Molecular Analyses for Ancient Parchment Documentary Materials

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Contributor: Guadalupe Piñar , Federica Cappa , Wilfried Vetter , Manfred Schreiner , Heinz Miklas , Katja Sterflinger

A new trend is molecular analysis, which has given rise to the emerging field of biocodicology, comprising protein and DNA analysis for the identification of the biological origin of the skins used for their manufacture. In addition, DNA analysis can identify the microbiome present in the object under investigation, which adds value by providing information on its history and state of preservation.

parchment animal skins inks biocodicology microbiome

1. Introduction

Novel molecular analyses have been applied in recent years to the study of ancient parchment documentary materials. The choice of the molecular technique that is used depends to a large extent on the question that is posed. There are two types of molecular analysis which have been applied to the study of ancient parchment documentary materials. Those that focus on the proteins [1] and those focusing on the analysis of the genetic information that is contained in the parchment, which includes the ancient DNA (aDNA) of the original animal skin [2] and the DNA of the microorganisms that make up the microbiome of the parchment material [3].

Molecular analyses applied to cultural heritage have taken advantage of the enormous development in the field of so-called “omics” analyses [4][5], which now also offer suitable tools to decipher the biological information that is contained in the ancient parchments. These approaches allow for the analysis of all of the biomolecules (proteins and total DNA), thereby delivering a kind of “bio-archive” of the material that are investigated. In addition, this approach confers a more reliable quantitative analysis of the data. Nevertheless, the biggest challenge in the molecular analysis of the manuscripts and other objects of cultural heritage has been the development of non-invasive or minimal invasive sampling techniques [6]. These techniques include different tools that are aimed at obtaining the maximum amount of information with the minimum amount of invasiveness that is employed when one is studying the objects. The use of a destructive method is permissible only on the fragments that cannot undergo conservation or cannot be reunited (for example, the fragments from the margins of pages, the bore dust that is produced by insects and the parts that will certainly be eliminated during restoration such as parts of the binding or cover leaves). Especially in the case of the documentary material, the development of non-invasive sampling techniques allows access to previously unanalyzed documents with the permission of conservators and archivists. To this end, a minimally invasive method has recently been developed for the sampling of parchment using conventional PVC rubber bands [1][7]. Documents can be sampled in situ by gently rubbing the eraser across

the surface of the material (**Figure 1**), and then, collecting the eraser fragments, without the need for abrasion or cutting. This sampling can easily be done by the conservators themselves without the need for specialized tools or equipment, and the sample can then be sent to the research laboratory for its analysis. Depending on the biocodicological analysis that is to be carried out (proteins or DNA), different amounts of eraser crumbs have to be collected, ranging from a 20–100 µL volume [1].

Figure 1. Sampling of an ancient parchment manuscript using PVC erasers by gently rubbing the frame of the manuscript.

2. Protein Analysis and Applications

The study of proteins, or proteomics, is a powerful tool for the study of a parchment material, as it can provide not only a sensitive approach to characterize the protein composition of a given sample, but also to identify the species of the proteins themselves and thus, the protein-based material that was used to manufacture a specific object [8]. This molecular approach is gradually becoming a recognized method in archaeological research, as one of the major advantages of this technology is that it requires a small sample and it is less laborious and costly than DNA

analysis is. This approach includes basic protein analyses or more in-depth proteomic analyses to identify the additional proteins on the surface of the parchment material [1].

Specifically, in the study of ancient parchment, a basic protein analysis has been successfully applied to determine the species of animal that was used in the parchment's manufacture. This method is called peptide mass fingerprinting (PMF) and it uses enzymatic digestion to cleave the proteins at specific amino acid sites, thereby forming a peptide mixture. After the collection has been performed, each sample is chemically treated to allow for the extraction of collagen from the skin. Although all of animal skin contains collagen, the mixture of peptides in the collagen of each species is unique. Using mass spectrometry techniques, the molecular weights of the individual peptides in each sample can be determined, thereby allowing a peptide mass fingerprint (PMF) to be generated for each of them. The fingerprint of one particular protein that has been obtained using mass spectrometry can identify the species of the animal that it derived from [8].

Until recently, the protein analysis of parchment required its destructive sampling, which consequently severely limited the number of analyses that could be performed on a given document or on several documents in an archive. As an example, in the study by Toniolo et al. [9] on the 13th century "Marco Polo Bible", the authors needed 5 mg of single-leaf parchment to identify that it was made of calfskin by the analysis of the peptide sequences. Thus, the same authors pointed out that the study was biased because more sheets could not be analyzed due to the destructive technique that was used and that it could have been made from different types of parchment, depending on what material was available at the time of its production. A further study by Kirby et al. [8] also showed the use of destructive sampling techniques to identify a Qur'an folio that is dated to the 9th c. as being made of sheep skin. Authors have also analyzed and compared some other objects that are made of animal skins, and they have stated that in all of the objects that were investigated, the ages and sample conditions had no significant effect on the resulting protein spectra and their subsequent identification. In order to avoid destructive techniques being used in the study of ancient manuscripts, Fiddyment et al. [7] went a step forward, using the non-invasive sampling method that is based on PVC polymer rubbers, which has the additional advantage of preserving the proteins in the PVC polymer residues at room temperature and without the need for the special storage of them. These authors performed a comparative analysis of the same sample using conventional MS techniques that require destructive sampling to occur, as well as the non-destructive electrostatic MS using the PVC eraser sampling method. They obtained the same, if not better, results with the PVC sampling than they did when they were using a real fragment of parchment. Thanks to the development of this non-invasive approach, the authors were able to extract the proteins from 513 parchment samples to resolve the animal origin of the suspected tissue paper-thin "uterine" vellum that was used in 13th century pocket Bibles and some additional parchment samples spanning this time period. The authors have found no evidence of the use of unexpected species, such as rabbit or squirrel, and they suggested that uterine vellum was the result of the technological production of it using the available animal resources, and that it would not have required unsustainable farming practices [7].

