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Fourier transform infrared spectroscopy studies of lipid domain formation in normal and ceramide deficient stratum corneum lipid models

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ABSTRACT

The current work describes thermotropic and kinetic Fourier transform infrared (FTIR) spectroscopy studies of lipid dynamics and domain formation in normal and ceramide (CER) deficient lipid samples designed as simple models of the stratum corneum (SC). For the first time, this work focuses on the time dependence of lipid reorganization and domain formation in CER deficient models. By utilizing deuterated fatty acid (FA) and simultaneously monitoring the methylene vibrational modes of both CER and FA chains these experiments follow the time evolution of lipid organization in these SC lipid models following an external stress. Kinetic and thermotropic experiments demonstrate differences in both CER and FA chain fluidity and ordered domain formation with decreased levels of CER. In the CER deficient model, the formation of CER orthorhombic domains is retarded compared to the normal model. Furthermore, there is little evidence of hexongally packed (or mixed) FA domains in the CER deficient model compared to the models of normal SC. These data demonstrate that barrier lipid organization, in terms of ceramide domain formation, is altered in the ceramide deficient model. This work highlights the successful development of an experimental methodology to study time dependent changes in lipid biophysics in simple SC model membranes and suggests this approach will prove useful for understanding some of the biophysical changes that underlie impaired physiological barrier function in diseased skin.

1. Introduction

The stratum corneum (SC), the outer stratum of the epidermis, is composed of keratin filled corneocytes within a lipid matrix of ceramides, free fatty acids, and cholesterol. In healthy skin the extracellular lipid species are present in approximately equimolar concentrations. The SC provides the primary barrier to water loss from the body, as well as providing protection from external insults. As such, a healthy functioning SC is essential to human physiology.

In healthy skin, SC lipids have been shown to exist in orthorhombic and hexagonally packed lamellar phases that coexist with more disordered (liquid–crystalline) lamellar phases (Forslind, 1994; Pilgram et al., 1999; Bouwstra et al., 2002; Moore, 2002; Mendelsohn et al., 2006; Bouwstra, 2009).

A wide variety of biophysical techniques including X-ray diffraction, transmission electron microscopy (TEM), differential scanning calorimetry (DSC), atomic force microscopy (AFM), nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy have been deployed to study intact and isolated SC, as well as various *in vitro* models (Bouwstra et al., 1991; Naik and Guy, 1997; Moore et al., 1997b; Lafleur, 1998; Pilgram et al., 1999; Moore and Rerek, 2000; Percot and Lafleur, 2001; Rowat et al., 2006; Pensack et al., 2006; Chen et al., 2007; Gooris and Bouwstra, 2007). Depending on the questions being asked these models have ranged from single ceramide systems to complex multi-component mixtures of fatty acids, ceramides, cholesterol and cholesterol esters, as appropriate (Moore et al., 1997a,b; Chen et al., 2001; Gooris and Bouwstra, 2007; Caussin et al., 2008; Groen et al., 2011).

The current work employs *in vitro* SC lipid models in combination with FTIR spectroscopy methods to probe the organizational changes in SC lipids associated with dry (xerotic) skin, a condition of significant interest in dermatology, pharmaceutical and cosmetic research. It is well documented that changes in SC lipid composition, including reduced levels of ceramides, are associated with dermatological diseases such as atopic dermatitis, psoriasis, lamellar ichtyosis, and winter xerosis (Fulmer and Kramer, 1986; Imokawa et al., 1991; Rawlings et al., 1994a,b, 2002; Rogers et al., 1996; Pilgram et al., 2001; Denda, 2002; Rawlings and Matts, 2005; Harding et al., 2005). In general, these conditions are associated with reduced barrier function, as determined by trans epidermal water loss (TEWL), and with changes in the lamellar lipid matrix (Rawlings et al., 1994a,b).

Abbreviations: SC, stratum corneum; CER, ceramides; FFA, free fatty acids; CHOL, cholesterol; FTIR, Fourier transform infrared.

