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Mechanical, compositional, and microstructural changes caused by human skin maceration

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ABSTRACT

We quantify the effects of prolonged exposure to water on the mechanical, compositional and structural degradation of human skin. This environmental exposure is associated with maceration and the pathogenesis of dermatitis, immersion foot syndrome, and trench foot. Uniaxial tensometry reveals that water immersion of isolated stratum corneum (SC) ranging in age across 27 to 87 years can notably stiffen the tissue and cause both reductions in plasticity and rupture energy when allowed to equilibrate to a high relative humidity. Subsequent water sorption studies reveal that water immersion induced decreases in plastic extensibility are caused by a reduction in the tissue's ability to hold water. In turn, this retention decrease is caused by a depletion in both lipids and natural moisturising factors. Further scrutiny of SC mechanical properties further reveals a clear delineation around 70 years. Employing this age-based categorisation, we establish that the degradative impact of water immersion is greatest for SC aged over 70 years. Histological examinations of full-thickness skin further reveal that prolonged water immersion for 7 days causes epidermal delamination, SC cavitation, and degradation in dermal collagen integrity. Immersion of skin in water at colder temperatures however reduces this degradation.

1. Introduction

The epidermis acts as a physical, chemical and microbial barrier to the external environment [1]. When ruptured, this barrier function is lost. Overexposure of the skin to wet and unsanitary environments, that can occur through occupational risks, flooding disasters, or drowning events of interest to forensic police investigations, can result in skin conditions characterised by erythema, blisters and tissue maceration; eventually leading to skin rupture, open sores and necrosis in severe cases. Conditions associated with the prolonged water immersion of skin range from subacute dermatitis [2] to more severe immersion-foot syndromes [3]. Yet, to date, the mechanistic cause of how skin tissue is affected by prolonged exposure to wet conditions remains unclear.

The most superficial stratum corneum (SC) skin layer experiences the greatest impact from the external environment. Previous studies have revealed numerous factors that alter the mechanical properties and integrity of this tissue layer. These include ageing [4], ultraviolet radiation [5,6], temperature [7], hydration [8,9] and chemical treatment [10,11]. Of these, hydration has the greatest impact on SC mechanical properties. At ~70% relative humidity (RH), the SC transitions from a ductile material, capable of undergoing significant plastic deformation prior to rupture, to a more brittle material [12,13]. Significant reductions in SC lipids, that are ordinarily present to minimise trans-epidermal water loss, and hygroscopic Natural Moisturising Factors (NMFs) that help to preserve the tissue's hydration, are associated with tissue stiffening, reductions in tissue extensibility [13], and increased trans-epidermal water loss [14]. While compromised SC barrier functionality has been attributed to changes in compositional, mechanical and water retention properties, to date, a comprehensive correlation amongst these three factors has not yet been made. Furthermore, the effects of prolonged or accumulated exposure of human skin tissue to wet environments has not yet been extensively studied. In this article we first quantify how prolonged water immersion for periods of up to 7 days impacts the mechanical properties of SC, then identify the underlying causes of this mechanical degradation in terms of the tissue's water retention, and changes in lipid and NMF content. Finally, we study the impact of water immersion on the integrity of full-thickness skin tissue.

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Abbreviations

SC	Stratum corneum
DI	Deionised
RH	Relative humidity
h	Hour
min	Minute
NMF	Natural moisturising factor

