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A versatile and user-friendly approach for the analysis of proteins in ancient and historical objects

Georgia Ntasi [a], Daniel P. Kirby [b], Ilaria Stanzione [a], Andrea Carpentieri [a,c], Patrizia Somma [c], Paola Cicatiello [a], Gennaro Marino [a,c], Paola Giardina [a], Leila Birolo [a,*]

[a] Department of Chemical Sciences, University of Naples Federico II, Naples, Italy
[b] Private Practice, Milton, MA, USA
[c] Department of Humanities, University Suor Orsola Benincasa, Naples, Italy

A B S T R A C T

Identification and characterization of ancient proteins still require technical developments towards non-invasiveness, sensitivity, versatility and ease of use of the analyses. We report that the enzyme functionalized films, described in Cicatiello et al. (2018), can be used efficiently on the surface of different objects ranging from fixative-coated paper to canvas to the coating on an albumen photograph, as well as the much harder surfaces of ivory objects and the proteinaceous binders in the decoration of a wooden Egyptian coffin. The mixture of digested peptides that are efficiently captured on the functionalized surface are also amenable to LC-MS/MS analysis, which is necessary to confidently identify chemical modifications induced upon degradation, in order to characterize the conservation state of proteins. Moreover, in a two-step procedure, we have combined the trypsin functionalized film with a PNGaseF functionalized film, which adds a deglycosylation pretreatment allowing improved detection of glycosylated proteins.

Significance: User friendly trypsin functionalized films were implemented to expand their potential as versatile, modular tools that can be widely exploited in the world of diagnosis of cultural heritage objects, ancient proteins, and palaeoproteomics: a procedure that could be carried out by conservators or archaeologists first *on-site* and later analysed with standard MS techniques.

1. Introduction

Any new information on the chemical composition of artistic objects or archaeological remains provides keys to decipher ancient and historic materials revealing new insights and/or serving cultural heritage conservation.

Recent technical advances in analytical chemistry and, in particular, the astonishing improvements in mass spectrometry (MS), have allowed adaptation of methods for the study of ancient proteins in artworks and objects of cultural heritage [1]. It is now possible to detect proteins with extreme sensitivity (below femtomoles) and characterize them in detail [2–4]. However, most classical procedures and strategies are considered to be invasive even when only micro sampling is required. In this respect, the technical and methodological challenges are still huge considering the complexity of the artwork samples and the microscopic amount often allowed for analyses. A novel method towards the non-invasive analysis of proteinaceous materials from artworks was recently developed, based on a protease functionalized sheet of cellulose acetate to directly digest proteins *in-situ* on painted surfaces without sample removal from the artefact [5]. This work demonstrated that, at least on a short term, temporary scale, the painted surfaces were not altered. Trypsin was readily immobilized on the sheet, taking advantage of the adhesive properties of the previously formed layer of a self-assembling protein, the class I hydrophobin Vmh2 from the fungus *Pleurotus ostreatus* [5]. The *in-situ* digested peptides can then be remotely analysed by standard mass spectrometric approaches to identify the proteins from which they arise, and a number of peptides sufficient for a confident identification of the proteins was obtained. The benefit of this functionalized film is that, unlike similar, existing film/resins [6–9], this is the only one, to the best of our knowledge, that directly allows the digestion of proteins and, due to the adhesive properties of Vmh2, extracts the peptides from the object. In contrast, other film/resin techniques remove intact proteins from the artefacts, but the proteins have to be digested afterwards before MS analysis and identification. Major advantages of the present bioactive films are: i) they can be used without the need to transport the works of art; ii) there is no need for specific technical skills to collect the sample since functionalized sheets can be provided as dried films and moistened just before use and then simply put in contact with the work of art. The bioactive film was developed as a user-friendly device, since sampling could eventually be carried out by

* Corresponding author.
E-mail address: leila.birolo@unina.it (L. Birolo).

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conservators; after use, the sheet can be stored, and peptides recovered just before mass spectrometric analysis.

The proof of concept was shown with paint replicas and ancient paintings [5]. We herein demonstrate: i) the effectiveness of the functionalized film approach for identifying proteins on a wider range of historical objects and supports; ii) the possibility of using these films for characterizing their conservation state since this experimental protocol is amenable to the analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) that is routinely used to confidently identify and localize chemical modifications in proteins; iii) that the use of trypsin functionalized films can be integrated with sheets that have been functionalized with other auxiliary enzymes, such as glycosidases.

Although intrinsically limited to the analysis of the top or immediately next layers, since immobilized trypsin will likely not migrate into the lower layers, the use of functionalized films can be a method of choice whenever physical sampling is not allowed or where information on the lower layers is not needed.

Thus, the overall goal of the present work is to determine i) if relevant analytical information can be obtained and whether the obtained data is comparable to data obtained with other sampling methods, and ii) what, if any, surface alteration occurs to provide guidelines to users as to the suitability of the method in a particular circumstance.

2. Materials and methods

2.1. Samples

A list of the samples herein analysed, with the details of the scope of the specific analysis and the technique is given in Table 1.

Paper samples (Barcham & Green Langley Handmade paper) were brush coated with isinglass, albumen and casein fixatives prepared according to historical methods. The albumen photograph was from a study collection and known to have an albumen coating. Ivory samples from walrus, mammoth and elephant were obtained commercially (Boone Trading Company, Brinnon, WA) and were not prepared in any way except to clean the surface with an alcohol swab prior to analysis. Pictures of the sampled objects are shown in the supplementary materials Figs. S1–S5.

A sample of the lining of historical canvas was taken from the back of an oil painting of "The Virgin and Child, Saints and Cherubins" (an artwork confiscated from Neapolitan mafia) stored in the storehouse of the Cultural Heritage Superintendence of Naples. The painting is attributed to the school of Solimena and can be dated back to 1700. A picture of the sample is reported in the supplementary materials Fig. S6.