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To characterize barrier lipid organization in diseased skin the current work compares *in vitro* SC lipid models with reduced ceramide levels to models of normal SC containing equimolar levels of ceramides, fatty acid, and cholesterol. The thermotropic and kinetic response of these models following perturbation is characterized utilizing FTIR spectroscopy methods we have previously described (Moore et al., 2006; Pensack et al., 2006). We note that our model systems do not exactly replicate the organization of lipids within intact human SC. However, the model lipid approach has been validated in many studies and provides a useful foundation before progressing to more complex experimental models. For the first time the current work focuses on the time dependence of lipid reorganization in lipid models of compromised skin. This allows us to characterize how changes in lipid composition, such as those associated with diseases conditions or external environmental stresses, lead to alterations in lipid dynamics and domain formation in our model systems.

2. Materials and methods

Perdeuterated stearic acid (octadecanoic-d35-acid) was purchased from CDN Isotopes (Quebec, Canada). Cholesterol and non-hydroxy fatty acid bovine brain ceramide powder were purchased from Sigma–Aldrich (St. Louis, MO). These lipids constitute a simple model of the major lipid classes of the skin barrier (fatty acids, cholesterol and ceramide) and have been used in many previous studies.

2.1. Preparation of SC lipid samples for thermotropic and kinetic studies

Two different SC lipid models were investigated in this study. The model of normal SC has a 1:1:1 molar ratio composition of free fatty acids (FFA):cholesterol (CHOL):ceramides (CER) while the ceramide deficient lipid model has a composition of FFA:CHOL:CER in a 1:1:0.5 molar ratio. Samples were prepared by co-dissolving the lipids in dichloromethane and methanol (2:1, v/v), evaporating the solvent at 50 °C in an oven for 24 h then hydrating the lipid film in excess pH 5.5 citrate buffer (to mimic SC pH) by repeated heating/cooling cycles and mixing with vortex action. The maximally hydrated lipid samples were sandwiched between AgCl IR windows and placed in a temperature controlled transmission cell (Harrick Scientific, Ossining, NY, USA). Sample temperature was controlled with a circulating bath and monitored with a thermocouple placed in the sample holder. Samples were heated to 85 °C to ensure maximum sample mixing and then cooled to 19 °C after which spectra were acquired at 3 °C intervals to 85 °C. To study the time dependence of lipid reorganization samples were heated to 80 °C to ensure maximum lipid disorganization and mixing after which the samples were rapidly cooled to 31 °C (to approximate skin surface temperature) and spectra were collected every hour for 11 h. All experiments were repeated in triplicate with new lipid samples prepared for each experiment. Equivalent results were obtained for each experimental sample. The presentation of FTIR spectroscopy data from the current studies follow standard biophysical spectroscopy protocols for membrane lipid studies (Moore et al., 1997a,b, 2006; Mendelsohn and Moore, 1998; Moore and Rerek, 2000; Moore, 2002) showing a representative data set in a figure (since all experimental results were very similar).

2.2. FTIR data analysis

All spectra were acquired with a FTIR Nicolet™ 6700 spectrometer equipped with a sample shuttle and a deuterated triglycine sulfate (DTGS) detector. Spectra were collected in transmission mode and generated from 30 co-added interferograms collected at 2 cm^{-1} resolution and analyzed using software GRAMS 32/AI version 7.0 (Thermo Galactic, Salem, NH, USA) and OMNIC™ version 7.0 (Thermo Electron Corporation, Madison, WI, USA). Peak positions were determined from the true maximum of second derivative spectra generated in GRAMS. Plots were generated using Sigma Plot (Systat Software Inc., San Jose, CA, USA) and Excel (Microsoft Office, 2007, WA, USA). For the kinetic studies a macro was written to collect 12 spectra, which included the t_0 spectrum, therefore spectra were acquired every hour for 11 h after the sample reached 31 °C.