2. Results and discussion

2.1. Global changes in mechanical properties of SC with water immersion

Uniaxial tensometry was used to quantify changes in the mechanical properties of isolated human SC samples (20 × 5 mm), induced by immersion in deionised (DI) water for periods of 30 min, 6 h, 12 h, 36 h, and 7 days. 30-min immersion was used as a control so that all samples were immersed in water. However, this treatment time does not permanently damage intercellular lipid lamellae, which has been shown to impact SC's ability to retain water and alter mechanical properties [11]. Tissue specimens ranged in age across 27–87 years. Details of the deidentified specimens are provided in supplemental Table S1. Fig. 1 shows changes in the average (a,e) Young's modulus, (b,f) modulus of toughness, (c,g) fracture strain, and (d,h) ultimate tensile strength of SC samples with increasing water immersion period. Fig. 1(a)–(d) show that water immersion causes no significant change in the mechanical properties of SC, equilibrated to 32% RH for 24 h prior to testing. At this RH condition, SC deforms elastically up until fracture, and this behaviour appears unaffected by water immersion. In contrast, Fig. 1(e)–(h) show that SC equilibrated to 100% RH for 24 h prior to testing exhibits notable mechanical changes with water immersion period. Relative to SC equilibrated to 32% RH, Fig. 1(e) highlights that equilibration to 100% RH exhibits notably reduced Young's moduli, in agreement with previously reported changes [8,13]. Relative to controls, water immersion for 36 h or greater induces monotonic increases in the elastic modulus of SC. Fig. 1(f) however highlights that a 7-day immersion period is required to cause significant decreases in SC toughness. Further, Fig. 1(g) highlights that the fracture strain is most significantly and rapidly affected by water immersion, with significant reductions first occurring after 12 h, and monotonic decreases thereafter until 7 days. Fig. 1(h) further highlights that the ultimate tensile strength of the tissue also varies with water immersion, with significant increases occurring after a 36 h immersion period. Collectively, water immersion-induced increases in elastic modulus and fracture stress, along with concurrent decreases in toughness and fracture strain, indicate that SC is becoming more brittle.

2.2. Age-based changes in SC mechanical properties

The results in Fig. 1 ignore the potential influence of gender, ethnicity, anatomical site and age of the skin. However, recent evidence suggests that ageing can notably influence the mechanical properties of SC [4]. While this previous study proposes that changes in SC mechanical properties occur linearly with age, the data used to perform regression analyses exhibit high variance with numerous outliers. In order to evaluate if water immersion impacts skin of different ages to different extents, we re-examine the data in Fig. 1 to elucidate aged-based changes in the mechanical properties of SC.

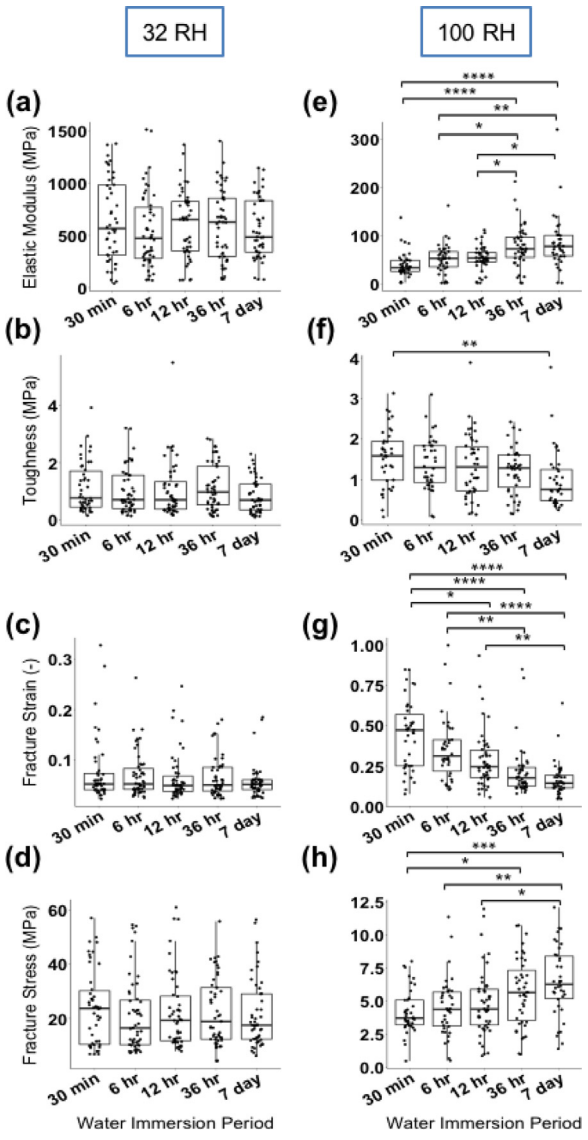

Fig. 1. Changes in SC mechanical properties with water immersion. Change in (a) Young's modulus, (b) modulus of toughness, (c) fracture strain and (d) fracture stress of SC samples equilibrated to 32% RH. Equivalent water immersion induced changes in SC mechanical properties after equilibration to 100% RH are shown in panels (e) through (h). Each bar spans the interquartile range. The horizontal line within each bar denotes the median of $38 \leq n \leq 51$ independent measurements of SC from skin ageing in range between 27 and 87 years. Whiskers denote the range excluding outliers. Significance levels are denoted by horizontal lines and asterisks.