A fragment was collected from the painted external layers of a yellow coffin that belonged to the Drosso-Picchianti Collection, at Museo Archeologico Nazionale di Napoli (MANN) [10]. The artefact is an inner anthropoid coffin and belongs to a specific type known as a "yellow coffin" because of a rich decoration painted on a yellow priming layer. A picture of the coffin can be found in [10]. The realization technique of the yellow coffin under study involves different layers; starting from the internal wooden parts there is a first layer (called *mouna*) essentially made of vegetable fibres, then the white preparative layer (made of anhydrite mixed with arabic gum), and finally the yellow background and the external layers of polychromies (analysed in the present study). Coating were probably made of natural (non colored) varnishes [10]. According to its iconography and inscriptions, the coffin is datable by the end of the XXIst and the beginning of the XXIInd Dynasty. (Niwiński, Nicola, and Egizio 2004; *AA.VV. Guida alla collezione egizia del MANN* 2016; Cantilena and Rubino 1989). We analysed a fragment from the surface layer of the yellow coffin (sample A from [10]) to simulate the possibility of *in situ* analysis of the surface of the coffin without removing the pieces from the coffin itself.

3. Procedures

3.1. Hydrophobin production

The protein Vmh2 was extracted from the mycelium of *Pleurotus ostreatus* as reported by Gravagnuolo et al. [11].

3.2. Enzyme immobilization

Trypsin was immobilized on Vmh2 coated cellulose acetate surface as previously reported [5]. Peptide:N-glycosidase F (PNGaseF) was immobilized on Vmh2 coated cellulose acetate surface following a very similar procedure. Briefly, 200 μl of 500 U/ml of PNGaseF solution in 10 mM of sodium phosphate pH 7.5 were deposited for 5 min on the Vmh2 coated surface (0.5 × 0.5 cm). After incubation, the surface was washed with 500 μl of 10 mM of sodium phosphate and left to dry at room temperature. As for trypsin activation, a solution of 50 mM of ammonium bicarbonate pH 7.8 (AMBIC) was sprayed on the PNGaseF biofunctionalized surface to restore the optimal environment for the enzymatic activity.

Table 1
Summary of samples, type of functionalized films, their dimension, the mass spectrometric analysis and its scope.

Samples	Dimension of functionalized surface	Type of functionalized film	Analysis	Scope of the analysis
Casein fixed paper	1st set: 400mm^2 2nd set: 100 and 25mm^2	Trypsin functionalized film	MALDI-TOF	Protein identification
Isinglass fixed paper	1st set: 400mm^2 2nd set: 100 and 25mm^2	Trypsin functionalized film	MALDI-TOF	Protein identification
Mammoth, ivory samples	1st set: 400mm^2 2nd set: 100 and 25mm^2	Trypsin functionalized film	MALDI-TOF	Protein identification
Walrus ivory samples	1st set: 400mm^2 2nd set: 100 and 25mm^2	Trypsin functionalized film	MALDI-TOF	Protein identification
Elephant ivory samples	1st set: 400mm^2 2nd set: 100 and 25mm^2	Trypsin functionalized film	MALDI-TOF	Protein identification
Lining of Canvas "The Virgin and Child, Saints and Cherubins"	400mm^2	Trypsin functionalized film	LC-MS/MS GC–MS	Protein identification Chemical modifications identification Glycation products
Fragment A of a yellow coffin [10]	400mm^2 (trypsin), 400mm^2 (PNGaseF)	Trypsin and PNGaseF functionalized films	LC-MS/MS	Protein identification Chemical modifications identification Test of the PNGaseF functionalized film for the removal of glycosilation
Egg based painting mock-up with azurite [5]	400mm^2 (trypsin), 400mm^2 (PNGaseF)	Trypsin and PNGaseF functionalized films	MALDI-TOF	Test of the PNGaseF functionalized film for the removal of glycosilation

3.3. In situ trypsin hydrolyses on fixative-coated paper, the albumen photograph, and ivory samples

First set: Large film pieces, ~400mm^2 (20x20mm), were spray-moistened with 50 mM AMBIC and applied to the surface being analysed for 10 min. For irregular surfaces, the films were held in place with tweezers to maintain as much surface contact as possible. Films were removed and air dried, and peptides were solubilized from the surface with 25 μl 50 mM AMBIC. Second set: Smaller film pieces were cut from the original 400mm^2 pieces into ~100mm^2 and ~ 25 mm^2 pieces. Rather than spray moistening the films, 5 μl AMBIC (100 mm^2 films) or 2 μl AMBIC (25 mm^2 films) was pipetted onto the surface being sampled, and the film were placed on top for 10 min. Films were then placed into Eppendorf tubes with 25 μl AMBIC, vortexed to extract peptides, dried under air and the films removed. The solubilized peptides in Eppendorf tubes were taken to dryness under a stream of air and resolubilized with 10 μl 0.1% TFA (trifluoroacetic acid). For both sets, MALDI samples were prepared by the addition of 3 μl of the peptides in 0.1% TFA to 20 μl of matrix solution (40% acetonitrile, 0.1% TFA, saturated alpha-Cyano-4-hydroxycinnamic acid) and then spotted onto the MALDI plate.

3.4. In situ hydrolyses on paint replica, historical canvas and fragments from the surface of yellow coffin

When requested, pretreatment of samples with PNGaseF functionalized surface was carried out, similarly to the procedure for tryptic digestion, by putting the sample in direct contact with the PNGaseF functionalized surface for 10 min at room temperature. Then, samples underwent tryptic digestion with the trypsin functionalized film. Digestion was carried out as reported in [5] and peptides recovered in 25–50 μl of AMBIC. For irregular surfaces, the films were held in place with tweezers to maintain as much surface contact as possible. PNGaseF and trypsin hydrolyses in heterogeneous phase (enzyme solution in AMBIC on solid samples) were carried out as reported in [12] on 10 μg of scraping of the lining of the canvas.