2.3. IR spectral regions used to monitor lipid organization and dynamics

FTIR spectroscopy provides a direct measurement of the conformational order (membrane "fluidity") and chain packing (lateral in-plane membrane "domains") of hydrocarbons chains in lipid membranes (Moore, 2002). The most intense chain modes in the IR spectra of lipids are generally derived from the methylene (CH_2) stretching vibrations, or CD_2 modes in deuterated chains (Mendelsohn and Moore, 2000). The symmetric (ν_{sym}) and asymmetric (ν_{asym}) CH_2 modes occur at ~2850 cm^{-1} and ~2920 cm^{-1} in the protonated chains of ceramides, whereas the symmetric (ν_{sym}) and asymmetric (ν_{asym}) CD_2 occurs at ~2090 cm^{-1} and ~2195 cm^{-1} in the perdeuterated chains of the fatty acids (Mendelsohn and Moore, 1998). Generally, the methylene stretching modes provide information on chain intra-molecular conformational order, or fluidity (Snyder et al., 1978; Naik and Guy, 1997; Mendelsohn and Moore, 2000). The symmetric and asymmetric methylene stretching modes generally provide equivalent information on chain conformational order (fluidity). Following this convention we have plotted the symmetric CH_2 and asymmetric CD_2 stretching modes in the current work. In addition, inter-molecular chain packing can be directly monitored via methylene scissoring (δCH_2 and δCD_2) and rocking (ρCH_2 and ρCD_2) modes, these modes exhibit a characteristic band splitting in crystalline orthorhombic phases that is diagnostic for the presence of homogenous fatty acid or ceramide domains (Dluhy et al., 1985; Moore et al., 1997b; Mendelsohn et al., 2006; Boncheva et al., 2008).

A representative FTIR spectrum of a SC lipid model of ceramide, deuterated fatty and cholesterol is shown in Fig. 1a with the above peaks indicated. No unique cholesterol peaks are indicated. A schematic of lipid inter- and intra-molecular organization in bilayers relevant to SC lipid organization is displayed in Fig. 1b. Fully extended *all-trans* orthorhombically packed chains (crystalline arrangement) are shown on the left side, *all-trans* hexagonally packed chains (gel phase) are in the middle, and on the right lipid bilayers with disordered packing (liquid–crystalline arrangement) (Moore, 2002).

3. Results and discussion

3.1. FTIR thermotropic studies on SC lipid models

The thermotropic response of the ν_{asym} CD_2 stretching mode from deuterated octadecanoic acid and the ν_{sym} CH_2 stretching mode arising primarily from ceramide in the normal SC lipid model are plotted in Fig. 2a. In the normal lipid model both the fatty acid and ceramide chains are conformationally ordered up to ~50 °C as indicated by a ν_{sym} CH_2 of ~2849 cm^{-1} for CER and a ν_{asym} CD_2 of ~2193.5 cm^{-1} for the FA. At ~55 °C a transition occurs in the ν_{asym} CD_2 to a higher frequency of ~2196.5 cm^{-1} as the FA chains undergo a transition to liquid crystalline (disordered) bilayers. Interestingly, at 55 °C the CER chains remain ordered (~2849.50 cm^{-1}) and do not undergo a transition to disordered chains until ~60 °C (Fig. 2a). The separate phase transitions of CER and FA in the normal lipid

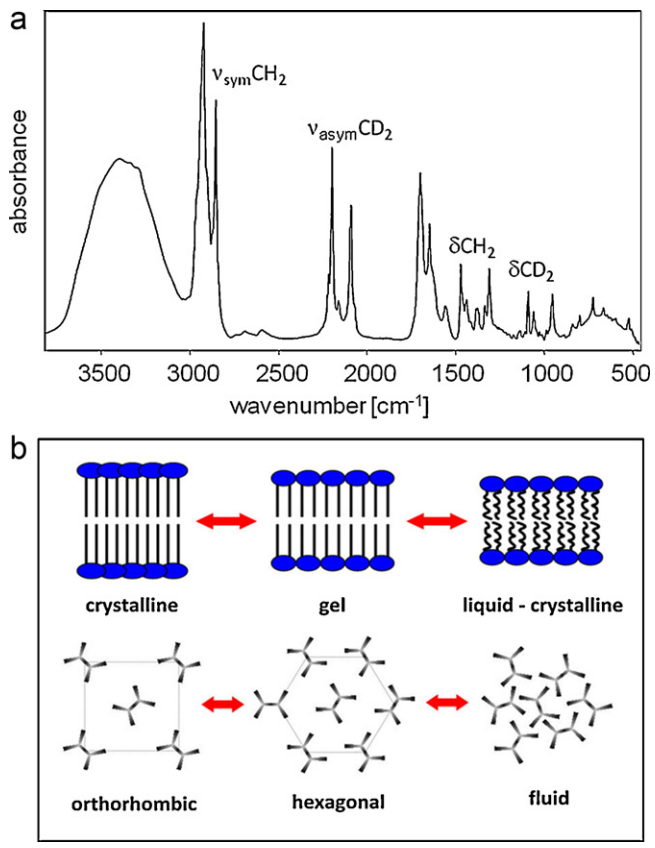

Fig. 1. (a) FTIR spectrum of a lipid model indicating major peaks of interest in this work and (b) a schematic illustration of inter- and intra-molecular lipid organization in SC lipid lamellae.