We first re-evaluate if a linear model can capture age-based differences in the mechanical properties of SC under both dry and fully hydrated conditions. Mechanical results from Fig. 1 are first divided into three age ranges: 27–49 ($10 \leq n \leq 11$ from 4 specimens), 50–69 ($10 \leq n \leq 11$ from 4 specimens) and 70–87 years ($19 \leq n \leq 24$ from 6 specimens). Supplemental Figure S1 shows that for SC equilibrated to 32% RH, all mechanical properties excluding toughness exhibit statistically significant differences between the 50–69 and 70–87 age ranges, and monotonic changes across the full age range. These age-based changes however are clearly nonlinear. Results from SC equilibrated to 100% RH in supplemental Figure S2 highlight that no statistically significant difference in mechanical properties exist. As such, a linear model does not appear to accurately capture changes in SC mechanical properties with ageing. As an alternative testable

hypothesis, we anticipate that a critical age must be reached before the mechanical properties of SC begin to notably change. Re-examination of the mechanical results delineated into two age ranges 27–69 and 70–87 in supplemental Figure S3 clearly shows significant differences in all mechanical properties for SC equilibrated to 32% RH. However, only significant differences in fracture stress occur for SC equilibrated to 100% RH, as shown in supplemental Figure S4. Collectively, these results indicate that ageing primarily impacts the elastic regime and does not notably impact the ability of the tissue to plastically deform. This degradation will be further enhanced given the predilection towards dryer skin conditions in aged tissue [15,16]. The results also suggest that the onset of age-based changes in SC mechanical properties occurs around 70 years. Subsequently, we employ this age-based demarcation to examine the impact of water immersion period on the two age groups, both at 32% and 100% RH conditions.

2.3. Age delineated changes in mechanical properties of SC with water immersion

Supplemental Figure S5 highlights that water immersion for up to 7 days has no effect on the mechanical properties of either SC aged between 27–69 years, or greater than 70 years, when subsequently equilibrated to 32% RH. Prolonged immersion therefore does not appear to impact the elasticity of dry SC tissue. In contrast, Fig. 2 reveals that water immersion induced changes in SC mechanical properties occur in both age groups when SC samples are equilibrated to 100% RH post immersion. Fig. 2(e)–(h) further highlights that changes occur more rapidly and by a greater magnitude in the aged skin group relative to the younger group (Fig. 2(a)–(d)). SC from younger skin therefore appears to be more resistant to immersion induced damage.

2.4. Changes in water sorption of SC with water immersion

With hydration acting as a major factor in altering the mechanical properties of SC [7,8,13,17], a potential cause of water immersion-induced mechanical degradation is an altered tissue water holding capacity. Reductions in tissue water content are associated with increases in the elastic modulus of the tissue [13, 18] and decreases in the plastic deformability of SC prior to rupture [8], consistent with the results of Fig. 2. Fig. 3 compares the maximum average ($n = 3$) absorbed water mass of SC, scaled by the dry tissue mass, prior to and post 7-day water immersion, for both young (white) and aged (grey) specimens. Prior to water immersion, aged SC retains notably more water than younger tissue. This correlates with known increases in SC thickness with ageing [19,20]. After a 7-day water immersion, the maximum water holding capacity reduces for both age groups, which would explain the changes in SC mechanical properties. Moreover, the more aged tissue experiences a significantly larger loss in its water holding capacity, relative to younger tissue. This would additionally explain the larger magnitude changes in SC mechanical properties in the more aged skin group (Fig. 2).

