm<sup>-1</sup>) and off-resonance (1802 cm<sup>-1</sup>). In this case, x-y planar images (353 μm x 353 μm) were captured every 1 μm in the z-direction at each measurement time. The scan time for each frame was 18.4 seconds.

The time-course of PG-d<sub>8</sub> and DMSO-d<sub>6</sub> absorption into the nail as a function of depth are shown in Figs. 3A and 3C, respectively. While the -CH<sub>2</sub> signal from the nail is relatively constant, the shorter time measurements (t ≤ 8 hours) reveal that uptake of PG and DMSO occurs only into the outer 15-20 μm of the nail. Only after about a day have the two solvents reached a depth of about 40-50 μm into the nail. Figs. 3B and 3D illustrate alternative, cross-sectional (x-z) views of PG and DMSO penetration

that enables direct visualization of the solvents on and within the nail. Notably, and self-evidently, the rate of diffusion of PG and DMSO is substantially less than that of D<sub>2</sub>O (which had permeated 100 μm in only ~30 minutes), the molecular size of which is about one-quarter of that of the two other solvents: the molecular weights of water, PG and DMSO are 18.0, 76.1 and 78.1, respectively; the corresponding molar volumes are 18.0, 73.4 and 71.0 cm<sup>3</sup>. The relatively poorer uptake of PG and DMSO into the nail has been reported (16, 17) and their penetration-enhancing abilities are less than clear-cut (18). Rotating 3D composite images showing the progressive penetration of PG-d<sub>8</sub> and DMSO-d<sub>6</sub> into the nail are presented in Movies S2 and S3, respectively.

### **SRS signal analysis and interpretation**

To better interpret the results obtained, an attempt to more quantitatively analyse the SRS signals (specifically, the measured pixel intensities) from the three solvents was undertaken. To do so required a number of potentially confounding factors to be addressed, including: (a) definition of the nail surface, (b) fluctuations in SRS laser intensity, (c) movement of the sample (e.g., due to swelling), (d) artefacts caused by residual particulate matter on the nail, (e) variable off-resonance background signal, and (f) confirmation that no significant depletion of solvent at the nail surface had occurred by the end of the experiment.

Because of the natural curvature of the human nail, it is clear that a z-series of x-y planar images will not sample the same depth across the entire sample (see Fig. S2) and a virtual surface was therefore defined using the intensity of the -CH<sub>2</sub> signal from nail protein. To do so, 5 regions of the examined nail (35.3 x 35.3 μm<sup>2</sup> x-y planes for PG-d<sub>8</sub> and DMSO-d<sub>6</sub>, 20 μm sections for D<sub>2</sub>O) were delimited avoiding those where either particulate matter or an air bubble in the solvent on the nail clearly interfered with the image (see Fig. S3 for an illustration). For each selected region, the nail surface was defined when the -CH<sub>2</sub> signal had reached 90% of its maximum value, thereby aligning the 5 surfaces on one horizontal line (Fig. S2). This procedure also allowed for correction of any sample movement (typically no more than 1-2 μm) to be made as well. The small background off-resonance signal, when present, was subtracted from -CH<sub>2</sub> and O-D/-CD<sub>2</sub> signals in each image. The average pixel intensity of the solvent (as a function of depth into the nail) was then normalized by that at the defined surface (z = 0 μm), i.e., all signals from the solvents were then expressed as a fraction of that at the surface reflecting, in theory at least, a relative concentration profile of the compound across the nail. The solvent signals at the nail surface did not decay significantly over the time-course of the experiments confirming that no appreciable depletion had occurred and that an effectively infinite dose had been applied to the nail.

The average pixel intensity data extracted from the SRS images as a function of time and position are presented graphically in the left-hand column of Fig. 4 (A: D<sub>2</sub>O; B: PG-d<sub>8</sub>; C: DMSO-d<sub>6</sub>). The error bars (standard deviations) reflect the variability observed across the 5 sampled regions of the nail. A logical starting point for the