model indicates that separate CER and FA domains exist in the normal SC model which is consistent with previous studies (Moore et al., 1997a,b; Moore, 2002; Chen et al., 2001; Rerek et al., 2005). It should be noted, however, that the transition temperatures for both fatty acid and ceramide chains are reduced from those of the pure lipid species (data not shown). This indicates that there are some mixed phases in the models and the lipids not completely phase separated.

In Fig. 2b the equivalent parameters are plotted for the ceramide deficient lipid model and show the CER ν_{sym} CH_2 stretching mode and the FA ν_{asym} CD_2 stretching mode undergo a synchronous frequency increase around 58–60 °C as both lipid components undergo a phase transition to disordered (fluid) bilayers at the same temperature. This raises a question as to whether the CER and FA components are predominantly packed homogenously in the ceramide deficient model (with few or very small separate domains) resulting in a single phase transition. However, the lack of a separate T_m for CER could indicate that as the FA chains melt the CER chains are miscible in the excess FA fluid phase and thus no separate CER phases exist once the FA is disordered.

To investigate the inter-molecular packing of the FA chains in both normal and ceramide deficient SC lipid models, and to address the question above as to whether mixed domains are present in the ceramide deficient model, the thermotropic response of the deuterated FA δCD_2 scissoring mode is plotted in Fig. 2c and d. These data clearly indicate the presence of separate orthorhombic FA domains in both lipid models as indicated by the presence of two peaks (splitting) in the FA CD_2 scissoring mode. The presence of two peaks is diagnostic of orthorhombic phases in which neighboring molecules are of the same isotope (in this case deuterated FA). The two SC lipid models, however, do exhibit differences in packing behavior. In the normal model, the FA orthorhombic domains collapse at ~55 °C (as expected given the increase in chain fluidity at 55 °C apparent in Fig. 2a). In the ceramide deficient model the