2.5. Changes in lipid and NMF composition with water immersion

Prior studies have revealed that both changes in lipid and NMFs can notably impact the amount of water skin tissue can hold [21,22]. Moreover, immersion of skin tissue in water has been demonstrated to cause extensive lamellar lipid bilayer disruption after 6 h [23]. We further evaluate changes in both the composition of lipids and NMF in SC resulting from prolonged water immersion. Fig. 4 shows a comparison of the average fluorescence density of SC samples immersed in water for 7

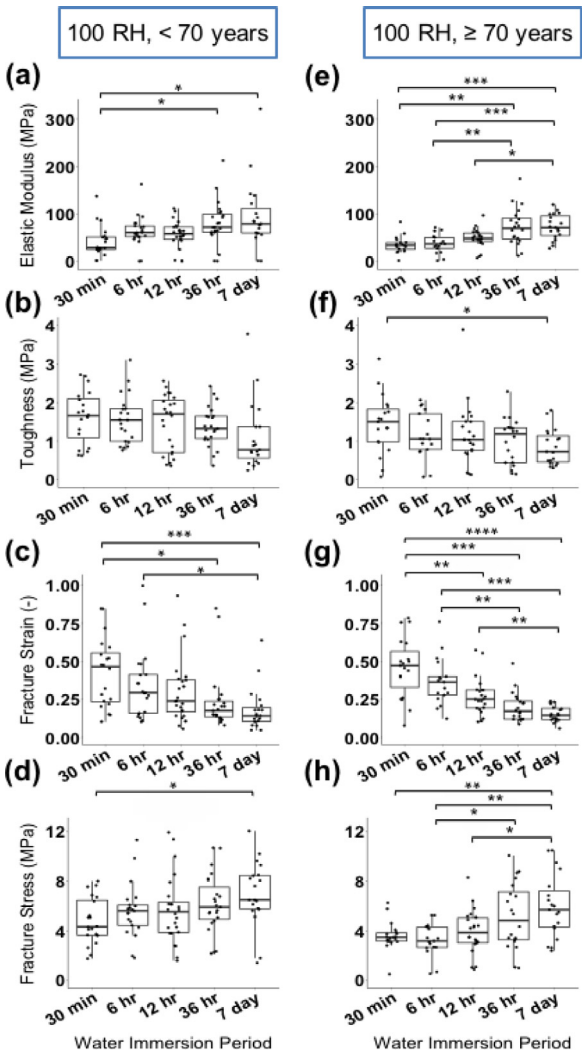

Fig. 2. Changes in the mechanical properties, of age delineated SC samples equilibrated to 100% RH, with water immersion time. Samples aged less than 70 years (left column) and greater than or equal to 70 years (right column). (a), (e) Elastic modulus (b), (f) modulus of toughness (c), (g) fracture strain and (d), (h) fracture stress. Each bar spans the interquartile range. The horizontal line within each bar denotes the median of $17 \leq n \leq 24$ independent measurements of SC from skin ageing in range between 27 and 87 years. Whiskers denote the range excluding outliers. Significance levels are denoted by horizontal lines and asterisks.

days, then stained with a BODIPY 558/568 C_{12} fatty acid dye, compared to unsubmerged controls. SC samples in both cases are from identical sources that range in age across 27–87 years. SC samples undergoing 7-day immersion show a significant reduction in fluorescence relative to controls, demonstrating that prolonged water immersion results in leaching of ceramides and free fatty acids from SC across all ages. This depletion is associated with barrier dysfunction, which will impact the water holding capacity of the tissue [24,25]. Fig. 5 shows variations in the ratio of NMFs to protein content of young (white) and aged (grey) SC tissue for different water immersion periods. Relative to SC from younger skin, aged SC exhibits reduced NMFs prior to water immersion, consistent with previous studies [26,27]. 7-day water immersion causes a significant decrease in the NMF quantity for both young and aged tissue, reducing the NMF composition to a similar level in both age groups. This suggests that the effect of prolonged water immersion on NMF composition outweighs any age-based effects. Fig. 5 shows no significant change in NMFs