interpretation of the results is to test the hypothesis that the uptake of solvents into the nail during the experiments performed follows non-steady state diffusion into a semi-infinite medium (19), i.e., treating the nail as a homogenous plane sheet (through which solvent transport is characterized by a constant diffusivity,  $D_i$ ), with the boundary conditions: (i) the normalized solvent signal ( $S/S_{z=0}$ ) at the nail surface ( $z = 0$ ) equals 1 at all times,  $t \geq 0$ , (ii) at  $t = 0$ ,  $S/S_{z=0} = 0$  at  $z > 0$ , and (iii) at  $t \geq 0$ ,  $S/S_{z=0} = 0$  at  $z = \infty$ . In other words, first, during the course of the experiment, there is a constant source of solvent on the nail surface. Second, initially, there is no solvent in the nail. And, third, the nail can be considered infinitely thick such that no solvent diffuses all the way through during the observation period. The analytical solution to Fick's 2<sup>nd</sup> law then predicts that SRS profiles to evolve as a function of time and position according to the following expression:

$$\frac{S}{S_{z=0}} = 1 - \operatorname{erf}\left(\frac{z}{2(D_i t)^{1/2}}\right) \quad (\text{Eq. 1})$$

Using  $D_i$  value derived from fitting the data of the profile measured at the shortest time for each solvent, Eq. 1 can then be used to predict the evolution of the SRS signal as the experiment proceeds. These predictions are shown in the right-hand column of Fig. 4 (D:  $D_2O$ ; E: PG-d<sub>8</sub>; F: DMSO-d<sub>6</sub>) and demonstrate very clearly how the experimental results deviate substantially from the classic model as the time of diffusion increases. Distortion of the water concentration profile occurs very rapidly and well within the 35-minute duration of experiment; for the other solvents, the onset of anomalous behaviour is slower but is nonetheless clearly perceptible within 6 hours for DMSO and certainly by 24 hours for PG.

Insight into the cause underlying the greater level of solvent uptake into the nail than that anticipated by the simple model is revealed when the normalized SRS signals are re-plotted as a function of  $z/t^{1/2}$ . If  $D_i$  is constant, then this transformation of the data should collapse all the concentration profiles (at different times) onto a signal curve (19). Fig. 5 (A:  $D_2O$ ; B: PG-d<sub>8</sub>; C: DMSO-d<sub>6</sub>) shows unequivocally that this is not the case and the behaviour is characteristic of a time- and, most commonly, concentration-dependent diffusion coefficient. The evolution of this dependency for the three solvents may be illustrated by plotting, as a function of time, the areas under the SRS signal profiles, normalized by the square root of time (i.e.,  $AUC/t^{1/2}$ ), as shown in Fig. 5D. The shaded areas on this graph represent the ranges of  $AUC/t^{1/2}$  (mean  $\pm$  SD) that would have been anticipated if the solvent diffusivities were constant. However, divergence from this situation is seen within 18 minutes for water, 6 hours for DMSO, and within a day for PG. These times reflect rather well the relative nail penetration rates of the three solvents and imply that the transport of water is at least an order of magnitude greater than those of DMSO and PG. The behaviour observed is consistent with the diffusivity of water increasing with its increasing uptake into the nail. Rapid nail hydration when immersed in water is well known, and the resulting swelling/opening of the keratin structure is manifested by an uptake-dependent increase in diffusivity (2). Similarly, the results for PG and DMSO suggest strongly that the progressive uptake of these solvents also impacts upon the

nail structure and facilitates enhanced diffusion. The much swifter passage of water, relative to the other two solvents, despite only a 4-fold difference in molar volumes, implies that there must be a strong size-dependence to at least one transport pathway across the nail that is accessible to water but excludes PG and DMSO.

Finally, it should be noted that the uptake of the solvents observed and analysed here is very likely less than the real amount for the reasons of light scattering and absorption discussed before. To take this phenomenon into account, using PG-d<sub>8</sub> as an example, the -CD<sub>2</sub> signal from the solvent was normalized by the corresponding -CH<sub>2</sub> resonance (Fig. 1D) from the nail at each position and time, and the 'corrected' profiles are in Fig. S4. While this results in a (relatively) modest adjustment to the data, most clearly evident at the longest exposure time, the correction in no way alters the interpretation of the data presented above. Nonetheless, it must be recognized that this approach represents only a step towards a truly quantitative measurement. The latter obviously requires appropriate validation with an independent analytical methodology and a more complete understanding of both inter-sample variability and the effects of different solvents (or more complex formulations) on the optical properties of the nail.