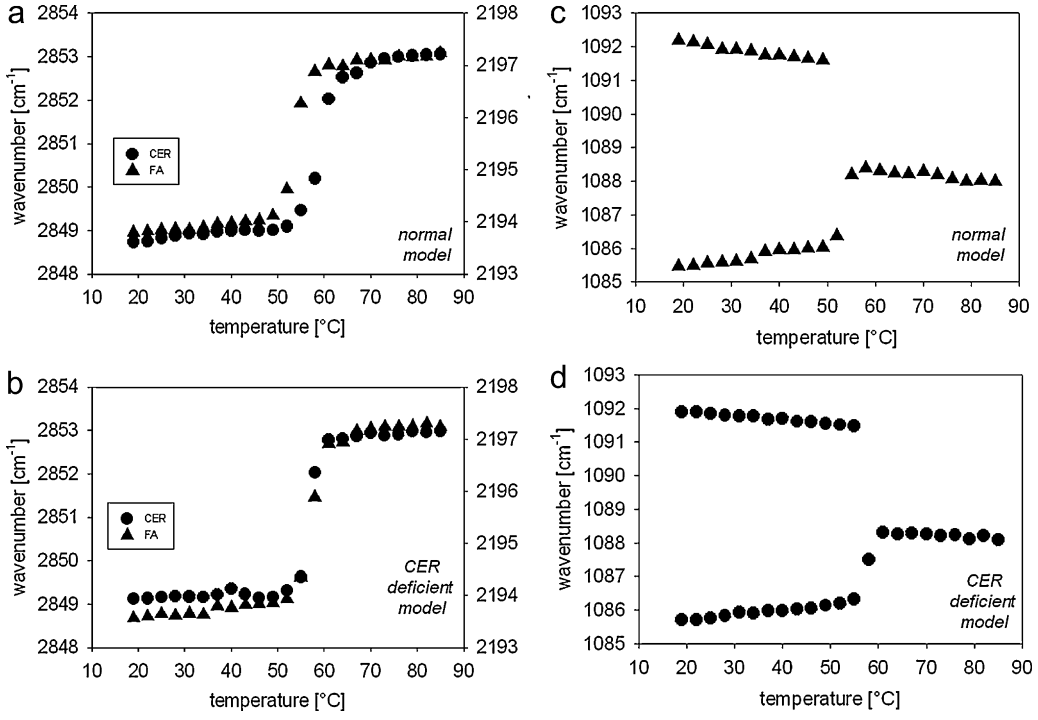

Fig. 2. (a) Thermotropic response of ν_{asym} CD_2 stretching mode from deuterated octadecanoic acid and ν_{sym} CH_2 stretching mode primarily arising from ceramide in the normal SC model. (b) Thermotropic response of the ν_{asym} CD_2 stretching mode and the ν_{sym} CH_2 stretching mode in the ceramide deficient SC model. (c) The CD_2 deuterated octadecanoic acid scissoring peak showing a singlet characteristic of hexagonal packing at 1088 cm^{-1} and a doublet characteristic of orthorhombic packing at 1092 cm^{-1} and 1085 cm^{-1} in the normal lipid model. (d) The CD_2 deuterated octadecanoic acid scissoring splitting showing hexagonal packing at 1088 cm^{-1} and orthorhombic packing at 1092 cm^{-1} and 1085 cm^{-1} in the ceramide deficient model.

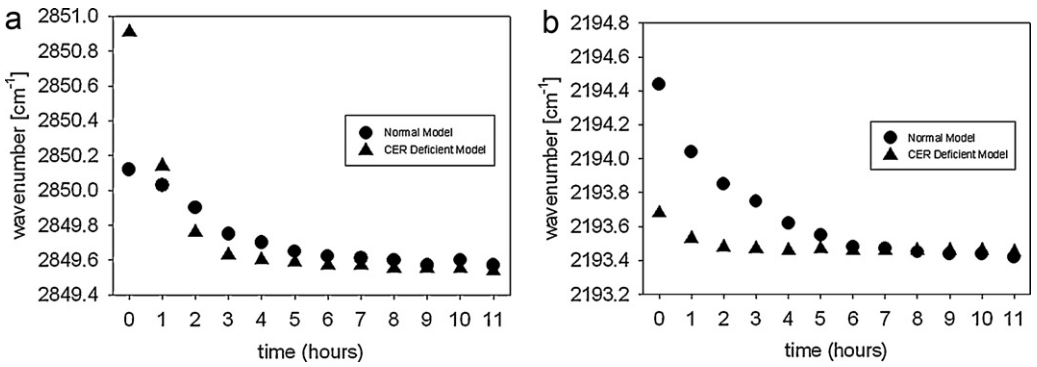

Fig. 3. (a) Chain conformational order (primarily ceramide) in the normal and ceramide deficient lipid model (a), fatty acid chain fluidity in the normal and ceramide deficient lipid model (b).

crystalline orthorhombic phase persists to 60 °C, as indicated by the strong splitting in the CD_2 scissoring mode, then collapses abruptly (Fig. 2d). The observation of splitting in the CD_2 scissoring mode in the ceramide deficient model is a direct evidence of significant FA domains. Indeed, the lack of a peak at 1088 cm^{-1} indicates there is little FA in hexagonal or more disordered domains. One explanation of the above would be that CER chains are distributed in very small clusters throughout the membrane, or mixed with cholesterol rich phases. When the purer FA domains melt, these other phases are miscible into the new fluid phases.

3.2. FTIR kinetic studies on SC lipid models

To investigate the kinetics of lipid reorganization in response to a physical stress SC lipid models were heated to 80 °C to achieve maximum homogeneity and then rapidly cooled to the surface