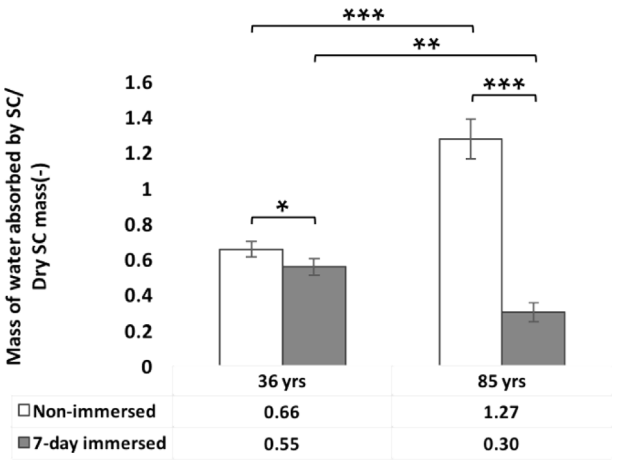

Fig. 3. Changes in water holding capacity of SC tissue with age and water immersion. Average mass of water absorbed per unit dry SC sample mass for $n = 3$ cycles of drying and hydrating. SC samples from 36 and 85-year-old skin immersed in water for 7 days (grey bars) are compared with equivalent samples not immersed in water (white bars). Error bars correspond to standard deviations. Significance levels are denoted by horizontal lines and asterisks.

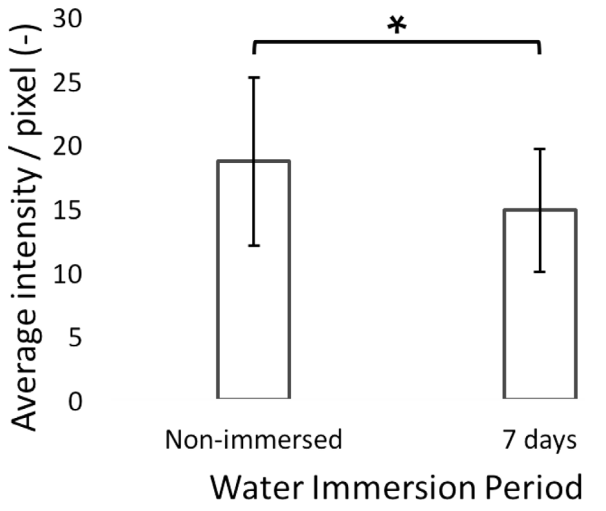

Fig. 4. Changes in lipid content of SC tissue with prolonged immersion in water. Average fluorescent density of BODIPY 558/568 C_{12} ceramide and free fatty acid stained SC samples across the age range 27–87 years after immersion in water for 7 days ($n = 6$ individual samples each from 5 skin specimens) compared with non-immersed controls ($n = 6$ individual samples each from 5 skin specimens). Error bars correspond to standard deviations. Significance levels are denoted by horizontal lines and asterisks.

between non-immersed and 30-min immersed SC, suggesting that a 30-min immersion period does not significantly alter SC NMF composition. This *ex-vivo* study however does not account for NMF replenishment, which is known to occur *in-vivo* [14].

2.6. Changes in full-thickness skin structure and integrity with water immersion

Studies further examine the impact of prolonged water immersion on the structure and integrity of full-thickness human skin. Fig. 6 illustrates representative histological cross-sections of 34, 52 and 72-year-old skin, unsubmerged (left column) and after immersion in water for 7 days (right column) at laboratory conditions (22 °C). Images focus on the epidermal and papillary dermal layers. Tissues were stained for keratin (red), nuclei (blue–black), elastin (blue–black fibres) and collagen (blue fibres).

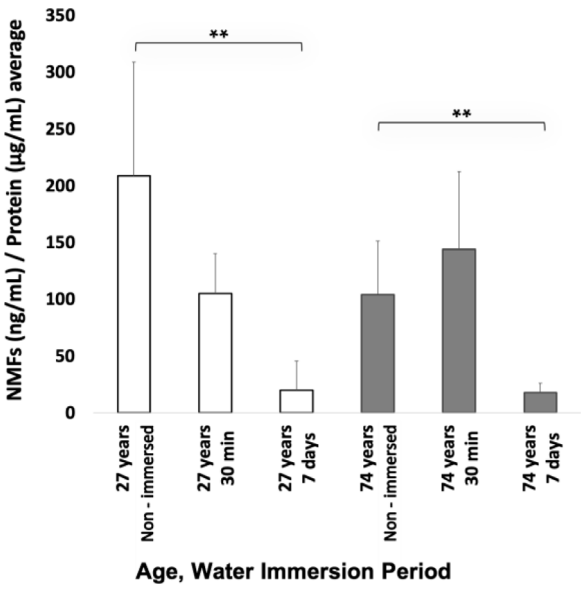

Fig. 5. Changes in SC NMF concentration with prolonged immersion in water. NMF mass to total protein mass in SC samples isolated from 27-year-old (white bars, $n = 3$ individual samples) and 74-year-old (grey bars, $n = 3$ individual samples) skin samples when not immersed in water, after 30 min water immersion, and after 7-day water immersion. Error bars correspond to standard deviations. Significance levels are denoted by horizontal lines and asterisks.