3.5. Polysaccharides analysis

The lining of historical canvas was subjected to ammonia extraction following the protocol by [13] with slight changes. Briefly, 400 μl of 2.5 M NH_3 were added to 1 mg of scraping of the lining of the canvas and incubated in an ultrasonic bath at RT for 30 min and afterwards in a water bath for 120 min. This step was repeated twice, and the supernatants combined. The extract is evaporated to dryness under vacuum and a further liquid-liquid extraction is carried out by the addition of 100 μl 1% TFA and 200 μl of diethyl ether (three times). The acid solution (containing proteins, polysaccharides, and free organic salts) is combined with the insoluble extract from the first step. Subsequently, proteins are separated from polysaccharides with MeOH precipitation by the addition of 200 μl MeOH at −20 °C for 120 min. 100 μl of the polysaccharides containing fraction were dried under vacuum and submitted to methanolysis. The reaction was performed by combining the samples with 500 μl of methanolic HCl (125 μl of acetyl chloride in 2.5 ml of anhydrous methyl alcohol); the re-*N*-acetylation of the monosaccharides mixture was performed by adding 500 μl of methanol, 10 μl of pyridine and 50 μl of acetic anhydride at room temperature for 15 min. Sugars were finally trimethylsilylated in 200 μl of *N*,*O*-bis-(trimethylsilyl)-acetamide (TMSA) at 70 °C for 15 min. The sample was dried under nitrogen, dissolved in 50 μl of hexane and centrifuged to remove the excess of solid reagents. The hexane supernatant (1/60) was used for the GC–MS analysis.

4. Mass spectrometric analyses

4.1. Gas chromatography-mass spectrometry (GC–MS)

GC–MS analyses were performed on an ISQ-QD quadrupole mass spectrometer (Thermo Fisher scientific) equipped with a TRACE™ 1300 Gas Chromatograph using a Zebron ZB-5HT Inferno (5%-Phenyl-95%-Dimethylpolysiloxane) fused silica capillary column (Column 30 m × 0.32 mm × 0.10 μm) from Phenomenex.

The injection temperature was 250 °C, the oven temperature was held at 70 °C for 2 min and then increased to 230 °C at 20 °C/min, increasing to 240 °C at 20 °C/min and finally to 270 °C at 20 °C/min and held for 3 min. Electron Ionization mass spectra were recorded by continuous quadrupole scanning at 70 eV ionization energy, in the mass range of *m/z* 30–800. Mass spectra assignment was generally based on the direct match with the spectra of NIST library. If the correlation match index was higher than 95%, the identification was considered reliable.

MALDI-TOF analyses were carried out on a 5800 MALDI TOF/TOF instrument (Sciex, Framingham, MA) as reported in [5]. Data analysis was done using the mMass freeware program [14]. Marker ions used for material identification are collected and validated from several sources including published research data and protein sequences, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) data from known samples, and analysis of known reference samples. See Supplementary Material Table S-1 for reference details.

4.2. Peptide mass fingerprint (PMF) analysis

PMF is used to identify proteins and simple protein mixtures through the use of marker ions produced by peptides, which are validated indicators for specific proteins. Markers for common proteinaceous materials found in artworks, such egg tempera, casein and animal glues, have been published [5,15] and are shown in Table S-1. These have been used in several recent applications [15–17] as well as in the first development of this functionalized film methodology [5]. PMF is also used routinely in archaeology and cultural heritage to identify mammalian sources of collagen-based [18] materials, wherein marker ion can identify to the family level, in some cases, and to species level in others.

In case of the egg based painting mock-up sample, PMF was carried out with a licensed version of Mascot software (www.matrixscience.com) version 2.4.0. Proteins were identified using a `Gallus gallus` database (29,476 sequences; 15,469,207 residues). Mascot search parameters were: peptide mass tolerance 100 ppm, allowed trypsin missed cleavages up to 3. No fixed chemical modification was inserted, but possible oxidation of methionine residues and deamidation of asparagine and glutamine were considered as variable modifications.

4.3. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

For LC-MS/MS analysis, the recovered peptides were filtered on 0.22 μm PVDF membrane (Millipore), and peptides were desalted and concentrated on in-house made C18 extraction stage tips [19]. To increase peptides recovery, the unbound fraction from the stage tips was loaded on a reverse-phase C18 Zip Tip pipet tip (Millipore). Peptides were eluted with 20 μL of a solution made of 50% acetonitrile, 50% formic acid 0.1% in Milli-Q water and combined with the eluted fraction from stage tips, dried under vacuum, and finally dissolved in 0.1% of formic acid.

LC − MS/MS analyses were carried out on a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent *Technologies*) and on a LTQ Orbitrap-XL (ThermoScientific, Bremen, Germany) as reported in [20], and raw data analyses as reported in [12]. Each LC − MS/MS analysis

was preceded and followed by blank runs to avoid carryover contamination. MS/MS spectra were transformed in Mascot Generic files (.mgf) format and routinely used to query the SwissProt database 2015_04 (548,208 sequences; 195,282,524 residues), with Chordata as the taxonomy restriction for protein identification. A licensed version of Mascot software (www.matrixscience.com) version 2.4.0 was used. Standard parameters in the searches were trypsin as the enzyme; 3, as allowed number of missed cleavages; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from 2+ to 3+. In all the database searches, no fixed chemical modification was inserted, but possible oxidation of methionine residues and deamidation at asparagines and glutamines were considered as variable modifications. When collagen proteins were identified, further identification runs were carried out with the insertion of hydroxylation on lysine and proline as variable modifications, since more confident identifications are commonly obtained for these proteins by taking into consideration their extensive posttranslational modifications. Moreover, to reduce the search space, these runs were carried out using a homemade database, which we named COLLE (60 sequences; 88,859 residues), that collects all the common domesticates used for animal glue. When egg proteins were detected, identification was carried out using a *Gallus gallus* database (29,476 sequences; 15,469,207 residues) with sequences provided from the UniProtKB/Swiss-Prot protein database (www.uniprot.org).

Other parameter changes for the specific database searches are indicated in the Table captions. Only proteins presenting two or more peptides were considered as positively identified. Protein scores are derived from ions scores as a non- probabilistic basis for ranking protein hits. (http://www.matrixscience.com/help/interpretation_help.html). Ions score is −10*Log(P), where P is the probability that the observed peptide match is a random event. Individual ion score threshold provided by Mascot software to evaluate the quality of matches in MS/MS data was used for confidence threshold in protein identification.

