Imaging the Prodrug-to-Drug Transformation of a 5-Fluorouracil Derivative in Skin by Confocal Raman Microscopy

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The widespread adoption of transdermal drug delivery has been limited by the barrier properties of the outermost layer of the epidermis, the stratum corneum (SC). A variety of approaches have been developed to overcome the barrier, including the use of a prodrug form of an active therapeutic agent to enhance transdermal delivery. Once in the epidermis, the pro-molecule is converted to the active drug by endogenous enzymes or simple chemical hydrolysis. The prodrug selected for the current studies, 1-ethyloxycarbonyl-5-fluorouracil, is known to enhance transdermal delivery of 5-fluorouracil, an important systemic antitumor drug. Using confocal Raman microscopy on pigskin biopsies treated with prodrug, we are able to image the spatial distribution of both prodrug and drug in the SC and viable epidermis, thereby providing information about permeation and metabolism. This approach may readily be extended to a variety of dermatological processes.

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INTRODUCTION

Transdermal delivery of drugs offers several important advantages for therapeutics, including ease of access, control of incorporation kinetics, and the relative non-invasiveness of application procedures (Barry, 2004; Prausnitz *et al.*, 2004). Various methods based on transient physical modification of the stratum corneum (SC) barrier have been developed to increase delivery of active agents, for example, iontophoresis, electroporation, and the use of chemical permeation enhancers. An alternative strategy, using prodrugs, entails modification of the physical and chemical properties of the active molecule. Evaluation of the success of transdermal delivery is limited by the absence of techniques that monitor *in situ* the spatial distribution of the drug, prodrug, or delivery vehicle. Any such method must be able to examine the outermost layers of intact skin in a non-perturbing manner and to distinguish closely related chemical species in the skin.

The Raman spectrum of a molecule provides a useful fingerprint for identification of substances without the necessity for external labels (which may perturb the physical properties of the molecules under study). Modern technology permits the acquisition of Raman spectra from skin in a microscopically resolved confocal manner (Caspers *et al.*, 2001), thereby providing a convenient, non-destructive method for monitoring the spatial distribution of exogenous materials and their biochemical transformations in intact skin.

5-Fluorouracil (5FU), an important systemic antitumor drug (Longley *et al.*, 2003), is also used to treat cancerous or precancerous conditions in skin including solar keratoses, actinic keratosis, superficial basal cell carcinoma, and Bowen's disease. Furthermore, 5FU is used to treat non-cancerous conditions in which cells are dividing rapidly, for example, psoriasis (Pearlman *et al.*, 1986). The delivery of 5FU in conventional topical preparations has been suboptimal, requiring methods such as light curettage to improve therapeutic utility (Epstein, 1985). A variety of prodrugs from 5FU have been prepared, one of which, 1-ethyloxycarbonyl-5FU (pro-5FU), provided a 25-fold increase in transdermal delivery (Beall *et al.*, 1994). The molecular structures of both prodrug and drug species are shown in the inset to Figure 1a. In this article, we describe the application of confocal Raman microscopy to image the spatial distribution of pro-5FU to 5FU conversion in skin.

RESULTS AND DISCUSSION

Differences in the Raman spectra (Figure 1a) of aqueous solutions of the prodrug and drug are significant in the low frequency region. Several bands likely arising from ring vibrations are diagnostic for each molecule, illustrating the potential for *in situ* differentiation of these molecules in the confocal Raman experiment. Included in Figure 1a is a spectrum of isopropyl myristate (IPM), the delivery solvent for the pro-5FU suspension. Only a few Raman bands in the

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Abbreviations: 5FU, 5-fluorouracil; IPM, isopropyl myristate; pro-5FU 1-ethyloxycarbonyl-5-fluorouracil; SC, stratum corneum