Fig. 6(a)–(c) highlight the relatively intact epidermis and papillary dermis of skin specimens not exposed to water. In these samples, epidermal–dermal interfaces are well integrated, have dense and conformally oriented collagen fibre networks in the papillary dermis, and exhibit a mostly cohesive SC–epidermal interface. Further, Fig. 6(c) shows a flattened basement membrane with smaller amplitude papillae, characteristic of aged skin [28,29]. In contrast, Fig. 6(d)–(f) show that after a 7-day water immersion, the dermal collagen network density decreases, and the orientation of the fibres becomes less pronounced for all skin ages. Further, the youngest tissue in Fig. 6(d) and oldest skin in Fig. 6(f) exhibit extensive or full epidermal–dermal and SC–epidermal delamination, with the 52-year-old skin in Fig. 6(e) exhibiting moderate dermal–epidermal delamination in the form of cavitation at many points in the interface (highlighted in red). Cavitation and delamination at the epidermal–dermal interface suggests a degradation of the hemidesmosomes binding the basement membrane to the extra cellular matrix. Further, Fig. 6(d)–(e) highlight that cavitation appears to occur first in proximity to the dermal papillae, suggesting these locations may act as nucleation sites for epidermal–dermal delamination. These representative results are strongly supported by a quantitative damage scale analysis performed across all fifteen donor samples, as detailed in the supplemental Figures S6–S7 and supplemental Tables S1–S2. These results also highlight that a lower water temperature of 4 °C reduces the structural degradation from immersion to a great extent, as shown in supplemental Figure S8.

In this article, we attribute the mechanical degradation of skin tissue predominantly to water immersion. However, there is the potential here for degradation to also be caused by microorganisms on, or within the skin, when the samples were received. Through both washing and tissue treatment protocols using deionised water, we anticipate that this influence is minimised. Bacteria are notably more populous on skin than fungi [30,31], and while bacterial permeation into skin tissue does occur, full permeation across the SC only occurs when the tissue exhibits similar lipid levels to patients with atopic dermatitis [32,

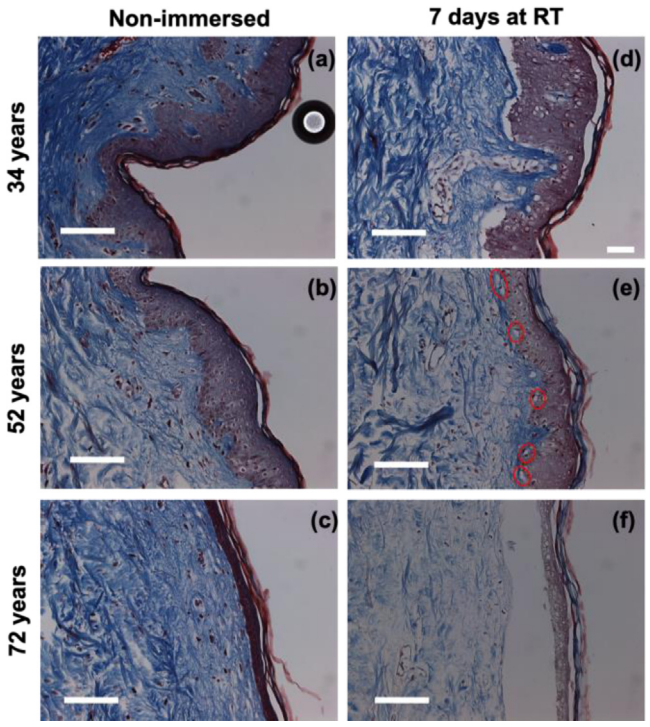

Fig. 6. Structural degradation of human skin with prolonged water immersion. Histological images of 34 (upper row), 52 (middle row) and 72-year-old (lower row) male abdominal skin tissue when (a)–(c) not immersed in water and (d)–(f) immersed in water for 7 days. Samples are stained with a modified Masson's trichrome stain. Red denotes keratin and cytoplasm, blue denotes collagen and blue–black denotes elastin and cell nuclei, *Scale bars:* 125 μm. RT denotes room temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