Deamidation rates for Asparagine (N) and Glutamine (Q) for individual samples was semi-quantitatively evaluated as variable modifications from MaxQuant's "evidence.txt" file with a code that is freely available on GitHub (https://github.com/dblyon/deamidation). Semi quantitative evaluation is based on calculation of all modification states of all identified peptides that contain N and Q [21].

5. Results and discussion

5.1. Use of trypsin-functionalized films for protein identification of different types of samples

The trypsin functionalized films were evaluated for their ability to detect and identify proteinaceous materials on objects from cultural heritage, including fixative-coated paper (albumen, casein and isinglass), an albumen photograph, and ivory from elephant, mammoth and walrus. These samples were chosen because they are sample types of interest and importance in art and cultural heritage where minimal sampling is usually a necessity. Moreover, species identification of elephantid ivory and their products is still a challenging question and is essential to combat illegal ivory trade [22]. Molecular approaches as simple as the functionalized film combined with PMF are very attractive. Also, the same samples have been used previously to evaluate other minimally invasive techniques, so there was a basis for evaluating the results with the functionalized films. For these initial evaluations, alteration of the sampled surface was observed without magnification: paper and photograph with raking light, polished ivory surfaces with strong reflected light. Ultimately, more stringent observation will be necessary to accurately inform users of any consequences of this minimally invasive procedure.

A first set of specimens (photograph, fixed paper and ivory) was analysed by PMF using 400 mm^2 (20 x 20 mm) film pieces, and proteins were identified by visual inspection of MALDI spectra for marker ions.

Intense spectra were obtained for all the objects and surfaces tested, and all spectra were comparable to those obtained with other sampling methods on the same specimen. No visible alteration of the surfaces of the ivory objects was observed. With the fixative coated papers, the area under the film became slightly swollen, and this mitigates as the paper dries. For the albumen photograph, the surface under the film was heavily distorted, as anticipated, because albumen coatings on prints are known to be very moisture sensitive. Subsequent analyses with small film pieces lessened the effect on paper and the photograph.

The spectrum reported in Fig. 1, which is from the casein-fixed paper with casein markers indicated [15,18], is an example of the high quality of the MALDI data obtained with the first set of samples. The other samples gave results of similar quality. Keratin was noted in some of the samples. This contaminant largely originates from dust/dirt accumulation, typically epithelial keratin, and does not interfere with data interpretation.

Given the high detection sensitivity observed with the first set of samples, a second set was analysed with smaller film pieces (100mm^2 or 25mm^2) and reduced quantities of AMBIC (5 μl for 100 mm^2 films and 2 μl for 25 mm^2 films, respectively). The AMBIC solution was pipetted directly onto the surface/object being sampled, and the film was placed on top for 10 min. As noted above, with the smaller film formats the film was then placed directly into the Eppendorf tube thus improving the overall recovery of produced peptides. The results for 100mm^2 and 25mm^2 films were very similar, and the high quality of the spectra at these reduced film sizes indicates that further size reduction will be possible, further minimizing alteration of the surfaces being sampled. Again, spectra from the second set were comparable with spectra from the same specimen with other sampling methods (see Fig. 2).

With this second set using smaller film pieces, there was no visible alteration of the ivory surfaces, very slight "pillowing" of the paper surfaces at the point of contact that quickly disappeared, and moderate swelling of the albumen photograph at the point of contact that persisted. With even smaller film pieces as a possibility, the effect on the paper and photograph would be expected to diminish further. Although surface alteration might not disappear completely with these specimens, there may be situations where sampling could still be an option. Fig. 2 compares the results from 100mm^2 and 25mm^2 film samples of the casein-fixed paper surface. The inset compares the relative signal-to-noise ratio (S/N) calculated by the mMass program observed for two of the weaker ions with the different film sizes.

Figs. S7–S9 are MALDI spectra from ivory, the albumen photograph, and the isinglass-fixed paper, respectively, obtained using the smallest (25mm^2) film pieces. Albumen and isinglass are identified with markers from Table S1. Elephant ivory is identified with marker ions from a published reference [23].

It is worth noting the high quality of the spectra obtained, considering that no sample cleanup or concentration, for example using Zip Tips, was done before analyzing the analysis. The peptide mixtures were simply collected from the trypsin functionalized film, dried, resolubilized, mixed with matrix and spotted on the MALDI plate. Given the high sensitivity observed with the smallest film pieces (25mm^2), it seems very likely that even smaller formats will be possible, further limiting the impact on sensitive surfaces and allowing analysis of smaller features.

5.2. The trypsin-functionalized film can be used for the characterization of ancient proteins

The trypsin functionalized film has been shown to be amenable for protein identification in several artistic objects, even by a simple MALDI-TOF analysis, without requiring fragmentation spectra, thus showing up as an extremely interesting and powerful tool for all stakeholders in cultural heritage interested in understanding the composition of works of art.

However, the story and the interest in ancient proteins might not stop at the identification level. It is now more and more common that, beside understanding the composing material, chemical analyses must