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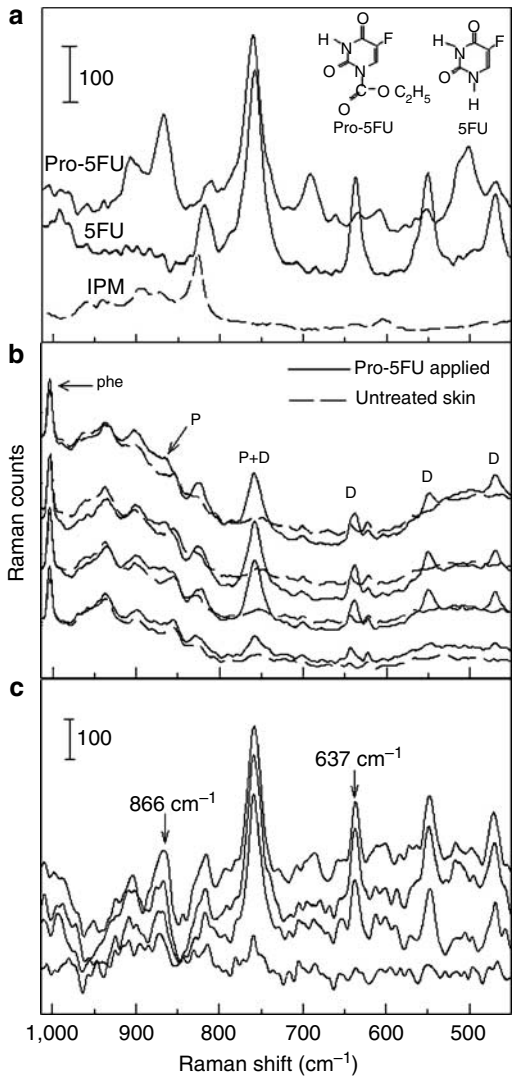

Figure 1. Raman spectra (450–1015 cm^{-1} region) probing pro-5FU (in IPM suspension) delivery of 5FU in pigskin. (**a**) Raman spectra of neat IPM (dashed line) and prodrug and drug solutions (solid lines, as noted). The molecular structures of pro-5FU and 5FU are shown in the inset. (**b**) Confocal Raman spectra of untreated (dashed lines) and pro-5FU-treated (solid lines) pigskin at different depths (2, 7, 12, and 17 μm) under the skin surface from top to bottom, respectively. Raman bands specific to prodrug (P) and drug (D), along with the phe ring-breathing mode are marked. (**c**) Confocal Raman spectra of pro-5FU-treated skin after subtraction of untreated skin spectra at equivalent depths. Spectra are stacked top to bottom as in (**b**). Raman bands used to quantify relative concentrations of prodrug (866 cm^{-1}) and drug (637 cm^{-1}) are annotated.

spectrum of IPM are observed in the same region as pro-5FU or 5FU. Bands at 866 and 637 cm^{-1}, free from interference from IPM bands, were chosen to monitor the relative concentrations of prodrug and drug, respectively. Spectra are presented over a higher wavenumber range in Figure S1a and S1b, supporting the selection of these two discrete bands in the low wavenumber region.

A suspension of pro-5FU was applied to intact pigskin (22°C) for 20 hours. Subsequently, Raman spectra were acquired at increasing depths beneath the skin surface in 5 μm increments. These were compared to spectra of untreated pigskin at equivalent depths (Figure 1b). In the Raman spectra of treated skin, features from prodrug and drug are observed along with Raman bands of the protein and lipid constituents of the SC. In a control experiment, pro-5FU was determined to be chemically stable in an IPM suspension monitored over a 1-month period. In an additional control experiment, when a suspension of 5FU in IPM was applied to skin, using the current experimental conditions, the drug permeated to a maximum depth of 5 μm. Thus, the presence of several bands arising from 5FU within the skin (Figure 1b) clearly indicates that *in situ* hydrolysis of the prodrug has taken place.

Evaluation of the spatial variation of the relative prodrug and drug concentrations requires an internal standard for Raman intensity. A useful spectral feature for this purpose, arising from endogenous skin proteins, is the ring-breathing mode of phe at 1,004 cm^{-1} (Figure 1b). This band diminishes in intensity with depth in the skin, and serves to calibrate for confocal scattering losses within the tissue (Figure S2). To determine the relative concentrations of pro-5FU and 5FU in skin, spectra of untreated skin are subtracted from spectra of treated skin at equivalent depths (see Materials and Methods). The difference spectra are shown in Figure 1c. The presence of both pro-5FU and 5FU is evident in spectra acquired at depths of 2, 7, and 12 μm beneath the skin surface. At a depth of 17 μm, bands arising from the drug are no longer visible, whereas the prodrug feature at 866 cm^{-1} is close to the noise level and is not considered significant. It is noted that the band parameters (positions and widths) of pro-5FU and 5FU in skin remain essentially unchanged from those in aqueous solution (Figure 1a). As Raman band parameters are often sensitive to environment, for example, solvent or intermolecular interactions, the similarity between the spectral features of the drug and prodrug in skin and in aqueous solution suggest that each molecule is located in an aqueous environment in skin. Consistent with this suggestion is the correlation between increased prodrug solubility in water and increased 5FU delivery through hairless mouse skin reported for this particular 5FU prodrug (Beall *et al.*, 1994).

