**Miniaturized Biosensors to Preserve and Monitor Cultural Heritage: from Medical to Conservation Diagnosis**

# Miniaturized Biosensors to Preserve and Monitor Cultural Heritage: from Medical Diagnosis to Conservation Diagnosis

Giorgia Sciutto, Martina Zangheri, Laura Anfossi, Massimo Guardigli, Silvia Prati, Mara Mirasoli, Fabio Di Nardo, Claudio Baggiani, Rocco Mazzeo,* and Aldo Roda*

**Abstract:** We have exploited the point-of-care testing concept to design and develop portable and reliable analytical systems, which can be used on site by not specialized operators, supporting restoration campaigns at a limited cost. These miniaturized devices for lateral flow immunoassays can simultaneously detect two proteins (ovalbumin and collagen) in artworks. In order to carry out an in-deep study on the application of portable biosensors, both chemiluminescent and colorimetric detections have been developed and compared in terms of sensitivity and feasibility. To simplify the use of the chemiluminescent system, which displayed two orders of magnitude higher detectability than that of the colorimetric system, a disposable cartridge has been designed ad hoc for this specific application. These results point to the enormous potential of these cheap, easy-to-use, and minimally invasive diagnostic tools for conservators and restorers in the cultural heritage field.

One of the most challenging issues in the science of cultural heritage conservation is to create an open dialog between scientists and conservators, in order to generate technological developments and advanced methodologies to meet specific needs.[1]

In recent decades, chemistry has played a crucial role in this field. It has provided tools to obtain information on multidisciplinary questions, such as the artistic technique, attribution, provenance, and alteration processes.[2] Furthermore, to select the appropriate conservation procedures, it is necessary a comprehensive characterization of the artwork materials and conservation state. As such, a work of art can be seen as a kind of patient that requires an anamnesis (e.g. cultural context, characterization of materials), diagnosis (e.g. evaluation of conservation state, identification of degradation processes), prognosis, and therapy (e.g. cleaning, restoration).

Until now, research has focused on developing non-invasive[3,4] or micro-invasive[5,6] techniques to avoid or minimize the size of samples that must be taken from valuable and unique heritage items. These techniques include non-invasive portable systems, which can provide extensive information on the artefacts, while safeguarding their integrity. Moreover, because these systems can be used on site, the artefacts do not have to be moved to a specialized laboratory. This minimized the risk of damage for the artwork. Nevertheless, these techniques usually involve advanced instrumentation, and require highly specialized personnel for the data acquisition and processing. Moreover, although conservators and curators recognize the importance of scientific analyses in restoration campaigns, the time and financial costs are often prohibitive.

An alternative approach is to develop analytical kits for easy use on site. These powerful tools would allow restorers to rapidly obtain information to guide their restoration strategy. The restorer would thus become a kind of primary care physician, performing the anamnesis, diagnosis, and therapy of the artefact. With its miniaturization of instrumentation and its delocalization of analyses, the point-of-care testing (POCT) approach is revolutionizing clinical diagnostics and therapy. Taking inspiration from these methods, we have developed miniaturized analytical biosensors, which can be used by not specialized operators to identify proteins in artworks. Proteinaceous materials have been used as fixatives or binders in paintings since ancient times and their characterization is still a challenge for diagnostic studies.

Proteins are common clinical biomarkers, so it is not surprising that clinical chemistry techniques can be adapted to analyze proteins in cultural heritage materials. In this regard, immunological approaches are particularly powerful. They are simple, highly specific, can be implemented with relatively inexpensive instruments, and require minimal sample processing.[7] Indeed, while originally developed for medicine, immunoassays have been used to study proteins in paintings for over 45 years.[8] They can even recognize denaturized proteins. In particular, immunofluorescence microscopy[9] (IFM) and chemiluminescent (CL)[10] detection have been used for the single or multiplexed detection of proteins in artwork samples. More recently, we demonstrated in a pioneering study that a CL immunochemical contact imaging portable device[11] could be used to detect ovalbumin in paintings. The analytical prototype used a silanized glass slide with an array of immobilized antibody spots and a polydimethylsiloxane (PDMS) fluidic element.

Here, we have designed new portable devices to simultaneously detect two proteins in situ. These devices exploit the lateral flow immunoassay (LFIA) system to obtain maximum user-friendliness, as the operator does not need to manipulate any reagent.

