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The influence of manufacturing and alterations on skin-based artifacts as characterized by nonlinear optical microscopy

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ABSTRACT

Nonlinear optical (NLO) microscopy is an efficient technique to investigate artworks as it allows non-invasive, three-dimensional imaging with micrometer-scale resolution. This technique can combine two-photon excitation fluorescence (2PEF) from exogenous and endogenous fluorophores and second harmonic generation (SHG), which enables the visualization of unstained fibrillar collagen. The application of NLO microscopy to different skin-based materials encountered in cultural heritage showed the influence of manufacturing, in particular the type of tannin and fats introduced, on the morphological structure and collected signals. The data also showed that NLO microscopy was well-suited to non-invasive examination of the degradation state of collagen in all skin-based materials, particularly based on the SHG images, and could be used to identify artifacts most at risk in collections so suitable conservation measures could be taken.

INTRODUCTION

In museums, libraries, and archives, skin is largely present in collections since it can be found in utilitarian objects, writing supports, musical instruments, and in decorative artifacts, as well as in natural history specimens. To preserve the skin, different manufacturing techniques have been used depending on the properties or appearance required for the final material. Except for natural history specimens where the hair is kept, the hypodermis and epidermis together with the hair are eliminated from the skin so as to retain only the dermis layer. The main constituent of skin dermis is fibrillar collagen, which exhibits a hierarchical organization from a triple helix to a fibril and a fiber that is responsible for skin's mechanical strength.

Different treatments are then applied, such as drying and stretching for parchment, or chemical treatment called 'tanning/tawing' for leather or alum-tawed skin. The aim of tanning is to convert the skin into a stable material that resists putrefaction by using vegetable or mineral tannin. Vegetable tannin is the most ancient tanning material. It relies on fixing collagen with polyphenol compounds extracted from plants. Among mineral tannins, chrome tanning, which came into use at the end of the 19th century, is currently the most commonly used to make leather because it is fast, reliable, and produces a highly stable end product. Alum has been used for a long time to preserve skin, but the material obtained cannot be called leather because the aluminum-collagen interaction is reversible in the presence of water. This treatment is therefore called 'tawing' (Covington 2011). Other preservation treatments, such as oils or fumes, have also been used in the past, but these are less widespread and will not be examined here.

Depending on the treatment used, the stabilization of the collagen molecule differs, which is reflected in the hydrothermal stability or shrinkage temperature (T_s), i.e. the measurement of the resistance of a wet material to heat. The measurement of this temperature, by differential scanning calorimetry (DSC) or the micro-hot table (MHT) method, is commonly used in the cultural heritage field to assess the degradation state of collagen-based materials. However, the technique is invasive, as it requires a sample, and destructive, which in the case of some historical artifacts is an issue.

Nonlinear optical (NLO) microscopy is an efficient technique for investigating artworks as it allows non-invasive, three-dimensional imaging with micrometer-scale resolution (Cormack et al. 2007, Filippidis et al. 2008, Latour et al. 2012, Villafana et al. 2014). This technique can combine two-photon excitation fluorescence (2PEF) from exogenous and endogenous fluorophores and second harmonic generation (SHG), which enables the visualization of unstained fibrillar collagen. Furthermore, the use of near-infrared excitation has the advantage of reduced photo-damage and increased penetration depth within the sample. In biomedical research, SHG microscopy has emerged as a powerful technique to quantify collagen 3D organization in tissues, in particular in skin dermis. Recently, the technique was successfully applied in combination with infrared nanoscopy (nanoIR) to investigate collagen degradation in parchment (Latour et al. 2016). This work showed that alteration of the collagen in the parchment resulted in changes to the signals collected by NLO, either in the form of morphological modifications to the SHG images or modifications in the ratio of SHG and 2PEF signal intensities. The correlation with nanoIR (AFM topography coupled with IR illumination to collect IR absorption spectra at nanometer-scale resolution) was used to identify the chemical origin of the structural changes at the scale of the collagen fibers or fibrils.

The aim of the present work is to extend the scope of NLO microscopy by examining the potential of this technique for other skin-based materials found in cultural heritage. The paper will focus in particular on parchment, leather, and alum-tawed skins with different types of degradation in order to discuss how the NLO signals can be used to characterize the influence of the preservation technique and the alteration to these skin-based artifacts.

