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Identification of proteinaceous binders in paintings: A targeted proteomic approach for cultural heritage

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ABSTRACT

Identification of proteins in paintings and polychrome objects is a challenge, which requires the development of tailored analytical approaches. In the present study, a targeted proteomics approach was developed for discriminating among the three most common proteinaceous materials used as paint binders, i.e. milk, egg, and animal glue. In this study a specific database of peptides was created based on tandem MS analyses of tryptic digests of several paint samples collected from a variety of art objects of different ages and conservation conditions. Specific peptide markers of each protein were then selected and monitored by LC-MSMS in Multiple Reaction Monitoring (MRM) ion mode, together with their specific precursor ion-product ion transitions, as defined by their unique amino acid sequence. The developed method enabled a sensitive and reliable detection of the target peptides in a selection of case studies, leading to the unambiguous identification of the proteins used as paint binders. The method showed greatly increased sensitivity compared to currently available strategies.

1. Introduction

The inherently multidisciplinary nature of proteomics has recently led to its application to assorted areas that include the field of cultural heritage [1] for the identification of protein-based materials in artworks and polychromies, archaeological remains, and paleontological objects [2].

The most up-to-date approaches for the characterization of proteinaceous materials in artworks and polychromies, and in general when dealing with ancient proteins, rely on mass spectrometry technologies that are able to identify proteins from the MS analysis of their digested peptides [2,3] (and references therein). Typically, MS-based proteomics pipelines aimed at identifying proteins in ancient samples follow the classical bottom up approach, where samples are enzymatically digested to peptides, which are either analyzed by simple MS, or separated by liquid chromatography and eventually analyzed by tandem mass spectrometry. Peptides and thereof proteins are identified by correlating experimental spectra to virtual ones in a protein sequence database [4]. In the classical full scan operating mode (typical of LC-MSMS analysis in discovery proteomics or shotgun proteomics, otherwise named global profiling proteomics or untargeted proteomics), the mass spectrometer continuously repeats the full scan mass spectrum and selects and fragments the n (typically $n = 1–10$) most abundant ions (information-dependent acquisition, IDA).

So far, the applications to the cultural heritage field are untargeted, discovery proteomics based experiments. The fascinating potential of this operating mode lies in the possibility of uncovering proteins without any "a priori" knowledge or hypothesis. It is therefore compatible with both well and poorly characterized systems in cultural heritage, the only basic requirements being the actual presence of proteins in the sample and the presence of the protein sequences or homologous sequences in a properly selected database [2,3].

Since the first report of LC-MSMS in 2006 [5], despite the tremendous success of these approaches in a wide range of works of art

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and archaeological remains [5–24] standard based proteomics strategies keep being developed to address the field-specific analytical challenges in samples from cultural heritage, by improving the steps in the standard proteomics protocols, from the solubilization step on [20,21,25–28]. So far, the greatest attention was addressed to the preparation of the sample, correctly considering the unusual nature/state of the sample itself as the biggest challenge in the analysis.

However, as far as the mass spectrometric analysis itself is concerned, some of the limits in the detection in a LC-MSMS analysis can also arise from the automated peak selection and the linked instrument's bias towards repeatedly selecting and fragmenting the species with the most intense MS signals. The complexity of these unusual samples in works of art and archaeological remains, in terms of chemical composition, the unavoidable presence of contaminating protein-based materials (even coming from the environmental dust), as well as the high levels of molecular damage found in ancient samples, can overwhelm even the most modern mass spectrometer [3,21,25], sometime making the identification of protein components in these samples extremely challenging. This because the mass spectrometer might miss signals coming from important, relevant proteins, but instead might “waste its time” on signals from uninformative contaminants, for instance. Searching for significative signals in a multitude of untargeted signals might then become like looking for a needle in a haystack with the naked eye.

There are cases, however, such as the identification of the protein-based binders in paintings, where, in principle, the search could be basically simplified to a discrimination between the most commonly used materials, i.e., animal glues, egg, and milk casein. Therefore, the analysis can be “targeted” towards a search for the protein components of these materials, within a limited range of possibilities, in an alternative, focused approach that by selectively searching for a restricted set of marker proteins, can eventually guarantee a higher sensitivity and reduce the interference from contaminations as lowest as possible.