An issue arises as to the depths and regions of the skin into which the prodrug or drug may permeate. More specifically, it is of interest to ascertain whether permeation is limited to the SC or whether the exogenous substances penetrate to the viable epidermis or dermis. Univariate analysis of spectral features or multivariate statistical algorithms such as factor analysis can be used to delineate native skin regions, that is, the methods are capable of differentiating the SC from the viable epidermis (Xiao *et al.*, 2004; Mendelsohn *et al.*, 2006; Zhang *et al.*, 2007). Factor analysis was applied to a set of confocal Raman spectra acquired from an untreated skin sample over the 800–1,015 cm^{-1} spectral region. Factor scores are shown as image planes (Figure 2a–c) for loadings that clearly differentiate the SC, viable epidermis, and dermis, respectively. The pigskin sample used for this analysis was taken from a biopsy similar to those used for pro-5FU permeation experiments. From the factor score images, the SC appears to be ~10–20 μm thick. Thus, for the data shown in Figure 1c, in which treated pigskin was incubated at 22°C,

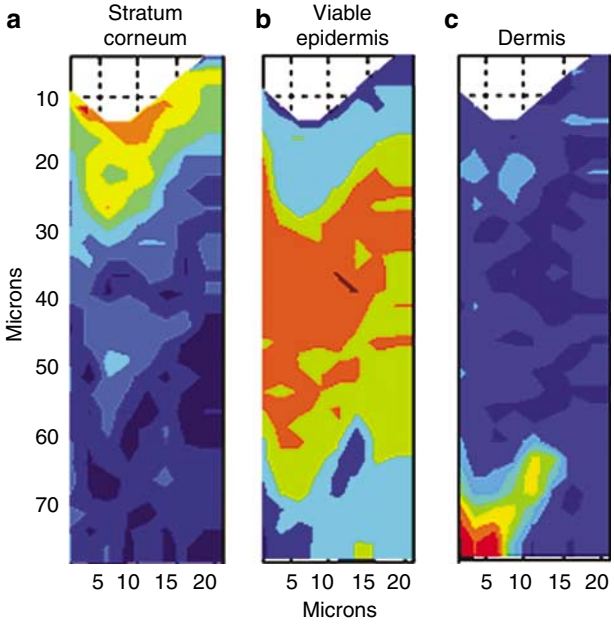

Figure 2. Factor analysis score image planes delineate skin regions. Factor analysis was conducted on baseline corrected confocal Raman image planes (800–1,015 cm^{-1} region) of untreated pigskin. The scores, red (highest) to blue (lowest), depict the fit of three different loadings (factors) to the original image plane and clearly differentiate the (**a**) SC, (**b**) viable epidermis, and (**c**) dermis.

we conclude that the majority of prodrug and drug reside in the SC and that permeation into the viable epidermis is not detected.

To evaluate the temperature dependence, permeation depths, and spatial distribution of the prodrug-to-drug conversion, confocal Raman images were acquired at both 22 and 34°C using a 5 μm step size. The higher temperature corresponds to the average surface temperature of human skin (Kraning, 1991). Image planes of the spatial distribution of the relative concentrations of pro-5FU and 5FU are presented in Figure 3 as band area ratios of the 866 and 637 cm^{-1} bands for pro-5FU and 5FU, respectively, each normalized to the phe band near 1,004 cm^{-1}, to correct for confocal losses as discussed above. The observed permeation differences between the two temperatures are striking. Consistent with Figure 1c, permeation of both species at 22°C is limited to the SC, whereas both are distributed throughout the SC and viable epidermis at 34°C. The distribution of pro-5FU at the higher temperature in Figure 3c shows a gradual decrease in concentration with depth for the top ~40 μm. In the lower half of the image (viable epidermis), a few low concentration pockets (marked with asterisks) are evident which correspond to higher concentrations of drug (Figure 3d). It thus seems likely that hydrolysis is taking place in both the SC and in the viable epidermis.