LFIA technology is one of the most commercially successful POCT techniques. It uses cellulose-based materials assembled in a strip format, which serves as a cheap and easy-to-use platform for performing immunological assays.[12] This format is used extensively in the medical, forensic, and environmental fields. It is extremely versatile, and provides specific analyte detection, even in complex matrices. The assay is performed on a strip (comprising sample pad, conjugate pad, membrane, and adsorbent pad) preloaded with suitable reagents, while capillary force drives sample and reagents to flow across the strip without the need of external pumps. Immunoreagents are immobilized in

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specific areas on the nitrocellulose membrane: one or more test (T)-line(s), in which the analyte(s) are bound and detected, and a control (C)-line, in which the excess labelled probe is captured to confirm the validity of the analysis.

The simplest LFIAs employ colorimetric detection, often based on gold nanoparticles (AuNPs) as labels, which leads to the formation of red bands visible at naked eye in correspondence of the T- and C-lines. The easiness of the method well fitted with the POCT application, offering the possibility to use the device where the patient (cultural heritage) is, by the local support team (restorers) in the proper way. On the other hand, colorimetric LFIA may suffer of poor detectability and limited quantitative performances.[13]

As an alternative, chemiluminescent (CL)-based LFIAs present higher performance, thus providing high detectability, rapidity, and a wide linear range in immunoassays for quantitative analyses.[14] In this case, the detection probe is often labeled with an enzyme, such as horseradish peroxidase (HRP), able to catalyze a CL reaction in presence of the proper CL substrate. On the other hand, the drawback of this approach, with respect to colorimetric detection, is the requirement of a more complex analytical device and protocol involving the addition of a series of reagents to produce the CL reaction.

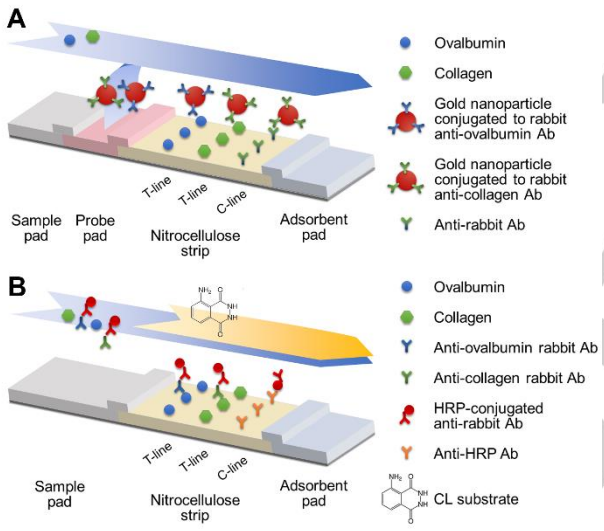


**Figure 1.** Formats for (A) colorimetric and (B) CL LFIA assays.

Here, we have developed POCT biosensors for diagnostic campaigns on paintings. For the first time, multiplex LFIA biosensors have been designed and compared for their application for in situ analysis of historical materials. In more detail, the two biosensor formats differ from one another by the detection principle: CL reaction catalyzed by enzymes in one case and AuNP colorimetric approach in the other. Their performances have been evaluated for the in situ identification of ovalbumin and collagen in microextracts obtained from 0.5 mg of paint samples. Therefore, the analysis protocol provides for the collection of a sample. Two main risks can be linked to sampling: it can damage precious objects and may not be representative of an entire work of art. [15] Despite this, the high complexity of the artworks often requires the use of micro-invasive and sensitive techniques for the complete identification of materials and degradation products. Here a rather small sample quantity ((collected directly by expert conservators or restorers) is required to be submitted to the extraction and analysis procedure. In addition, the samples to be analyzed may refer to materials to be removed (e.g. varnishes during the restoration campaigns). To further limit the amount of needed sample, multiplexing LFIA approaches were developed to simultaneously detect the two different proteins with a single analysis.

The multiplex LFIA biosensors formats developed are shown in Figure 1 and they have been designed as follows. For the colorimetric LFIA, the strip is inserted into a commercial plastic housing, which is designed for visual LFIAs (Figure 2). A drop of extracted sample (~60 µL) is added to the sample well and, after 10 minutes, the result is observed by the naked eye. If the sample contains ovalbumin and/or collagen, the color intensity of the corresponding T-line decreases proportionally to the protein amount, in accordance with the competitive assay mechanism.