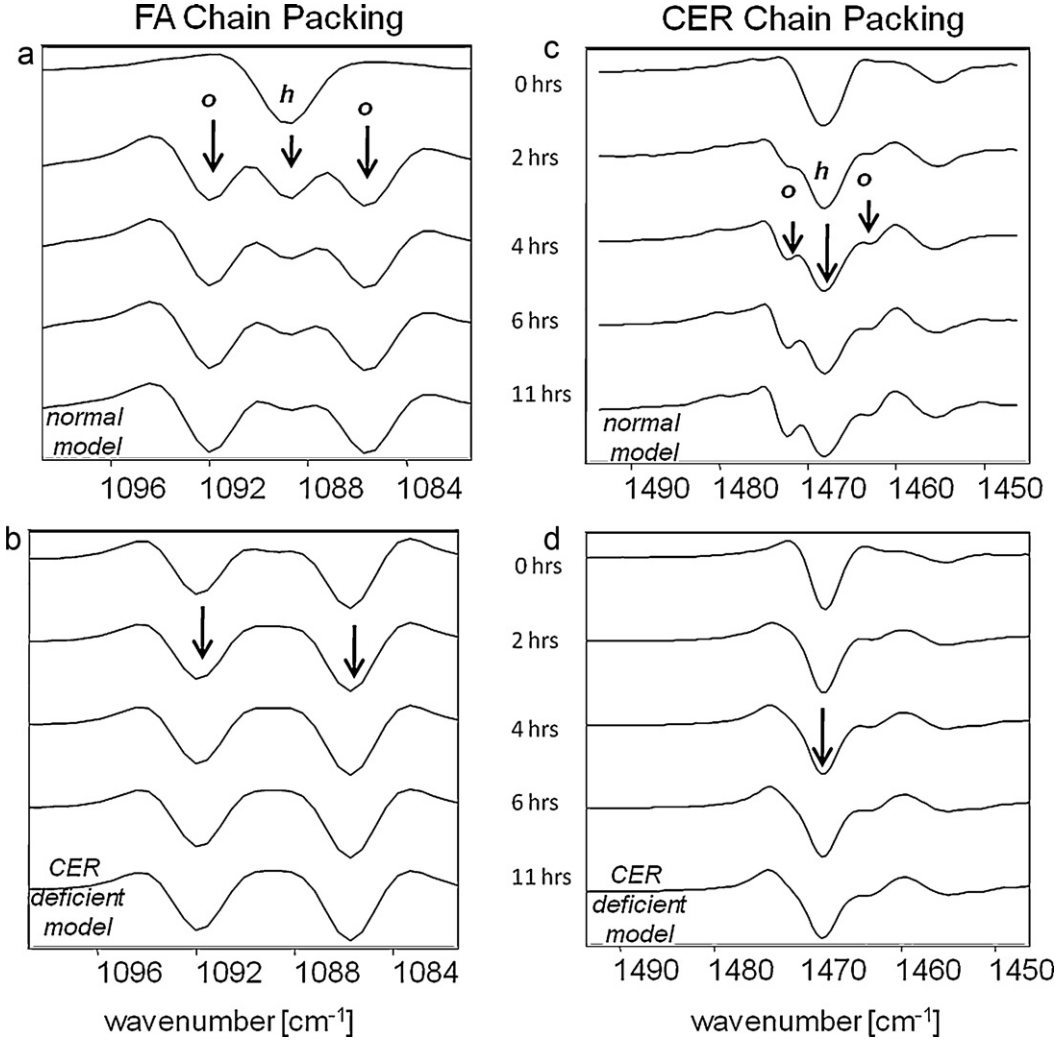

Fig. 4. Second derivative spectra showing the δCD_2 fatty acid (a, b) and δCH_2 ceramide (c, d) methylene scissoring modes. The arrows indicate peaks characteristic of orthorhombic (o) and hexagonal (h) inter-molecular packing for the fatty acid chains (1092, 1085 and 1088 cm^{-1}) and for the ceramide chains at (1463, 1473 and 1468 cm^{-1}) for both the normal and ceramide deficient lipid model.

temperature of skin (~31 °C). The intention is to model changes in lipid biophysics that might occur in the outer epidermis and how disease states impact this behavior. Inter-molecular coupling between deuterated fatty acid chains and between protonated ceramide chains was monitored as a function of time for 11 h via characteristic changes in the IR spectra. These temporal spectral data provide a kinetic description of increasing chain order, and separate orthorhombic domain formation as, and if, phase separation and domain formation of CER and FA occurs.