Nonspecific hydrolytic esterase and lipase activity have been observed in the intercellular spaces of the SC (Menon *et al.*, 1986; Beisson *et al.*, 2001) and throughout the viable epidermis (Sugibayashi *et al.*, 1999; Redoules *et al.*, 2005).

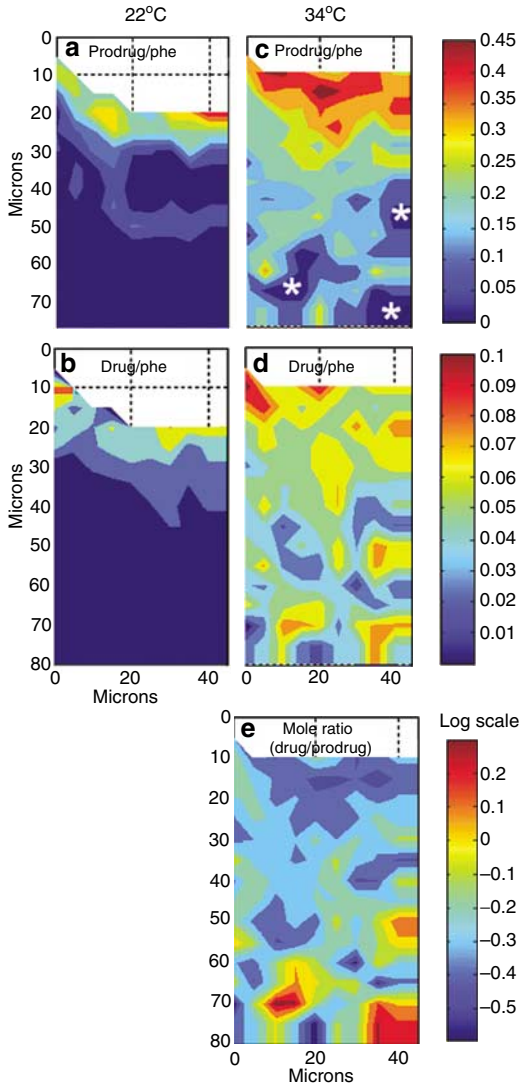

Figure 3. Confocal Raman image planes depict the temperature dependent permeation and hydrolysis of pro-5FU in pigskin. Pro-5FU treated pigskin incubated at (**a**) and (**b**) 22°C, and at (**c**) and (**d**) 34°C. The spatial distribution of pro-5FU concentration relative to phe present in endogenous skin proteins is measured by the band area ratio 866/1004 cm^{-1}, in (**a**) and (**c**), and similarly for relative 5FU concentration via the band area ratio, 637/1004 cm^{-1}, in (**b**) and (**d**). The scale bars (right) apply to both respective image planes (left). (**e**) Spatial distribution of the drug/prodrug mole ratio in pro-5FU treated pigskin with scale bar shown to right. Several regions in the viable epidermis have a relatively high concentration of drug (red) corresponding to low prodrug concentrations relative to phe as marked by the asterisks in (**c**).

Although a detailed comparison of the hydrolytic potential between the two skin regions has not been reported (to our knowledge), it seems reasonable to assume that conversion to 5FU would be more rapid in the viable epidermis owing to differences in water content and the presence of viable cells. The image of the drug-to-prodrug mole ratio (Figure 3e) supports this assumption. The highest relative amounts of drug with respect to prodrug are observed in the viable epidermis at depths between 40 and 70 μm from the skin surface (offset scale). Earlier reports of 5FU

delivery in mouse skin using the current prodrug suggested that the large amount of the promoiety delivered into the skin might saturate the enzymes (Beall *et al.*, 1994). In addition, the influence and extent of enzyme leaching could not be determined in earlier experiments where delivery was examined using a diffusion cell in which the skin is in contact with an aqueous receptor phase. Results from the current experiments where enzyme leaching is not expected suggest that while SC enzymes may be saturated, those in the viable epidermis do not appear to be at this level of prodrug dosing (see prodrug distribution in Figure 3c).