### **Nail morphology**

SEM images of control nail samples and of those exposed to the three solvents for 24 hours are shown in Fig. 6. The untreated nail surface is compact and relatively smooth, while solvent treatment appears to have loosened the structure and markedly increased surface roughness. It may be inferred, therefore, that the integrity of the outer nail has been compromised, at least to some extent, and this is consistent with recent research reporting an increase in surface porosity with hydration (20). Further precise details as to the molecular mechanism by which the uptake of a solvent facilitates its own diffusion across the nail cannot be deduced from the results obtained. Nonetheless, the SRS signal profiles, even with the important caveat that light absorption and scattering prevent any absolute quantification of the results, are consistent with the diffusivity of the solvents in the nail exhibiting some form of concentration-dependent behaviour, a phenomenon that appears to be common (at least for water) across other keratinized tissues, such as hair and the stratum corneum (2, 21). It is worth noting again that (because of the absorption/scattering limitation) that the effects observed and reported here are probably greater than those deduced from the results. Whether the solvent diffusional front proceeds uniformly and enhanced transport occurs in a similar fashion across the entire nail, or whether there are solvent 'channels' opened up at weak points in the barrier with increasing time of exposure to provide lower resistance pathways, remains to be seen. Nonetheless, it is clear from Fig. 6 that the solvents are in some way altering nail structure; the effect is not immediate, however, suggesting a process analogous, for example, to the penetration of solvent into glass polymers (19, 22) where, behind a moving front of the diffusing chemical, sufficient accumulation occurs to cause rapid relaxation and swelling. Further insight into the precise mechanisms involved in the non-classical

behaviour observed may be accessed by experiments proving the non-biomechanical properties of the nail after exposure as a function of time to the different solvents (e.g., using nanoindentation with atomic force microscopy (23) to determine hardness, viscoelasticity, etc.).

## Conclusions

SRS microscopy has been successfully used to unambiguously visualize the uptake of water, propylene glycol (PG) and dimethyl sulphoxide (DMSO) into the human nail plate and to characterize the diffusion of these solvents across the tissue. Analysis of the SRS signal profiles revealed the much faster transport of water through the nail, relative to PG and DMSO. Furthermore, the results demonstrate that all three solvents progressively enhance their own diffusion through the nail: as more solvent is taken up, there is a distinct deviation from ideal behaviour. This apparently concentration-dependent diffusivity is consistent with scanning electron microscopy of the outer nail surface that indicates each solvent's ability to undermine the integrity of the tissue. Unravelling the precise form of the concentration dependence observed for solvent diffusivity requires, *inter alia*, improved measurement reproducibility (particularly for water) and clarification as to whether it is appropriate to consider the nail as a homogenous membrane (24). An approach to account for signal attenuation with sample depth (due to light absorption and scattering) has also been proposed and illustrated, although further development of the idea will require independent validation and further characterization.

The research described is significant as it offers insight into the practical challenge of drug formulation for the treatment of nail disease, an important unmet medical need. The substantial barrier properties of the nail mean that the rate and extent at which topically applied drugs can reach (e.g., fungal) targets in the nail plate are very limited. The results presented here show that optimization of delivery platforms to the nail must prolong and sustain exposure of the barrier to excipients, such as common solvents like water, PG and DMSO, that can facilitate both drug and their own transport. In this way, it is envisaged, it should be possible to develop new and improved formulations that significantly increase the availability of drugs at their site(s) of action in and/or beneath the nail.

## Materials and methods

### Sample preparation

Deuterated water (D<sub>2</sub>O), PG-d<sub>8</sub> and DMSO-d<sub>6</sub> were purchased from Sigma-Aldrich Co., Ltd. (Gillingham, UK). Human fingernail clippings were obtained from healthy volunteers and stored at -20°C until use. The University of Bath Research Ethics Approval Committee for Health (REACH; EP 11/12 115) granted ethical approval for nail sampling, and all individuals donating nails gave informed consent. Each nail sample was carefully cleaned with deionized water and dried with absorbent tissue before each experiment. Prior to SRS imaging, 5 µL of neat solvent were applied over the nail (~16 mm<sup>2</sup> in area) which was then sandwiched between two glass coverslips within a Parafilm<sup>®</sup> frame, which acted as a spacer. The coverslips were sealed by melting the Parafilm<sup>®</sup> and further secured using double-sided tape. This ensured that the sample was tightly sandwiched to minimize solvent evaporation and sample dehydration during the time-lapse experiments.