33]. The time scale of this permeation (9 days) is also greater than the water immersion period tested in this article (7 days). As such, we do recognise that bacterial mediated mechanical degradation here will occur but is likely to be small over the time scales examined in this study. Future studies should however quantify this effect. Furthermore, unlike *in-vivo* behaviour, *ex-vivo* isolated SC does not undergo regeneration. By assuming that regeneration restores the mechanical function of water immersion damaged tissue, our *ex-vivo* results detail the most extreme mechanical changes that could occur with water immersion.

Overall, prolonged immersion in water impacts the mechanical properties, function, and composition of the superficial SC, and the structure and integrity of full-thickness skin. Leaching of lipids and NMFs causes a reduction in the water holding capacity of the SC, which results in the tissue becoming more brittle and prone to rupture. In addition to the possibility of being a helpful tool in forensic dermatology, we anticipate these results will help advance the development of in-vitro skin models, improve laboratory tissue storage procedures, and improve understanding of water associated skin conditions and occupational risks of prolonged water immersion that can lead to severe skin maceration.

3. Materials and methods

3.1. SC isolation

An exempt approval (3002–13) was obtained to perform research using de-identified tissue samples pursuant to the Department of Health and Human Services (DHHS) regulations, 45 CFR 46.101:b:4. De-identified full-thickness human skin tissue specimens were obtained both from Upstate New York Tissue Services (Buffalo, NY) and Yale Pathology Tissue Services, Yale University (New Haven, CT). Skin specimens originated from both elective surgery and cadavers. Specimens from elective surgery were received within 24 h of surgery. The skin samples tested varied in age across 27–87 years, gender, and body location (breast, abdomen or buttocks). Details of the skin samples used for each experiment are detailed in the supplemental Tables S3–S7. SC tissue was isolated from full-thickness human skin using well established heat and trypsin isolation protocols [34]. Once isolated and dried, SC tissue was stored under laboratory conditions (22 °C, 25% RH).

3.2. SC treatment and preparation for tensile testing

Rectangular 20 × 5 mm SC samples were cut out from the source SC using a scalpel. Details of the SC sources used are provided in supplemental Table S3. Isolated SC samples ($n = 4$) were immersed in DI water for periods of 30 min, 6 h, 12 h, 36 h and 168 h (7 days) followed by drying for at least 6 h in the laboratory environment at 22 °C. Dried samples were taped on both ends to prevent tearing of tissue in between the tensometer clamps, and equilibrated to either 32% or 100% RH for at least 24 h using an airtight humidity chamber with a base filled with either magnesium chloride salt solution [35] or water, respectively. Humidity was measured using a digital hygrometer (Humidity Alert II, EXTECH Instruments, China).

3.3. SC mechanical testing

Mechanical testing was performed under laboratory conditions using a uniaxial tensometer (CellScale UStretch, Waterloo, ON, Canada) with a 4.4 N load cell. Samples were stretched along the long axis of the sample with a grip separation rate of 50 μm s^{-1} until complete rupture, equivalent to a strain rate of $\dot{\gamma} = 0.005\ s^{-1}$ [36]. The tensometer records tensile force and grip displacement at a frequency of 10 Hz. These parameters and the sample geometry were then used to obtain engineering stress versus strain curves. For each sample, optical microscopy was used to measure the sample thickness far from the crack interface in order to avoid measuring reduced thicknesses arising from localised plastic deformation. Stress–strain curves were then used to establish the sample mechanical properties. For each immersion condition, samples were used to establish average mechanical properties and standard deviations.

3.4. Water sorption study

Two SC samples of dimensions 25 × 50 mm were taken from each skin source, detailed in supplemental Table S4. One control pair remained unsubmerged. The other pair were immersed in DI water for 7 days. All samples were then equilibrated to 23% RH for 24 h in a hermetically sealed airtight chamber containing a dessicant (Drierite, Xenia, OH). Dry mass values of individual samples were then recorded using an analytical balance with 0.1 mg resolution (Mettler Toledo MS-TS, Columbus, OH). Samples were then equilibrated to 100% RH for 24 h in a hermetically sealed airtight chamber containing tray of DI water. Individual hydrated sample masses were then recorded. This process was then repeated 3 times for each sample.