MATERIALS

Historic and modern skin samples preserved by different techniques and in different conservation states were studied. For each preservation technique, a minimum of three samples from different origins were examined. Only a selection of the representative samples are presented in this paper:

- Untanned skin: a dried calf skin from the STEP project (Larsen et al. 1994), a 17th-century parchment, parchment samples from the IDAP project (Larsen 2007) including a reference, a parchment aged for 16 days at 100°C, and a parchment aged for 32 days at 80% RH and 80°C.
- Alum-tawed skins: a modern sheep skin supplied by Lieutard (France) and a zebra specimen skin from the French Natural History Museum collections that suffers from hydrolysis. Samples of the zebra skin were exposed for three months to either 50% or 80% RH at ambient temperature.
- Leathers: a modern chrome-tanned sheep skin, a calf skin tanned with sumac vegetable tannin from the STEP project, and two ancient vegetable-tanned leather bookbindings – one from the 18th century and the second dated 1828 – suffering from “red rot.”

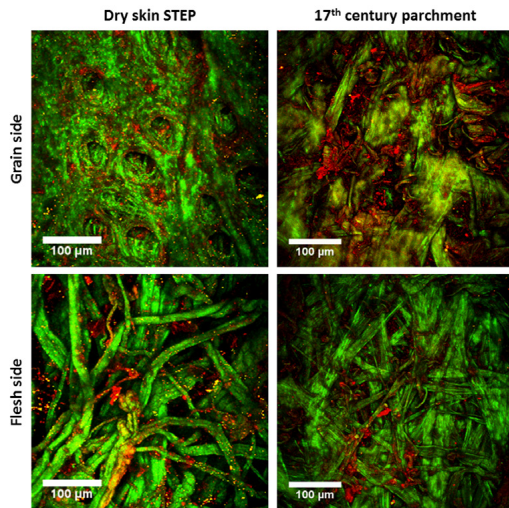


Figure 1. Nonlinear optical microscopy image of a dry skin and a 17th-century parchment measured from the grain and the flesh side. SHG signal in green and 2PEF signal in red

METHODS

Differential scanning calorimetry (DSC): the shrinkage temperature (T_s) and enthalpy (ΔH) were measured on a PerkinElmer DSC 8000. The samples were soaked in distilled water for one hour, enclosed in an aluminum capsule and analyzed at a heating rate of $5^\circ\text{C}/\text{min}$. For each sample, three measurements were collected and averaged.

Nonlinear optical (NLO) microscopy: the set-up was a custom-built upright microscope based on a femtosecond Ti:Sa laser, as previously described by Latour et al. (2016). A high-numerical-aperture air objective ($20\times$, NA 0.75) was used for non-contact imaging and $0.7\ \mu\text{m}$ lateral by $4\ \mu\text{m}$ axial resolutions near the sample surface were achieved with $860\ \text{nm}$ excitation. 2PEF and SHG signals were simultaneously epi-detected using two different spectral channels, represented in false colors, respectively red and green, with enhanced contrast. The acquisition pixel rate was $200\ \text{kHz}$, corresponding to around one frame per second for 640×640 pixel wide images ($480 \times 480\ \mu\text{m}^2$ at a $0.8\ \mu\text{m}$ pixel size). Laser power at the objective focus was 3 to 20 mW without any observable damage to the studied samples. For each sample, a minimum of three images were collected. All the images presented correspond to the stack of the images collected at the different depths.

RESULTS AND DISCUSSION

Manufacturing

The first manufacturing step, liming, which is used to remove the unwanted layers, is common to all skin-based materials. Subsequently, the skin is dried to make a parchment, or fixed with tannins to make leather. This preservation treatment modifies the skin's properties, such as its color, softness, smell, and stability. Additionally, it also changes the morphological structure and may affect the chemical structure of the collagen molecule.