The ongoing development of proteomic techniques is enabling researchers to conduct more extensive studies to infer the animal origin of a multitude of parchment documents. Although, in principle, the selection of the skins seems to reflect the available livestock, and thus, the preferences of a geographical region, recent studies have

deciphered that some animals were preferentially used for the production of certain types of documents. It has been shown that valuable books were made from a rather expensive calfskin, which was reserved for the highest quality books [7]. In contrast, Doherty et al. [10] analyzed 477 legal deeds from the sixteenth to the twentieth century in the United Kingdom using peptide mass fingerprinting, and they concluded that most of them were made from sheepskin. This was due not only to the abundance of sheepskin and the low cost of sheepskin parchment at this time in the UK [11], but also to the unique biological structure of sheepskin parchment, which consists of an inner layer of skin called the dermis. The authors conclude that the pre-modern writers preferred to use sheepskin parchment due to its inherent ability to make visible the fraudulent erasure of texts, which is attributed to the high fat content of the skin (30–50%) when it is compared to that of calfskin (2–3%) and goatskin (3–10%) [12][13]. The removing of the fat during the parchment-making process may cause the layers of sheepskin to detach more easily than those of other animals. In order to make fraudulent changes to the documents after signing them, the original text would have to be scraped off. This could cause the sheepskin parchment layers to separate, and it would leave a visible imprint on the document, thereby making the fraud easily noticeable [10].

3. DNA Analysis

DNA analyses have been applied for several years to recover the biological information that is contained in the ancient manuscripts on the parchment [14]. These analyses have been gradually developed to minimize the biases that are associated with destructive sampling in order to recover the genetic material that is contained in the parchment. Nevertheless, the amount of DNA that can be extracted from the parchment is generally low, and it may be degraded for a variety of reasons, such as the chemical processes that occur during its manufacture, the age of the parchment and the various alterations it may have undergone over the centuries due to the human manipulation or poor preservation of it. Several scientific publications have addressed this issue and all of them point to the need to develop accurate non-destructive and non-invasive sampling methodologies and precise DNA extraction protocols, as well as a careful DNA manipulation to avoid the occurrence of external contamination [2][15][16][17][18][19][20].

It is important to highlight that the field of biocodicology has experienced a unthinkable advance thanks to the development of next-generation sequencing (NGS) approaches, thus eliminating the intrinsic limitations of the methodologies that were used before NGS platforms became available. However, even before NGS analysis was widely used, numerous studies already highlighted the possibilities that are offered by genetic analysis of parchment [14]. Some of these studies focused only on ancient DNA (aDNA) for the sole purpose of identifying the animal skins that were used in the parchment production [17][19][21]. In contrast, other studies have focused on the microbiological research of manuscripts to identify the risk of potential damage that is exerted by microorganisms [22][23][24][25] as well as to establish restoration/conservation methods [26].

Nowadays, advances in non-invasive sampling techniques, together with the development of NGS approaches, are making it possible to investigate the genetic material of the parchment on a larger scale. These analyses are still far from being routinely applied on ancient parchment due to the laborious work that is required for sequencing techniques, but also due to the high price of such analyses, which can only be afforded within special research

projects. Nevertheless, there are already some studies in which high-throughput NGS analyses coupled with bioinformatics have been applied to parchment manuscripts, thus allowing the researchers to identify the aDNA [15]. The compilation of these data has resulted in a specific microbiome for each investigated parchment or "bio-archive" [3][27][28], which can be considered to be a historical added value. The recovered biological information offers new possibilities to answer two main questions: (a) the type of skin/animal that was used to produce the parchments, and (b) the composition of the microbiome that is colonizing their surface, but also other relevant questions that are related to the manufacturing process of the book, its state of preservation and its historical use as well as the animal breeding history [1][2][3][15][27][29][30].

The workflow to answer these questions includes several steps that can be summarized as the use of non-invasive or minimally invasive sampling techniques which are followed by the DNA's extraction, obtained directly from the sample material. This is a very delicate and difficult task that requires experience and care to be taken. There are strict criteria to ensure the reliability of the aDNA results. There are critical conditions for which the DNA extraction procedures can occur, such as it being performed in dedicated laboratories using special devices and equipment, and there are downstream steps that will depend on the selected sequencing platform. Primarily, the Illumina sequencing platform has been used for these analyses, as it is an ideal platform for sequencing short fragments of aDNA, it has good coverage and it shows a greater sensitivity and reliable results [2][31][32]. Bioinformatics analyses with different pipelines have also been developed to better analyze the huge dataset that results from this method [27]. Comparative analyses with DNA databases finally allow the identification of sequences and their affiliation to animals, bacteria, fungi, viruses and humans [15].

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