### Raman spectroscopy

To identify suitable vibrational bond resonances for each solvent prior to SRS imaging, their Raman spectra, and that from the dorsal surface of a nail sample, were acquired using a Raman microscope (Renishaw RM1000, Renishaw plc, Wotton-under-Edge, UK) and Renishaw v1.2 WIRE software. A 1200-line/mm grating providing spectral resolution of 1 cm<sup>-1</sup> was used with a diode laser operating at 785 nm. The Raman band (520 cm<sup>-1</sup>) of a silicon wafer was used for calibration.

Raman spectra of three nails were also obtained across their cross-sections using a long x50 working objective. The nails were first sectioned with a microtome (Reichert Jung UltraCut E, Leica Microsystems Ltd, Milton Keynes, UK) to create a clean, horizontal plane, which was then positioned in the microscope with the cut edge orientated towards the objective. Streamline spectral mapping was performed on the planar (x-y) surface of the cross-section, from the dorsal to the ventral side of the nail. A Raman spectrum was recorded for each pixel (2.8 x 2.8 µm<sup>2</sup>) and 13 spectra were collected parallel to the nail surface for each row of the map. The acquisition time was 12.5 seconds per spectrum. All spectra were baseline-corrected with a third-order polynomial prior to analysis.

### SRS imaging

The SRS microscope consisted of a picosecond laser system and a modified commercial inverted laser-scanning microscope with a confocal laser scanner (FV300/IX171, Olympus UK Ltd, UK). Synchronized, dual-wavelength picosecond excitation was provided by an optical parametric oscillator (OPO) (Levante Emerald, APE, Berlin) which was synchronously pumped at 532 nm by a frequency-doubled Nd:Vanadium laser (picoTRAIN, High-Q GmB), delivering a 7 ps pulse train at a 76 MHz repetition rate. The OPO consisted of a temperature-tuned, non-critically phase matched lithium triborate (LBO) crystal, which allows the OPO signal (employed as

the pump beam) to be continuously tuned from 690 to 980 nm by adjusting the LBO temperature and an inter-cavity Lyot filter. A Si PIN photodiode was used to record the intensity variations of the OPO signal. The pump-laser fundamental (1064 nm) was also available as a separate output and was used as the Stokes beam, which was amplitude modulated at 1.7 MHz with an acousto-optic modulator (3080-197 Crystal Technologies, West Chester, PA, USA).

The pump beam and the modulated Stokes beam were spatially overlapped using a dichroic mirror (1064 DCRB, Chroma Technology Corp, Bellows Falls, USA) and temporally overlapped using a delay stage. The collinear beams were directed into the microscope and focussed onto the sample using a 20X 0.75 NA air objective (UPlanSApo, Olympus) and scanned in two dimensions using a pair of galvanometer mirrors. The resulting SRL in the pump beam was collected in the forward direction via a 1.0 NA condenser lens (LUMFI, Olympus) and detected by a large area photodiode (FDS1010, Thorlabs, New Jersey, USA). A band-pass filter (850/90 nm, Chroma) was mounted in front of the detector to block the modulated 1064 nm beam. Finally, a lock-in amplifier (SR844, Stanford Research Systems, Sunnyvale, CA, USA) was used to detect the SRL signal with a time constant of 30-100  $\mu$ s.

### **Scanning electron microscopy (SEM)**

SEM was used to investigate solvent effects on the integrity of the nail surface. A nail sample was cut into four pieces of approximately 4 mm<sup>2</sup> in area. Three were placed in sealed vials containing 1 mL of either water, PG or DMSO for 24 hours at room temperature; the fourth piece of nail served as an untreated control. Post-treatment, the nails were dried with tissue, mounted on the aluminium stubs using double-sided tape, and imaged by SEM (JEOL SEM6480LV, JEOL Ltd., Tokyo, Japan).

### **Data analysis**

All acquired SRS images were processed using ImageJ (U.S. National Institutes of Health, USA). Each data point was normalized against the OPO signal recorded on the PIN photodiode to correct for laser intensity fluctuations. Images of different Raman shifts were presented using different color schemes for ease of interpretation. Signal quantification of the image was performed using the 'plot profile' or 'plot z-stack profile' plug-ins, overlaid images were obtained using the 'color merge' function, and 3D images were produced using the '3D viewer' function. Data fitting was performed using GraphPad Prism<sup>®</sup> version 5.00 (GraphPad Software, San Diego, CA, USA).