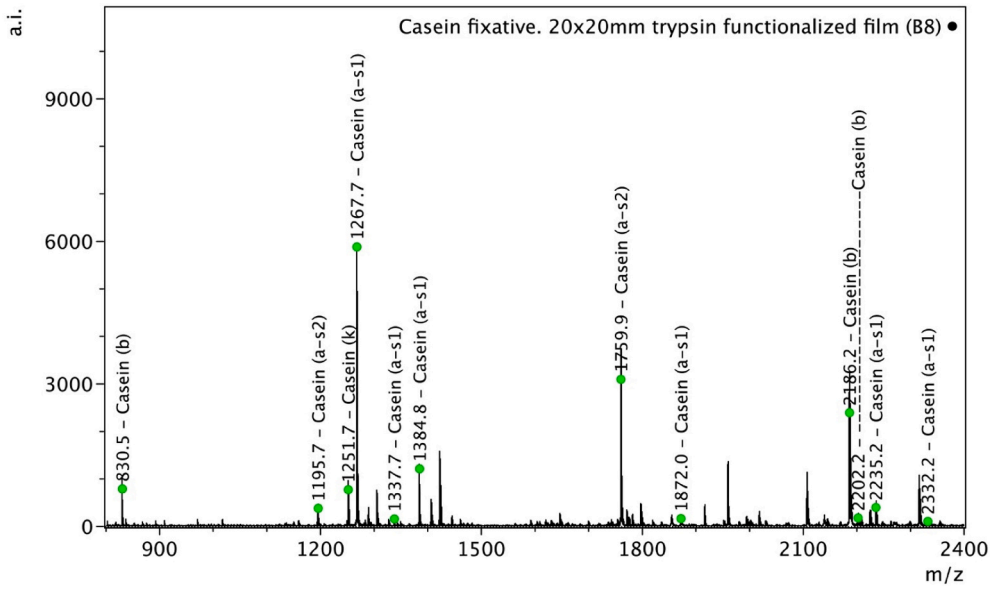

Fig. 1. MALDI spectrum from casein-fixed paper sampled with a 400mm^2 trypsin functionalized film. Major casein marker ions derived from published sequences (see Table S1) are indicated: a-S1: alpha casein S1 (P02662), a-s2: alpha casein S2 (P02663), b: beta casein (P02666) and k: kappa casein (P02668).

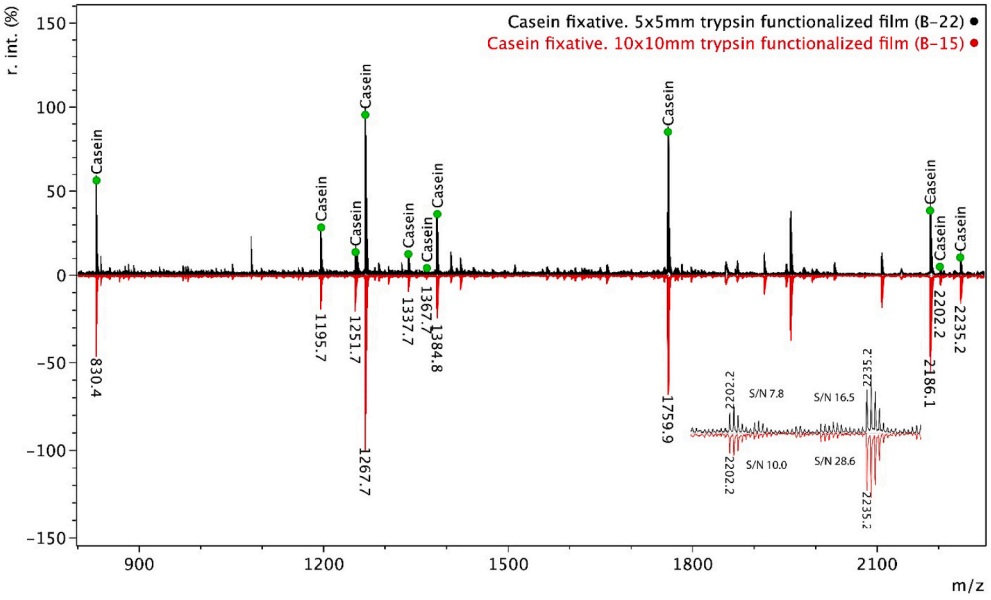

Fig. 2. MALDI spectrum from the casein-fixed paper. Upper, black, 25mm^2 film. Lower, red, 100mm^2 film. The inset shows the 2202 Da and 2235 Da ions to compare the relative S/N observed with the larger (lower) and smaller (upper) film sizes. Casein markers are the same as noted in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

face the challenge of understanding at the molecular level the conservation state of the work of art [2]. This is at the basis of a wide range of questions, from authentication to restoration interventions. To face this challenging issue, the peptide mixtures recovered from the film are usually analysed by LC-MS/MS, since unambiguous identification and localization in the peptide sequence of chemical modifications that can be considered as markers of degradations, such as oxidations, deamidations or spontaneous hydrolysis, strongly benefit from the analysis of fragmentation spectra [2]. Moreover, LC-MS/MS analysis is crucial in providing unambiguous identifications of complex samples with proteins in mixture, where MS analysis without MSMS, and thereof sequence information, can fail [2]. Therefore, to verify whether the peptide mixtures recovered from the trypsin functionalized films are amenable to LC-MS/MS analysis, a sample from a lining material of the historical canvas "The Virgin and Child, Saints and Cherubins", that dates back to 1700, that was under investigation for the presence of proteinaceous binder, was digested with the trypsin functionalized flexible sheet and the recovered supernatant analysed by LC-MS/MS. The results were compared to those obtained with a parallel experiment where an aqueous trypsin solution was directly added to an aliquot of the same solid sample in a conventional digestion procedure in heterogeneous phase [24], that requires microinvasive sampling of the object. A summary of the identification results is shown in Table 2, and the details of the identifications are reported in the supplementary results (Table S2-S3).

Hide glue from sheep was unambiguously identified since type I and type III collagens from *Ovis aries* were detected. Good confidence was obtained in both cases, although, not unexpectedly, identification was

Table 2
Identification of proteins in the lining of the historical canvas "The Virgin and Child, Saints and Cherubins" by LC-MS/MS.

		Digestion with the Trypsin functionalized film				Overnight digestion in heterogenous phase			
Sample	Protein (Uniprot entry)	Score	Sequence coverage (PSC) (%)	No. of peptides	No. of unique peptides	Score	Protein coverage (PSC) (%)	No. of peptides	No. of unique peptides
Lining of the canvas "The Virgin and Child, Saints and Cherubins"	Collagen 1(I) (W5P481)	1223	27	26	4	2621	53	53	2
	Collagen 2(I) (W5NTT7)	757	22	19	4	2847	43	36	5
	Collagen 1(III) (W5Q4S0)	597	15	14	5	1212	29	30	5

Comparison of results by LC-MS/MS of digestion with the trypsin functionalized film and standard digestion in heterogeneous phase. Raw data were searched by Mascot MS/MS Ion search using the homemade COLLE database. Identification details are reported in Table S2–S3.

on average better with the digestion in heterogeneous phase, possibly also because of the much longer incubation time (o/n digestion *versus* 10 min, roughly ground samples to expose larger surface, constant 37 °C incubation, *etc.*).