3.5. Lipid study

A circular 5 mm diameter hole punch (Harris Uni-Core, Redding, CA) was used to cut $n = 12$ individual SC samples from isolated SC sheets from 27 to 87-year-old donors, detailed in supplemental Table S5. For each skin source, $n = 6$ samples were immersed in water for 7 days, while the remaining half remained non-immersed. All samples were then stained with a free fatty acid and ceramide dye BODIPY FL 558/568 C_{12} (ThermoFisher Scientific, Catalog: D3835), using standard protocols [32], then laminated onto glass slides. Fluorescent images of samples were captured using a CCD camera (Andor Clara, Belfast, Northern Ireland) mounted to a Nikon eclipse-Ti inverted microscope (Nikon, Melville, NY) with 10X objective lens (Nikon Plan Fluor) when illuminated with a SOLA 6-LCR-SB (Lumencor light engine, Beaverton, OR) with TRITC filter. For each skin source, the integration time of recorded images was maintained across both unsubmerged and immersed samples. Images of complete circular specimens were created by stitching images across an area of 5.5×5.5 mm using image processing software (Nikon Elements, Nikon, Melville, NY). Fluorescent densities for each sample are obtained by summing the fluorescent intensity of pixels within the inner half radius of each SC sample, then scaling by the total number of pixels within that region.

3.6. NMF study

SC samples ($n = 12$) of dimension 10×20 mm were cut from SC sheets isolated from both 27 and 74-year-old specimens, as detailed in supplemental Table S6. $n = 3$ samples were immersed in DI water for 30 min and 7 days, each, while the remaining 3 samples were left non-immersed. All samples were then individually placed in a protein LoBind Eppendorf tube with 1.2 mL of a 25% aqueous solution of ammonia [37] and sonicated for 2 h, followed by 3 min centrifugation at 13200 rpm using a centrifuge (Eppendorf 5810, Hamburg, Germany). Each sample was portioned into three parts and evaporated using a MiniVap nitrogen evaporator (Porvair, Wrexham, U.K.) at 60 °C for 90 min. Two parts were used for protein analysis using High Performance Liquid Chromatography (HPLC) [37] while the third part was used for NMF characterisation using Liquid Chromatography–Mass Spectrometry (LC-MS) [38], as detailed in supplemental Tables S8 and S9.

3.7. Histology study

Paraffin embedded histology was performed on 15 full-thickness skin specimens, ranging in age across 34 to 82 years, as detailed in supplemental Table S7. From each skin specimen, circular 6 mm diameter samples were excised using a biopsy punch. Adipose tissue was then removed from each sample using a scalpel. Two samples from each skin specimen remain unsubmerged, two were immersed in 22 °C DI water for 7 days, and two were immersed in 4 °C DI water for 7 days by storing the samples in a refrigerator. Tissue samples were subjected to standard histological processing and paraffin embedding procedures [39]. Embedded samples were then cross-sectioned into 5 μm specimens using a microtome (Leica CM1850, Wetzlar, Germany). Cross-sections were stained using a modified Masson's trichrome staining protocol [40], further detailed in the supplemental methods, to visualise nuclei, keratin, elastin, and collagen. A microscope (Olympus BX43, Center Valley, PA) with 4X (Olympus Plan Objective 4x/0.10) and 20X objective lens (Olympus LUC PlanFLN 20x/0.45) was used to image the stained cross-sections under transmitted light. A digital camera (Olympus DP74) was used to capture histological images.

3.8. Statistical analyses

Levene's and Shapiro Wilk's tests were respectively performed on all data sets to test for homogeneity of variance and normality. A corresponding one-way ANOVA test – parametric, Welch's or Kruskal–Wallis – was used for results in Figs. 1–2. This was followed by corresponding post-hoc analyses — Tukey–Kramer, Games Howell, Kruskal–Nemenyi or Dunn's. Independent one-tailed t-tests were used to establish statistical differences between pairs of data sets in the mass balance, lipid and NMF studies (Figs. 3–5 respectively). In the figures, * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, and *** denotes $p \leq 0.001$.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eml.2020.101017.

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