One of the advantages of NLO microscopy is that it provides multimodal signals that are compound or structure specific. Due to its dense and non-centrosymmetric fibrillar structure, collagen in skin displays strong SHG signals; it also exhibits a slight 2PEF signal related to the molecular crosslinks. Thus, SHG images of the 3D organization of fibrillar collagen can be collected without disturbance from the surrounding materials that do not exhibit SHG signals. Moreover, any modification to the collagen molecule or organization may affect the collected signals, so NLO microscopy is expected to be sensitive to any structural modification in the collagen.

NLO images were collected from both sides of two untanned dried skins: a skin left to dry after the liming step and a parchment which had been scraped and dried under tension. Strong SHG signals were collected from both materials, while a 2PEF signal was only observed in localized spots associated with fluorophores from residues of fats, keratin, elastin, or dust. The difference in manufacturing between the two materials was clearly visible from the different structural organization of the collagen fibers in the SHG images (Figure 1). Whilst the images obtained from the dry skin were similar to those collected from the *in vivo* or *ex vivo* skin, with a clear distinction between grain and flesh sides and the presence of loose and thick fibers on the flesh side, the images from the parchment showed

collagen fibers under tension organized parallel to the skin surface with an increase in fiber density.

The NLO images collected from the chrome and vegetable-tanned leathers and the alum-tawed skin revealed differences in the collected signals and in the morphology of the collagen fibers (Figure 2). Like the dried untanned skins, the chrome-tanned leather displayed strong SHG signal and only localized fluorescence spots. The images of the organization of the collagen fibers on the flesh side of these two skins were, however, clearly different: the chrome-tanned leather exhibited a more open structure with smaller diameter fibers, which may account for the greater flexibility this leather is known for.

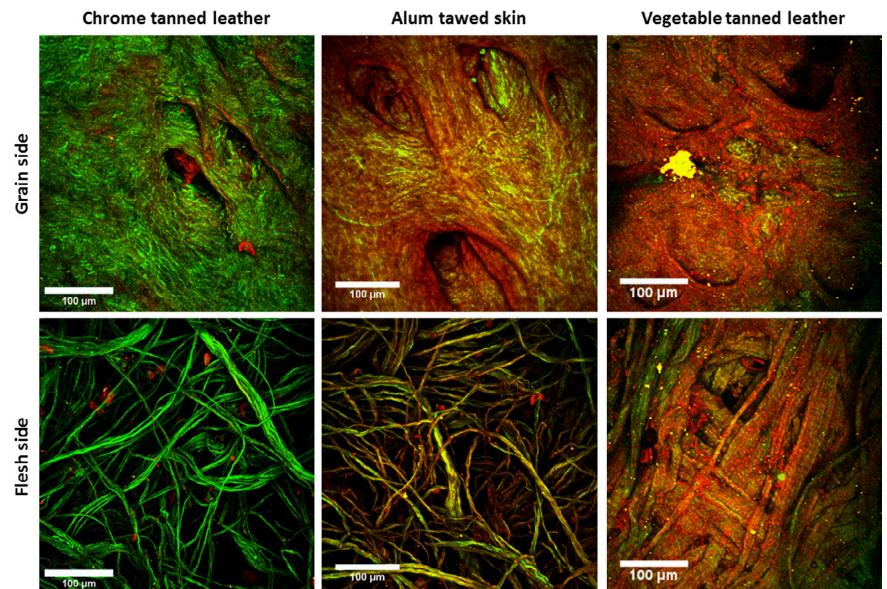


Figure 2. Nonlinear optical microscopy image of chrome-tanned leather, alum-tawed skin and vegetable-tanned leather

In the vegetable-tanned leather and alum-tawed skin, the SHG signal decreased compared to the previous skin samples and a strong 2PEF signal was collected all over the material (Figure 2). Whilst the fluorescence was greater on the grain side than on the flesh side for the alum-tawed skin, the fluorescence was strong on both sides of the vegetable-tanned leather. Due to the strong two-photon absorption by vegetable-tanned leather during the NLO measurements, the laser power had to be greatly reduced to avoid beam damage and the penetration depth was limited. In alum-tawed skin, preservation is obtained by using alum, a mixed salt of aluminum and potassium sulfate, together with egg yolk and flour. Alum alone does not give any SHG or 2PEF signal (data not shown), therefore the fluorescence increase likely originated from the fats introduced with the egg yolk. The overall fluorescence observed in the vegetable-tanned leather probably resulted from the polyphenol in the tannins and possibly also the fats introduced with lubricants. The reason for the decrease in the SHG signal, however, is less straightforward. It may have been due to a modification in the structural organization of the collagen or a modification in the nonlinear optical response on the molecular scale due to the collagen-tannin interaction. Indeed, polyphenols react through multiple hydrogen bonding with collagen, whilst metal ions interact with the collagen carboxyl group, by electrostatic interaction for Al^{3+} and covalent interaction for Cr^{3+} (Covington 2011).