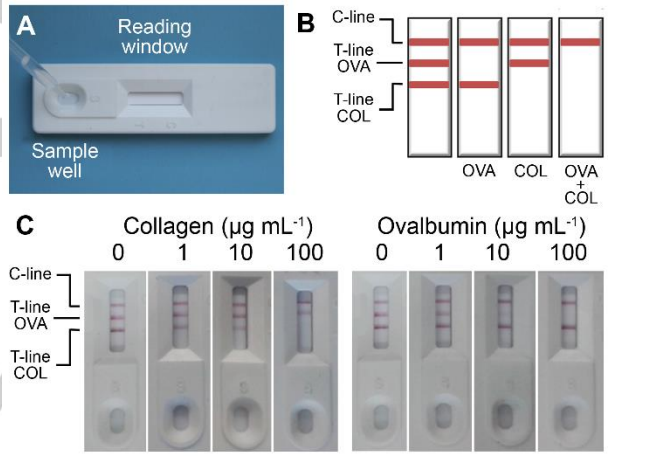


**Figure 2.** a) Device for colorimetric LFIA; b) Visual detection of ovalbumin and collagean according to the competitive LFIA format; c) Visual limits of detection (vLODs) for the two proteins.

The visual limit of detection (vLOD) is the minimum protein concentration that provides a T-line color visually weaker than that produced by a negative sample.[16] Here, the vLOD is about 10 µg $mL^{-1}$ for both proteins. To help the operator interpret the result, a negative sample should be analyzed in parallel in each experimental session.

The CL-LFIA method requires a multistep analytical protocol with sequential addition of sample and reagents. To simplify the procedure, we designed, ad hoc, a disposable analytical cartridge containing the LFIA strip and all the necessary reagents (Figure 3). Thus, only the sample must be added. The analytical cartridge comprises two parts: a fluidic element and a holder. The fluidic element prevents the chemical reagents from dispersing in the environment. It contains the fluidic channel network, the LFIA strip, pouches for the preloaded reagents, and

pressure-actuated polydimethylsiloxane (PDMS) valves, which allow the channels to be open and closed (Figure S1 in the Supporting Information).

The 3D-printed holder includes the fluidic element and allows performing the analysis through simple manual operations. It includes a port, where the sample can be loaded using a conventional hypodermic syringe and a series of buttons for actuating valves and pouches as required by the procedure. CL measurements can be performed on site thanks to the use of a 3D-printed mini dark box coupled to a thermoelectrically cooled, battery-operated, ultrasensitive CCD camera has been developed. The dark box encloses the analytical cartridge, preventing interference from ambient light, and aligns the LFIA membrane to the CCD sensor. Details on the procedure of use are described in Scheme S1 in Supporting Information.

To keep the device small, we used a "contact imaging" configuration, in which the CL signal is conveyed to the CCD sensor by a fiber optic faceplate (rather than by conventional lens-based optics).[17]

The relatively cheap CCD camera (starting from 1500 euros) and the materials employed for the cartridge (polypropylene for the fluidic element and acrilonitrile butadiene stirene (ABS) for the holder) were chosen to reduce costs.

It is thus a competitive candidate for further industrial development. The cartridge holder and the mini-dark box were prototyped using a commercial benchtop 3D printer. However, production could be scaled up using other inexpensive techniques, such as injection molding.

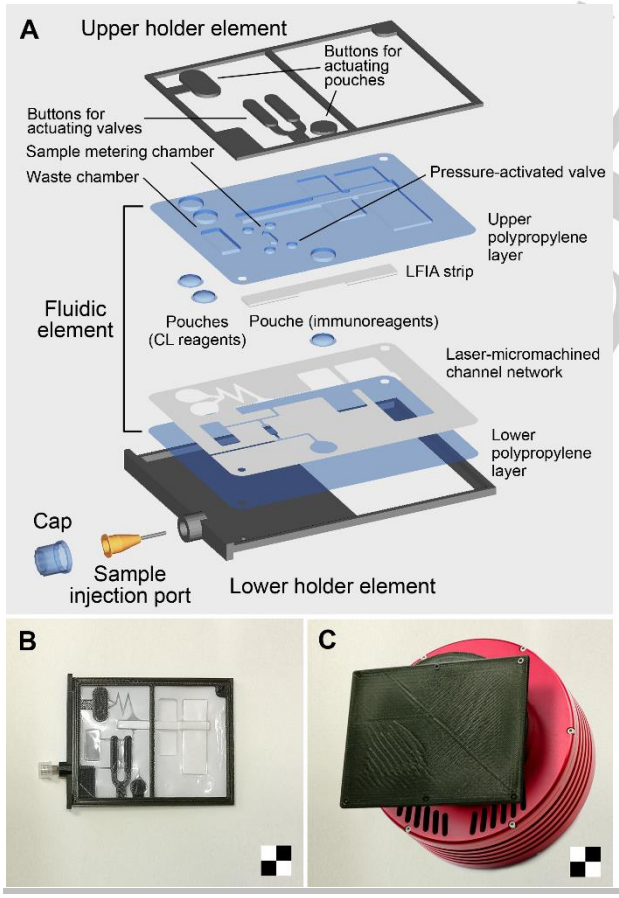


**Figure 3.** a) Layout and b) Image of the disposable analytical cartridge; c) CCD camera with 3D-printed mini dark box. Scale checkerboard is 2x2 cm.