Despite the lower yield in peptides, we demonstrated that data collected from LC-MS/MS analysis of the samples recovered from the trypsin- functionalized film are able to be used for characterizing the degradation state of the proteins inside the samples. MS/MS data were indeed used to search for common chemical modifications that are classically linked to molecular damage patterns, such as deamidation at asparagine (N) and glutamine (Q) residues [21,25], spontaneous hydrolysis [26,27], and glycation at lysine (Lys) and arginine (Arg) [21,26].

As it can be seen from Fig. 3, the levels of collagen deamidation that can be calculated are comparable between the two protocols, meaning that, despite the lower yield in peptides with the film, there is no bias in extracting deamidated rather than unmodified peptides in the samples. Interestingly, both protocols demonstrate a remarkably high level of deamidation both for N and Q. Glue preparation frequently involves prolonged boiling of animal connective tissue that would significantly increase the rate of deamidation [28]. The observed deamidation level is therefore the sum of heating during animal glue preparation and aging.

This result is extremely important since deamidation is actually considered an indicator of "authentical age" [2,25] of the sample, although we embrace the sense by Schroeter and colleagues [29] that deamidation should be rather viewed as a global indicator of their preservational quality, since deamidation rates and levels are greatly affected by numerous chemical and environmental factors.

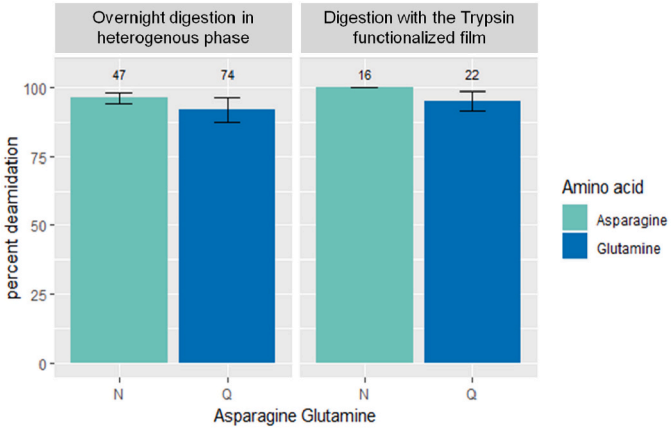

Fig. 3. Overall percentage of deamidation for asparagine (N) and Glutamine (Q) residues for the proteins identified on the surface of the lining material of the historical canvas "The Virgin and Child, Saints and Cherubins". Comparison of deamidation level between the peptide mixtures obtained with the trypsin functionalized film in comparison and a standard digestion in heterogeneous phase. Error bars represent standard deviation and numbers above the bars represent the number of peptides containing N and Q on which the data was based on [21].

We also searched for spontaneous hydrolysis and modifications such as glycation, both non-enzymatic modifications well known to occur upon aging/deterioration. Table 3 reports the results of the search for semitryptic peptides, which are the hallmark for partially hydrolyzed proteins [26,27].

It is worth mentioning that, when semitryptic peptides are considered for protein score computation, the results became almost comparable. However, if we go into the details, the trypsin functionalized film seems to extract a larger number of "damaged" peptides, since semitryptic peptides exceed 80% of the detected peptides with the film *versus* the 50% of the peptides observed with the standard protocol in heterogeneous phase. A plausible explanation might lie in the fact that, during the digestion in heterogeneous phase, trypsin in solution is likely to penetrate more deeply into the layer, whereas when immobilized on the film it might attack only the external, and therefore possibly more degraded, surface of the object. This aspect is beyond the scope of the present paper and deserves further investigation, both to understand how deep the trypsin can go and to explore the possibility of discriminating the protein content and degradation state among different layers.

Even more interesting is the observation that almost all the glycation products, arising from exposure of proteins to sugars, were detected on semitryptic peptides. Tables S6-S7 illustrate the peptides where advanced glycation end-products (AGEs) of lysine and arginine were found. A wide set of AGEs was detected in the sample from trypsin functionalized film: glycation of arginine with the formation of glyoxal and methylglyoxal adduct (mass shift of +39.99 and + 54.01 Da respectively), as well as formation of carboxymethyl and carboxyethyl lysine (with mass shift of +72.01 and + 58.01 Da, respectively). As an example, the fragmentation spectra of a glyoxal and methylglyoxal modified peptide is reported in Fig. S10.

Glycation products are consistent with the presence of sugars as identified by GC–MS analysis (Fig. 4). In particular, arabinose, xylose, mannose and glucose were detected, that, according to the literature [30], might be interpreted as the presence of some plant gum in the binding media of the historical canvas. We suggest that some protein glycation might arise from the binding of reducing sugars from gum to proteins in the complex mixture of the painting media.

Conclusively, we suggest that the trypsin functionalized film can be used to gain information on chemical modifications of proteins on the surface of an artistic object and therefore on its degradation state and not only to merely identify the protein component.

5.3. Development and implementation of the approach

The success of proteomics experiments in cultural heritage objects lies in the efficiency of either protein extraction from solid matrixes or direct digestion of proteins still incorporated inside the matrix itself. In this respect, we already observed that glycosylation of egg proteins created a significant molecular hindrance which hampered proteases to

Table 3
Comparison of spontaneous hydrolysis in proteins in a lining of the historical canvas "The Virgin and Child, Saints and Cherubins" by LC-MS/MS.