In these cases, a significant advance in the solution of the analytical challenge can be the multiple reaction monitoring (MRM) ion mode, an alternative mass spectrometric strategy, the actual gold standard in mass spectrometric technique for selectivity and high signal-to-noise ratio that selectively and non-redundantly search for specific ions of one or, more desirably, a few peptides that will unequivocally constitute protein markers to be identified as unique signatures for each binder. The mass spectrometer will therefore focus on selected signals, getting rid of the “noise” of the other ions from the background, thus sensibly increasing sensitivity. Quoting the commentary on Nature Methods on 2013 [29], MRM “can be thought of as the mass spectrometrist's ELISA. It targets proteins using a predetermined assay with high sensitivity and selectivity [30]: molecular ions of a target peptide are selected in the first mass analyzer, fragmented in the collision cell and one or several of the fragment ions uniquely derived from the target peptide are measured by the second analyzer. MRM is referred to as targeted approach as only predetermined ions are measured, and mass spectrometer acts as a mass filter selectively monitoring a specific analyte molecular ion and one or several fragment ions generated from the analyte. MRM has been first developed and applied for decades in the pharmaceutical industry to quantify small molecules [31] and evolved to be applied in the field of proteomics, to detect low abundant proteins in complex matrices, to verify biomarker candidates, and has proved to be successful in clinical settings [32–42].

Mass spectrometry-based strategies that search for specific peptides as markers in the analyses of protein-based binders have been reported [20,43–46]. However, in none of them, to the best of our knowledge, has ever been used to discriminate between collagen-, milk- and egg-based binders. We propose this technique as a probe to specifically identify peptide markers of proteinaceous materials in paintings. Herein, we developed and applied a method based on a targeted proteomic approach that makes use of tandem mass spectrometry in multiple reaction monitoring (MRM) ion mode to selectively monitor a number of specific peptides belonging to proteins markers of individual proteinaceous binders in paintings. This alternative strategy, by selectively and non-redundantly searching for specific signatures of the proteinaceous binders, could represent a significative advance in the solution of the analytical challenge set by ancient samples. Differently from previously reported methods [20,43,44,46] that also search for specific peptides but in untargeted, typical shotgun runs, where relative abundance of the single peptide ions can still strongly affect the results, MRM by its two-level mass filtering instrumental operating mode results in an increase of selectivity, and an incredibly high signal-to- noise ratio for the target analytes, getting rid of most of the nuisances arising from abundant contaminating proteins in the samples that can unavoidably affect, for instance, mass fingerprinting methods but also standard LC-MSMS approach.

In this work, the most prominent and specific protein markers for protein-based binder were chosen by screening a collection of LC-MSMS analyses of a panel of paintings/test samples that had been carried out during the years. Samples had different origin, had been prepared with different procedures, with different pigments, in order to be as general as possible in the selection of the target peptides, and provide a versatile tool that could be applied to any painting sample. Specific peptide markers of each protein were then selected on the basis of their frequency of detection in the set of samples and of their mass spectrometric behavior together with their specific precursor ion-product ion transitions as defined by their unique amino acid sequence. Then, a single MRM method was constructed to detect all the target peptides in a single analysis with high sensitivity, selectivity and accuracy, leading to the unambiguous discrimination among the different proteinaceous binders. The method was tested on a panel of samples of known composition and then successfully applied to a set of paint samples of unknown composition.

Results presented here demonstrate the unique ability of MRM method to selectively assess the nature of proteinaceous binders in paintings, and its successful application to samples where standard LC-MSMS analysis had failed, or to disclose previously undetected components.

2. Materials and methods

2.1. Reagents

Ammonium hydrogen carbonate (AMBIC), Ethylenediaminetetraacetic acid (EDTA); Tri(hydroxymethyl)aminomethane (TRIS), and TPCK-treated trypsin were from Sigma; recombinant Peptide N-Glycosidase F (PNGaseF) was from Roche. Formic acid and Acetonitrile (ACN) were purchased from Baker. Deionized water was obtained from Millipore cartridge equipment.

2.2. Painting samples

The list and the characteristics of paint replicas and historical samples used to build the database of selected peptides are reported in Table S1. Historical samples were from collections that have been published elsewhere [26,47–50], and criteria for sampling and description of the single cases are therein given.

Samples were motley analyzed in the course of the years, and were generally treated in heterogeneous phase with trypsin with variations in respect to the minimally [21] invasive protocol as reported in Table S1, and the resulting peptide mixtures were analyzed by LC-MSMS, as detailed in the therein indicated references [21,25,26,47–50]. For those samples that were used in the setup of the database that have not been published previously, digestion with trypsin, LC-MSMS analysis and data analysis were carried out as described in detail in the Supplementary information.

In order to build up the database, the proteins and the peptides most frequently detected in standard global profiling experiments were screened regardless the treatment procedures they had undergone and