The increase in permeation at the higher temperature may be caused by the direct effect of temperature on diffusion along with a temperature-induced change in the SC barrier. Since the lipid barrier in the standard model of the SC (corneocytes embedded in a continuous lipid-enriched matrix) is thought to be primarily responsible for barrier function, we speculate that temperature-dependent changes in the lipid are partially responsible for the increased permeation. A temperature-induced solid–solid phase transition (orthorhombic to hexagonal acyl chain packing) in the lipids of isolated human SC has been reported between 20 and 40°C using transmission infrared (Mendelsohn *et al.*, 2006) and similarly for (cryo-) electron diffraction studies (Pilgram *et al.*, 1999). This transition occurs over a slightly lower temperature range in isolated porcine SC (Figure S3), most likely due to slight differences in porcine SC lipid composition. The loosening in acyl chain packing may be partially responsible for the increase in the observed prodrug permeability. Unfortunately, the infrared parameters that monitor this transition in isolated SC do not display the same patterns of spectroscopic activity in the Raman measurement. Therefore, this lipid transition cannot be detected in intact skin using confocal Raman. The association of the permeants with corneocyte envelopes or within corneocytes must also be considered. The ability to obtain high quality infrared and Raman spectra from isolated corneocytes has been demonstrated (Zhang *et al.*, 2006, 2007) and work in this area is ongoing. It is possible that access to the cells increases with the change in lipid packing.

One unanticipated result with important pharmacological and therapeutic ramifications is the observation of solid drug beneath the skin surface. The physical state (solution versus solid) of permeated 5FU is easily identified in the Raman spectra shown in Figure 4. Marker bands specific for solid drug at 768 and 996 cm^{-1} differ from those observed for 5FU in aqueous solution and can be used to clearly differentiate the state of 5FU in skin. The confocal Raman spectra of three separate skin samples shown in Figure 4 were each obtained from a depth of $\sim$35 μm underneath the surface, within the viable epidermis. At this position within skin, the relative concentration of 5FU as measured by the band area ratio of 637/1,004 cm^{-1} for the spectrum noted as skin + solid 5FU (0.6) is well above the comparable maximum value shown in Figure 3d (0.1). In spectra where the bands specific for solid 5FU (768 and 996 cm^{-1}) are present, the area of the phe band (1,004 cm^{-1}) is obtained by doubling the integrated area of the high frequency half of the band. Thus, increased delivery

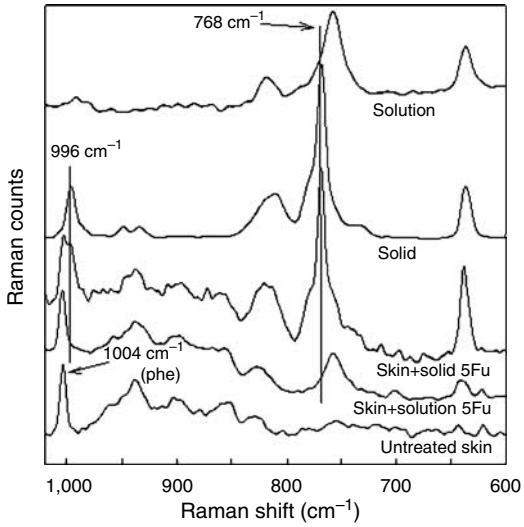

Figure 4. Evidence for the presence of solid 5FU within pigskin epidermis after treatment with pro-5FU. Raman spectra (600–1,020 cm^{-1} region) of an aqueous solution of 5FU, solid 5FU, and three separate pigskin samples acquired at a depth $\sim$35 μm from the skin surface (top to bottom). Bands marked at 996 and 768 cm^{-1} are specific for solid 5FU. The endogenous skin protein phe ring-breathing mode is also highlighted at 1,004 cm^{-1}.

of the prodrug and its conversion to its parent drug (5FU) resulted in a supersaturated solution of 5FU from which some solid 5FU precipitated.

The current confocal Raman investigation demonstrates the feasibility of monitoring *in situ* the spatial distribution of both prodrug and drug permeation in skin. The inherent sensitivity of the spectra permits the discrimination of closely related chemical species and physical states without the introduction of potentially perturbing probes or elaborate sample preparation. In addition, image planes of spectral parameters depict permeation gradients with respect to skin microanatomy (to depths of $\sim$70 μm). The techniques employed herein are likely to be applicable to various prodrug/drug topical skin treatments. Several combinations are currently being investigated, as is the influence of these agents on the conformation and distribution of endogenous skin constituents.