		Digestion with the Trypsin functionalized film				Overnight digestion in heterogenous phase			
Sample	Protein (Uniprot entry)	Score	PSC (%)	No. of peptides	No. of semitryptic peptides	Score	PSC (%)	No. of peptides	No. of semitryptic peptides
Lining of the canvas "The Virgin and Child, Saints and Cherubins"	Collagen 1(I) (W5P481)	3970	48	113	90	3852	55	120	55
	Collagen 2(I) (W5NTT7)	2326	43	75	57	2507	52	74	34
	Collagen 1(III) (W5Q4S0)	1286	40	55	35	1468	35	51	18

LC-MS/MS raw data were searched by Mascot MS/MS Ion search using the homemade COLLE database and considering semitrypsin as the enzyme. Identification details are reported in Table S4–S5.

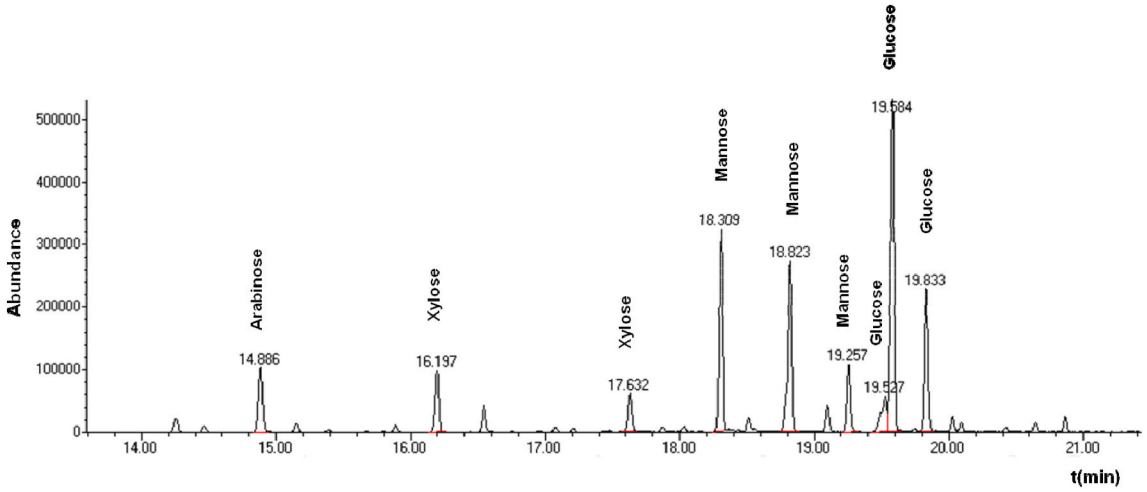

Fig. 4. Sugar analysis of the sample from the lining of the historical canvas "The Virgin and Child, Saints and Cherubins" by GC–MS. GC-TIC chromatogram of TMS sugars identified in the historical canvas. Annotation of the sugars was performed by comparing both the retention times and the fragmentation spectra of the sugars with analytical standards.

efficiently interact with the proteinaceous substrates, thus greatly decreasing proteolytic efficiency and therefore reducing the confidence of protein identification [12]. In these cases, in the standard solution approach, we bypassed the problem of obtaining a reliable identification by introducing a deglycosylation step before treatment with trypsin. We explored the possibility of introducing similarly a treatment with PNGaseF to get rid of the sugar coating that, as we suggested, seems to prevent the accessibility of proteases. We adopted a similar strategy by immobilizing PNGaseF on a separate cellulose acetate sheet to trim out the glycosidic decoration before proceeding with the trypsin functionalized film in a two-step procedure.

The PNGaseF functionalized film was first tested on an egg-based painting mock-up that contained azurite as pigment. Briefly, the sample was placed in contact with the PNGaseF functionalized film for ten minutes and subsequently in contact with the trypsin functionalized film for 10 min. As a control, the same sample was also analysed with only the trypsin functionalized film. MALDI-TOF analysis of the recovered supernatants was then recorded as a pre-screening, and Fig. S11 demonstrates a richer spectrum, in terms of number of ions and identified peptides, in the case where the PNGaseF functionalized film was used. The peptides in table S8 confirmed the presence of several proteins of *Gallus gallus* in the sample, digested with trypsin functionalized films

Table 4
Proteins identified in the painting egg-based painting mock-up sample by LC − MS/MS.

		PNGaseF functionalized film + Trypsin functionalized film				Trypsin functionalized film			
Sample	Protein (Uniprot entry)	Score	PSC (%)	No. of peptides	No. of unique peptides	Score	PSC (%)	No. of peptides	No. of unique peptides
Egg based painting mock-up with azurite	Ovalbumin (P01012)	446	47	15	9	252	30	9	8
	Ovotransferrin (P02789)	186	13	9	8	117	7	6	5
	Ovalbumin-related protein Y (P01014)	168	13	7	6	ND	ND	ND	ND
	Elongation factor 1-alpha 1 (Q90895)	74	7	4	4	57	4	2	2
	Ubiquitin (P79781)	49	19	2	2	ND	ND	ND	ND
	Ovalbumin (P01012)	446	47	15	9	252	30	9	8

Comparison of LC-MS/MS analyses with the trypsin functionalized film with or without pretreatment with immobilized PNGaseF. Raw data were searched by Mascot MS/MS Ion search using Chordata as the taxonomic restriction in the SwissProt protein database. ND: not detected. Details of the identification are given in Table S9 and S10.

after the treatment with immobilized PNGaseF. Therefore, even in this more elaborated configuration, it is possible to successfully analyze samples with a simple, rapid, sensitive and specific approach such as Peptide Mass Fingerprinting, amenable to a museum laboratory, and that can be practiced successfully even by non-specialists.

LC-MS/MS analysis (Table 4) confirmed the effectiveness of the deglycosylation pretreatment step with immobilized PNGaseF on functionalized films, since more proteins were identified when the sample was pretreated with PNGaseF and all more confidently. Details are reported in Table S9-10.