To monitor the response of intra-molecular chain conformational order in SC lipid model components, we investigated the kinetics of the ν_{asym} CD_2 stretching mode from deuterated FA (at ~2195 cm^{-1}) and the ν_{sym} CD_2 stretching mode, primarily arising from CER (at ~2850 cm^{-1}). The frequencies of both modes decrease as lipid chains become more ordered. The time dependent decrease in the ν_{sym} CH_2 stretching mode associated with CER is plotted in Fig. 3a and shows that in the normal lipid model the CER chains undergo a gradual ordering over 6 h. In the CER deficient lipid model, although the CER chains are initially more disordered, they rapidly order and reach their maximum conformational order within 3 h (Fig. 3a). The data shown are from one of the triplicate experiments and are representative.

Ultimately, the conformational order of CER chains is equivalent in both models and stabilizes at ~2849.6 cm^{-1}. An equivalent representative plot of the FA ν_{asym} CD_2 stretching mode is shown in Fig. 3b for both lipid models. The FA chains in the normal model show a gradual increase in chain order over 6 h to their stable state at ~2193.4 cm^{-1}. In contrast, the FA chains in the CER deficient model are significantly more ordered at time zero and reach the equivalent maximum conformational order within 2 h, after which the FA chains maintain their conformational order for the remaining 9 h.

To follow the kinetics of inter-molecular chain packing and the evolution of ordered separate domains the CH_2 scissoring mode (δCH_2) for CER (1462–1473 cm^{-1}) and the CD_2 scissoring modes (δCD_2) for deuterated fatty acids (1085–1092 cm^{-1}) were monitored. As discussed previously these modes are sensitive to subcell packing and are diagnostic for the presence of orthorhombic phases and separate domains of CER and FA. Second derivative spectra from the 1100–1080 cm^{-1} spectral region where the CD_2 scissoring modes (δCD_2) of FA occur were collected at 0, 2, 4, 6 and 11 h and are shown in Fig. 4a and b, for the normal and CER deficient model respectively. It is clear that 3 peaks are present for the normal lipid model (Fig. 4a) and only two peaks are present (Fig. 4b) for the CER deficient model. In both cases the outer peaks (~1085 and ~1092 cm^{-1}) show the splitting of the CD_2 scissoring mode (δCD_2) providing direct evidence for the presence of separate orthorhombic FA domains in both lipid models. However, in the normal model (Fig. 4a) the initial peak at 1088 cm^{-1} indicates hexagonally packed FA lipids and this peak persist even as orthorhombic domains emerge. In the CER deficient model there is no evidence of this FA phase.

Second derivative spectra from the 1445 to 1495 cm^{-1} spectral region where the CH_2 scissoring modes (δCH_2) of CER occur are shown at 0, 2, 4, 6 and 11 h in Fig. 4c and d, for the normal and CER deficient model respectively. It is very clear that 3 peaks are present for the normal lipid model (Fig. 4c) and only one dominant main peak is present (Fig. 4d) for the CER deficient model. In the normal model the outer peaks (~1464 and ~1472 cm^{-1}) represent the splitting of the CH_2 scissoring mode (δCH_2) that occurs in orthorhombic CER domains, providing direct evidence for the presence of separate orthorhombic CER domains. The center peak at ~1468 cm^{-1} in the normal model (Fig. 4c), which is the only clear peak in the CER deficient model (Fig. 4d) indicates hexagonally packed CER chains or chains present in mixed domains. This peak is present at all times in both models, although it is only in the normal model that it coexists with orthorhombically packed CER chains. We note there is some slight peak broadening in Fig. 4d at 11 h that may indicate some small domains beginning to form.

4. Conclusions

These initial FTIR thermotropic and kinetic studies of lipid organization and domain formation in simple lipid models of compromised and normal stratum corneum demonstrate measurable differences in CER and FA molecular organization, and re-organization following stress. Lipid organization in the SC is ultimately responsible for the water permeability barrier, which is the primary physiological function of the SC, and is necessary for terestrial life. Previous work from many laboratories has demonstrated that simple barrier lipid models are useful tools for understanding some of the changes occuring *in vivo* in SC lipid organization, and that these correlate to physiological barrier function. The successful development of a kinetic experimental methodology to study lipid reorganization following an external stress using such *in vitro* models suggests this approach will prove useful for understanding some of the stratum corneum barrier changes that occur *in vivo* in diseased or damaged skin conditions. Such experimental work is just beginning.

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