MATERIALS AND METHODS

Materials

5FU and IPM were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Pro-5FU was synthesized as described in Beall *et al.* (1994). Skin biopsies from Yucatan white, hairless pigs were purchased from Sinclair Research Center Inc. (Columbia, MO).

Sample preparation

The Rutgers University Internal Review Board has approved all protocols used herein. Aqueous solutions of pro-5FU (36 mM) and 5FU (22 mM) were prepared by warming to 60°C for 5 minutes. To keep the material dissolved, Raman spectra were acquired from solutions in glass capillary tubes warmed on a heated stage. Separate suspensions of pro-5FU and 5-FU in IPM were prepared by stirring mixtures for 48 hours at room temperature. The concentration of

each suspension was 6 M yielding enough excess solid to maintain saturation. Suspensions were topically applied to the SC of intact skin at a surface coverage of 5 μl/cm^2. Samples were sealed to prevent dehydration and held for 20 hours at either 22 or 34°C after which excess solid on the surface was rinsed off using fresh IPM. The treated skin samples, SC side up, were gently pressed into a milled brass cell, covered and sealed with a microscope coverslip for confocal Raman measurements. Several (5–7) separate skin samples were probed for prodrug/drug permeation at each temperature. In addition, numerous confocal lines and planes of spectra acquired from separate untreated skin samples were examined.

Confocal Raman microspectroscopy

Raman spectra were acquired with a Kaiser Optical Systems Raman Microprobe (Ann Arbor, MI). The instrument has been described previously in detail (Xiao *et al.*, 2004, Zhang *et al.*, 2007). Briefly, excitation is achieved with a solid-state diode laser at 785 nm. Approximately 12 mW of single mode power is focused with a 100 × oil immersion objective to a volume of ~2 μm^3 within the sample. The backscattered light illuminates a near-infrared CCD (ANDOR Technology, Model DU 401-BR-DD) with spectral coverage over 100–3,450 cm^{-1} at a spectral resolution of 4 cm^{-1}. Data are encoded every 0.3 cm^{-1} following linearization. Spectra are acquired using a 60-second exposure time, four accumulations, and cosmic ray correction. Confocal maps (axial lines and planes) were obtained at room temperature perpendicular to the skin surface using a step size of 5 μm. The time required to acquire an image plane varies with size and spatial resolution. For the images shown herein, acquisition time was 10 hours or less per sample. Previously published protocols (Xiao *et al.*, 2004, Zhang *et al.*, 2007) were used to evaluate axial characteristics of the current optical set-up. Briefly, the use of the oil immersion lens provides better refractive index matching with the skin sample than a dry objective, lessening depth distortion (to ~10%), and maximizing laser power. The actual axial spatial resolution in the skin samples cannot be directly ascertained.

Data analysis

Grams/32 AI software version 6.0 (ThermoGalactic, Salem, NH) was used for processing individual Raman spectra. Raman spectra were Fourier smoothed (80%) and a linear baseline was applied over the 800–1,014 cm^{-1} region in both treated and untreated skin spectra to better isolate the weak prodrug band at 866 cm^{-1}. The relatively large degree of smoothing could be applied without distorting spectral features. To evaluate the relative concentrations of pro-5FU and 5FU at different depths in skin, spectra of untreated skin are weighted (so as to normalize a pair of spectra to the phe band intensity) and subtracted from spectra of treated skin at equivalent depths.

ISys software version 3.1 (Spectral Dimensions, Olney, MD) was used to perform factor analysis, integrate band areas, and generate Raman image planes. Factor analysis was performed as described previously (Zhang *et al.*, 2007) over 800–1,015 cm^{-1} spectral region using the iterative score segregation algorithm available in ISys software.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. Raman spectra analyzing pro-5FU (in an IPM suspension) delivery of 5FU in pigskin.

Figure S2. Confocal Raman scattering loss with depth in pigskin demonstrated by an image plane of the integrated area of the phe ring-breathing mode (995–1010 cm^{-1}).

Figure S3. Transmission infrared measurement evaluating lipid acyl chain packing as a function of temperature in isolated SC.

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