Comparison of the NLO images collected on the flesh side revealed a strong difference in fiber organization. The morphological structure of the alum-tawed leather resembled that of the chrome-tanned leather with thin collagen fibers tangled in an opened structure, whilst the structure of vegetable tanned leather was much more compact with thick collagen fibers. These observations are consistent with the characteristics of these skins: vegetable-tanned leathers are known to be thick and strong so they are generally chosen for their resistance, whilst alum-tawed skins are known to be soft and flexible so they are used to make gloves.

Alteration

Skin-based materials react differently to the different agents of deterioration depending on their manufacture. The two alteration mechanisms in skin, acid hydrolysis and oxidation, both involve the breakage of bonds in the collagen molecule but in different areas. The final stage of deterioration is the denaturation also known as “gelatinization” which is when the collagen molecule unfolds to gelatin.

In the authors’ previous study on well-preserved and gelatinized parchments, it was shown that NLO imaging can map the state of local degradation (Latour et al. 2016). In NLO images, parchment alteration is associated with a decrease or loss of the SHG signal and a joint increase in the 2PEF signal. In the case of gelatinized parchment, the structural organization is also strongly affected, with the collagen fibers being disassembled and the formation of an amorphous and compact material providing a strong homogenous fluorescence signal.

In the present work, the aim was to extend knowledge on the alteration of other skin-based materials. Regarding parchment, two aging environments associated with different collagen alteration mechanisms were compared: dry heat conditions, expected to cause mostly oxidation, and humid heat conditions, which favor hydrolysis and gelatinization. The shrinkage temperature dropped from 55°C to 35°C and 38°C for the parchments exposed to dry heat and humid heat, respectively (Table 1). Again, in the NLO images of both aged parchments a more compact structure was observed with a loss of the fibrillar organization, a decrease of the SHG signal, together with an increase of the 2PEF signal (Figure 3). Nevertheless,

Table 1. Shrinkage temperature (Ts) and enthalpy (ΔH) measured by DSC on preserved and altered skin-based materials. (-) unmeasurable signal

| | Sample | Ts (°C) | ΔH (J/g) |
|-----------------------|----------------------------------|---------|------------------|
| Preserved skin | Dry untanned skin - STEP | 41.5 | 18.1 |
| | Vegetable-tanned leather - STEP | 74 | 27.5 |
| | Chrome-tanned leather - LCC | 110 | 30.5 |
| | Alum-tawed skin - Lieutard | 70 | 20.0 |
| | Parchment reference - IDAP | 53.3 | 50.3 |
| Altered skin | Parchment aged using dry heat | 35.4 | 31.9 |
| | Parchment aged using humid heat | 38.2 | 39.3 |
| | Hydrolyzed zebra skin at 50 % RH | 28.2 | 3.1 |
| | Hydrolyzed zebra skin at 80 % RH | - | - |
| | 18th-century leather bookbinding | 41.6 | 8.4 |
| “Red rot” leather | 37.5 | 2.7 | |

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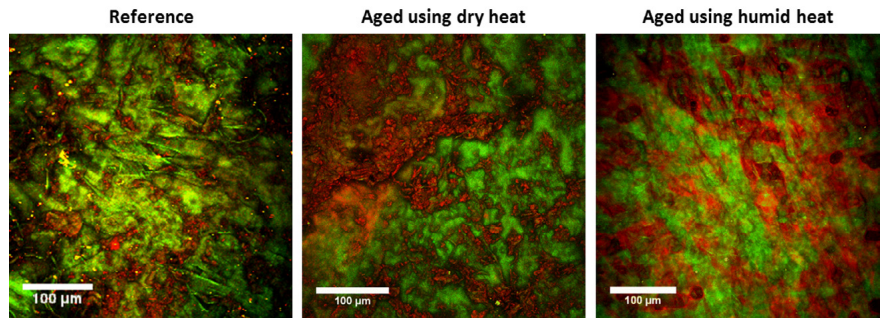