For Raman spectral mapping of the nail cross-sections, non-parametric Kruskal-Wallis analysis followed by a Dunn's post-test was used to compare the -CH-stretching signals obtained at different distances perpendicular to the dorsal surface (i.e., depths into the nail) for each sample. The level of statistical difference was set to  $p \leq 0.05$ .

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## Figure Legends

**Fig.1:** (A) An example of part of the Raman spectrum taken from a cross-section of human nail. The area shaded in grey indicates  $-\text{CH}_2$  stretching ( $2830\text{-}2900\text{ cm}^{-1}$ ). (B) Light microscopic images of nail samples from three donors (V1, V3 and V3); spectral data were acquired from the areas within the boxes. The scale bar is  $60\text{ }\mu\text{m}$ , and illustrates that the individual nails had quite different total thicknesses. (C)  $-\text{CH}_2$  signal ( $n = 13$ , mean  $\pm$  S.D.) versus normalized depth for three nail samples (red, orange and green symbols from donor nails V1, V2 and V3, respectively). The vertical dashed lines represent a depth of  $60\text{ }\mu\text{m}$  into each nail sample. (D) Normalized  $-\text{CH}_2$  signal from another nail sample acquired as a function of depth during imaging using SRS microscopy following treatment with PG- $\text{d}_8$  for different periods of time. Normalization is performed with respect to the signal at the nail outer surface.

**Fig. 2:** Composite SRS x-z orthogonal view images of the penetration of  $\text{D}_2\text{O}$  into 5 regions of the nail as a function of time (the 5 panels on the right of the figure labelled as O-D). The visible spectrum scale indicates the O-D signal recorded every 2.7 minutes, from  $t = 10$  minutes (red) to  $t = 34.3$  minutes (magenta) post-application. The SRS image from the keratin in nail is shown in the far left panel (labelled as 'Nail'), while the background, off-resonance control is to the immediate right (labelled as 'OR'). Scale bar =  $20\text{ }\mu\text{m}$ .

**Fig. 3:** SRS x-y planar images of the penetration of (A) PG- $\text{d}_8$  (blue), and (C) DMSO- $\text{d}_6$  (green) into the nail (red) as a function of time and depth. OR shows the off-resonance background. Depths of images are indicated along the top while the experimental duration is indicated down the left-hand column. Scale bars =  $50\text{ }\mu\text{m}$ . SRS x-z orthogonal sections showing the diffusion of (B) PG- $\text{d}_8$  (blue), and (D) DMSO- $\text{d}_6$  (green) into the nail (red) at various times post-application. Composite images show the superposition of  $-\text{CH}_2$  and  $-\text{CD}_2$  signals. The arrows represent the directions of solvent movement into the nail. Scale bars =  $50\text{ }\mu\text{m}$ .

**Fig. 4:** Experimentally measured, normalized SRS signal versus nail depth profiles for (A)  $\text{D}_2\text{O}$ , (B) PG- $\text{d}_8$ , and (C) DMSO- $\text{d}_6$  as a function of time post-application ( $n = 5$ , mean + SD), compared to the corresponding predicted profiles calculated from Eq. 1 and assuming a constant value of  $D_i$  derived from fitting the data measured at the shortest time for each solvent, (D)  $\text{D}_2\text{O}$ , (E) PG- $\text{d}_8$ , and (F) DMSO- $\text{d}_6$ .

**Fig. 5:** Normalized SRS signal of (A)  $\text{D}_2\text{O}$ , (B) PG- $\text{d}_8$  and (C) DMSO- $\text{d}_6$  as a function of the composite variable,  $z/t^{1/2}$  ( $n = 5$ , mean + SD). Panel (D) shows the areas under these SRS signal profiles (AUC), normalized by  $t^{1/2}$ , as a function of time. The shaded areas (black for  $\text{D}_2\text{O}$ , blue for PG- $\text{d}_8$  and green for DMSO- $\text{d}_6$ ) represent the ranges of  $\text{AUC}/t^{1/2}$  (mean  $\pm$  SD) predicted for constant values of solvent diffusivities.

**Fig. 6:** SEM images of the dorsal nail surface, either (A) untreated or exposed to (B) water, (C) PG or (D) DMSO for 24 hours. The scale bars are  $50\text{ }\mu\text{m}$  for the 4 panels on the left, and  $10\text{ }\mu\text{m}$  for those on the right.