The combination of PNGaseF and trypsin immobilized on cellulose acetate sheets was explored on the decoration of a micro-sample collected from of XXII dynasty wooden coffin (yellow coffin), where egg proteins had been recently identified with the standard protocol in heterogeneous phase, and GCMS analysis had also detected the copresence of rabic gum (reference: sample Sarcofago A in the paper by Melchiorre *et al* [10]). As a further control, the same sample was also digested only with the trypsin functionalized film. The recovered peptides were subjected to LC-MS/MS analysis, since preliminary PMF analysis did not provide satisfactory results, as could be expected from the complex mixture of proteins that LC-MS/MS analysis revealed (Table 5 and details reported in Tables S11-12). Identifications are in agreement with the already published data [10] where trypsin digestion was carried out in heterogeneous phase. We can say that the trypsin functionalized film, (even in the absence of the pretreatment with PNGaseF), is also effective on proteins in the decoration of a wooden surface, since the physical support of the coffin is wood, thus demonstrating again its versatility of use. However, most importantly, it is worth mentioning that trypsin functionalized films and, even more, the combination of PNGaseF plus trypsin functionalized films, produced more confident identifications compared to what we previously reported [10], since numerous proteins from *Gallus gallus* were identified, assessing an unambiguous chicken origin, while in previous experiments lysozyme was the only egg protein detected. Moreover, a clear identification of several collagen chains confirms that a mixture of egg and animal glue was present, accordingly to what had already been observed [10].

6. Conclusion

Trypsin very efficiently works when immobilized on a cellulose acetate sheet with the further benefit that digested peptides are thoroughly captured on the functionalized surface. These are the major advantages of the functionalized films, which we have demonstrated can work on the surfaces of different objects such as albumen, isinglass and casein used as fixatives on paper, the much harder surface of ivory objects, or the proteinaceous binders on a wooden Egyptian coffin. We simulated analyses that can now confidently be carried out directly on the surfaces of the objects in their actual location (namely museums or archaeological sites) and without sample removal from the artefact.

The obtained hydrolysate is definitely comparable to that which is obtained from a standard proteomic approach and is amenable to Peptide Mass Fingerprinting by simple MALDI-TOF analysis as well as to LC-MS/MS analysis of the same peptide mixture. PMF, one of the modern techniques for protein analysis recently introduced into conservation science, is advantageous because of its sensitivity and specificity, speed of analysis, and relatively low cost. However, for more challenging questions, such as the identification of chemical modifications, useful for characterizing the conservation state of proteins or the analysis of more complex protein mixtures, LC-MS/MS is required. We have demonstrated that protein digestion with the functionalized films is fully adequate to both mass spectrometric techniques. Moreover, the functionalized film is amenable to further implementation and modularity: herein we have combined the trypsin functionalized film with a PNGaseF functionalized film thus integrating the deglycosylation pretreatment to improve detection of glycosylated coated proteins. The impact of sampling on sensitive surfaces is extremely relevant in the field of cultural heritage. The minimal sample requirements for current methods of analysis are making it possible to study art and objects where the consequences of removing even micro-amounts of material can be prohibitive. Thus, developing methods for sampling sensitive objects is still an important issue for conservation, and the results from the use of trypsin-functionalized films show great promise. Further development of the present version will focus on further reduction of film dimensions to minimize surface contact and make the technique usable for smaller objects and features. Concomitant with smaller films, smaller volumes of liquid will be used, minimizing effects on sensitive surfaces such as paper and photographs.

In conclusion, we have demonstrated that the enzyme functionalized film is a versatile, user-friendly and modular tool that can be widely exploited in the world of diagnosis of cultural heritage objects. We have shown that relevant analytical information can be obtained, and we have indicated what the consequences of using the films on a variety of different surfaces may be.

Author contributions

G.N, L.B., P.G., D.K. G.M. Conceptualization; G.N., D.K., I.S., P.C. Investigation and Data curation; G.N., D.K., I.S., A.C., P.S., P.C., P.G., L. B. Formal analysis; G.N., D.K., I.S., A.C., P.C., P.G., G.M., L.B Writing - review & editing; L.B. Funding acquisition and Project administration.

Table 5
Proteins identified in the fragments of the painted surface of yellow coffin collected from the decoration of a XXII dynasty wood Sarcophagus (yellow coffin) with the combination of PNGaseF and trypsin functionalized films in comparison with the use of trypsin functionalized film alone.

Fragments of the painted surface of yellow coffin	Protein (Uniprot entry)	Score	Sequences	Unique sequences	Protein sequence coverage (PSC) (%)
PNGAseF + Trypsin functionalized films	Ovalbumin (P01012)	26	4	2	15
	Ubiquitin (P79781)	45	2	2	20
	ATP synthase subunit beta (Q5ZLC5)	34	2	2	5
	Vitellogenin 2 (P02845)	20	4	4	2
	Collagen 1(I) (*Bos taurus*) (P02453)	101	5	0	9
	Collagen 2 (I) (*Bos taurus*) (P02465)	78	5	0	9
	Collagen 2 (I) (*Gallus gallus*) (P02460)	37	2	1	2
	Collagen 1 (III) (*Gallus gallus*) (P12105)	32	2	1	3
	Collagen 1 (I) (*Gallus gallus*) (P02457)	15	2	2	4
Trypsin functionalized films	Ovalbumin (P01012)	69	3	3	6
	Collagen 2 (I) (*Bos taurus*) (P02465)	63	4	0	7

Sample fragments were digested *in situ* with the trypsin functionalized film with or without pretreatment with PNGaseF functionalized film and peptide mixtures were analysed by LC − MS/MS. Raw data were searched by Mascot MS/MS Ion search using Chordata as the taxonomic restriction in the SwissProt protein database. Since collagen was detected, further searches were carried out using the homemade COLLE database considering also hydroxylation at Pro and Lys as variable modifications. Collagen results therefore refer to the homemade COLLE database analysis. Identification details are reported in Table S11 and S12.

Declaration of competing interest

The authors declare to have no conflict of interest.

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LC-MS/MS data and COLLE database have been deposited to *Mendeley Data* (https://data.mendeley.com/datasets/nskhk333zp/1) with the dataset identifier 10.17632/nskhk333zp.1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.104039.

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