Figure 3. Nonlinear optical microscopy images of an IDAP parchment reference, artificially aged using dry heat and humid heat measured from the flesh side

the image morphologies and the extent of the signal changes were different between the two aging environments. Regarding the parchment exposed to dry heat, a high SHG signal was recorded in the background with a slight punctuate fluorescence signal for degraded collagen at the surface. In the parchment exposed to humid heat, on the other hand, the collagen damage seemed greater since the fluorescence signal dominated and there was only a small SHG signal. In addition, the material seemed to have melted and may correspond to denatured collagen.

The second alteration example concerned an alum-tawed skin from a natural history zebra specimen. To preserve natural history specimens, taxidermists in the past have often used alum salts because the skin retains a light color and great flexibility which is important to return a sense of life back to the animal. Nowadays, a large number of alum-tawed specimen skins in collections suffer from acid hydrolysis and the skin has lost its flexibility, become powdery and is at an advanced stage of turning into a brown gelatinous material (Robinet et al. 2014). The hydrolysis is induced by the high acidity of the skin, with a pH generally below 3, and relates to the presence of alum and seems accelerated by the use of fats. The zebra skin is a good example of acid hydrolysis alteration, with a dry powdery skin that breaks easily. This skin has been treated with alum, as well as sodium chloride to eliminate excess water, sodium sulfate and kaolin to dehydrate the skin, and fats to soften it. The skin has a very high acidity, with a pH of 2.78. To assess the sensitivity of the skin to humidity, one sample was stored at 80% RH and a second at 50% RH, both at ambient temperature. The skin kept at 50% RH remained unchanged, while the skin exposed to 80% RH transformed progressively into a sticky brown material identified as gelatin (Figure 4) (Robinet et al. 2014). The shrinkage temperature for the zebra skin kept at 50% RH was 28°C but was unmeasurable for the sample exposed to 80% RH since all the collagen had denatured (Table 1).

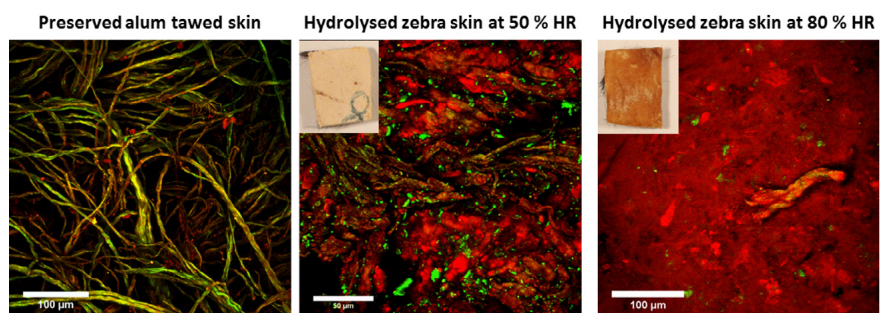


Figure 4. Nonlinear optical microscopy images of preserved alum-tawed skin, and hydrolyzed zebra skins kept at 50% RH and 80% RH, together with photographs of the zebra samples

The extensive alteration of the collagen was confirmed by the NLO images of the two samples (Figure 4). The image of the skin stored at 50% RH showed a few collagen fibers exhibiting only a weak SHG signal as well as an unstructured material emitting only a 2PEF signal. In the image of the skin exposed to 80% RH, the fiber structure had collapsed into a compact material emitting only fluorescence. A few SHG spots observed in this image likely originated from the only other compound in the skin with a non-centrosymmetric structure: kaolinite.

Finally, two examples of alteration in vegetable-tanned leather were examined by NLO microscopy. Both leathers had been used as bookbindings. The first, dating from the 18th century, appeared dry and had lost flexibility, whilst the second, dating from 1828, had suffered from “red rot,” a pollution-induced alteration causing hydrolysis of the leather and turning it into a red powder. The shrinkage temperature dropped from 75–80°C for a recent vegetable-tanned leather to around 42°C for the 18th-century leather and 37°C for the ‘red rot’ leather (Table 1). In the NLO images of the two leather samples, the organization of the collagen fiber was still clearly visible, but the SHG signal from the fibers was greatly reduced, particularly in the case of the ‘red rot’ leather (Figure 5).

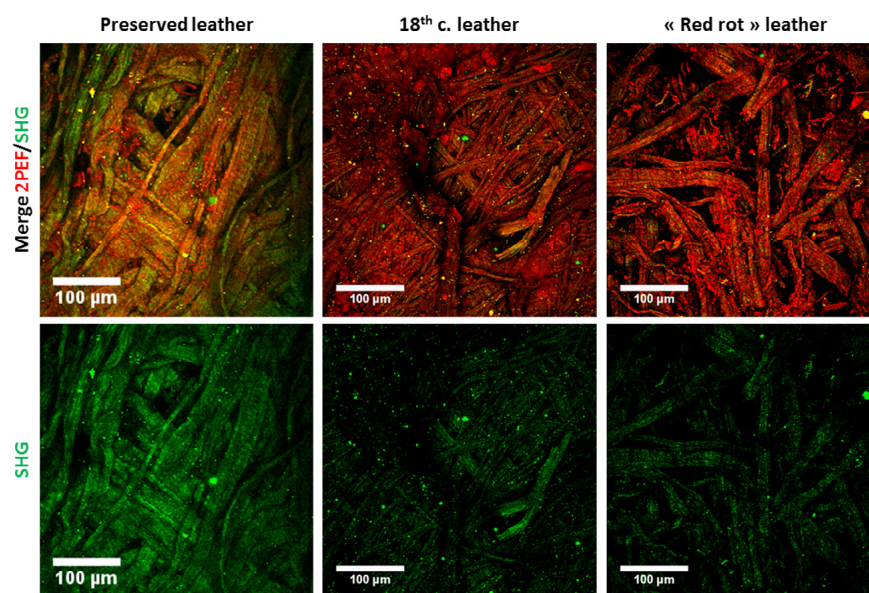


Figure 5. Nonlinear optical microscopy images of vegetable-tanned leathers: preserved, 18th-century bookbinding and “red rot” leather

In these three examples of alteration, similar observations were made by NLO microscopy for all the skin-based materials: a decrease or loss in the SHG signal, an increase in the 2PEF signal and, depending on the involved mechanism, some changes to the morphological structure, which became more compact and unstructured. NLO microscopy is therefore a technique well-suited to non-invasive examination of the degradation state of collagen in all skin-based materials, and could in the future overcome conventional invasive approaches using optical microscopy or DSC. The depth penetration is however limited in vegetable-tanned leather because of the strong absorption (resulting fluorescence signal) likely due to the presence of the vegetable tannins and lubricants. In general, this study shows close agreement between the DSC measurements of the degradation

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state and the evolution of the NLO signals. However, the two techniques do not agree when comparing different degradation types, as for parchments. This probably comes from the fact that the two techniques are not sensitive to modifications at the same structural scale. Whilst the measurement of the hydrothermal stability relates to a change in the hydrogen bonding in the collagen triple helix, the SHG signal is sensitive to the alteration of both the collagen triple helical structure on the molecular scale and the macro-molecular organization on the fibril and fiber scales.

CONCLUSION

This study demonstrates the high potential of NLO microscopy for the assessment of the conservation state of collagen-based materials in cultural heritage. Because of the non-invasive nature and rapidity of the measurements, it is possible to work directly on any ancient artifacts and examine several regions, mapping representative fields of view for a few square millimeters with sub-micrometer spatial resolution, which is crucial to take into account the heterogeneity of these materials. It should help to identify artifacts most at risk in collections and allow suitable conservation measures to be taken. This work will be extended in the future to a correlative approach with IR nanoscopy to help identify the different materials interacting with collagen within the skin and the degradation products formed on the micro and nanoscale. Finally, polarization-resolved SHG signals will be developed to extract quantitative information about the orientation of the fibrils on sub-micrometer to millimeter scales and allow a more precise ranking of the degradation.

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