The acquisition of the CL image and its elaboration provide quantitative information about the signal intensities of the T- and C-lines. Here too, the competitive assay format provides a CL signal intensity that decreases with the amount of analyte. We used standard solutions of ovalbumin and collagen to generate calibration curves, which we fitted using four-parameter logistic functions. These showed limits of detection (LODs) of 0.1 and 0.05 µg mL$^{-1}$ for ovalbumin and collagen, respectively (Figure 4). Prior to data analysis, the T-lines signals are normalized to the C-line signals. This increases assay robustness, since errors related to environmental and matrix factors (i.e. changes in ambient temperature or the presence of HRP inhibitors in the sample) might affect the CL signal's intensity.

The data analysis is automatically performed with a spreadsheet. To obtain the protein's quantitative value, the operator enters the CL intensity value (extracted from the image) into the spreadsheet's calibration curve equation. To assess potential interference due to other sample components, preliminary tests on paint mockups prepared using different pigments (red ochre, blue smalt, minium) and egg or animal glue as binders have been performed (Figure 5a and 5b). For each mockup, about 0.5 mg of sample were collected as powder or flake, extracted with an optimized protocol,[7] and analyzed.

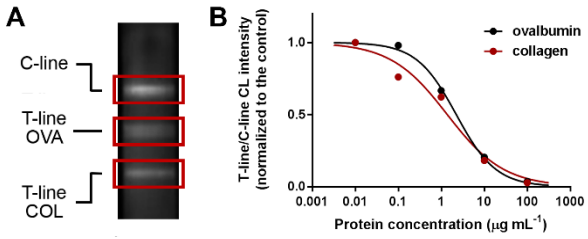


**Figure 4.** a) CL image of the LFIA strip showing the areas used for the evaluation of the CL signals of T-lines and C-line; b) CL calibration curves for ovalbumin and collagen.

Both colorimetric and CL systems correctly identified negative samples that did not contain the target analytes. The extracts measured with the CL-LFIA system had protein concentrations higher than 50 µg mL$^{-1}$ for ovalbumin, and ranging from 5 to 100 µg mL$^{-1}$ for collagen, depending on the protein amounts used in the painting mixtures. In agreement with the estimated vLODs, the colorimetric LFIA correctly identified ovalbumin in all the extracts from egg-based mockups, and correctly identified collagen in the extracts containing high amounts of protein. It failed to recognize the protein in the extracts containing low amounts of collagen (< 10 µg mL$^{-1}$).

Despite its significant advantages in terms of simplicity, its poorer LOD makes colorimetric LFIA less appropriate for cultural heritage investigations of small samples. Nevertheless, it may be suitable for heritage contexts where large amounts of sample are available (e.g. analyses of coatings prior to their removal, archeological excavations, stone monuments, and buildings).

Moreover, it is intrinsically user-friendly with a low environmental impact. These promising features should be considered in the development of future portable devices.

To increase the sensitivity of colorimetric detection, the color intensity of the lines can be measured using digital image sensors, such as a complementary metal-oxide-semiconductor (CMOS) sensor.[18] This approach[19] improved the LODs of the colorimetric LFIA (0.5 µg $L^{-1}$ and 1 µg $L^{-1}$ for collagen and ovalbumin, respectively). However, this improvement reduces the assay's simplicity and applicability in low-resource settings, making it comparable with the CL approach, which offers higher detectability.

To test the CL-LFIA biosensor's applicability, we used the device to analyze historical samples, in which ovalbumin and collagen were previously localized within the paint stratigraphies by microscopy immunochemical analyses.[20, 21]

In the first sample, collected from a Renaissance wood painting (second half of the fifteenth century), both ovalbumin (0.69 µg $mL^{-1}$) and collagen (1.22 µg $mL^{-1}$) were detected. The analysis of paint stratigraphies already showed that egg white and animal glue were used as a finishing layer or added in the ground, respectively. According to the previous stratigraphic analysis, a smaller amount of collagen (0.1 µg $mL^{-1}$) was detected in a pigment layer extract, which was collected from a mural painting in the Italian Abbey of S. Maria del Monte and dated to 1774. The proteinaceous binder was most likely used during a restoration following the World War II bombardments in 1944.

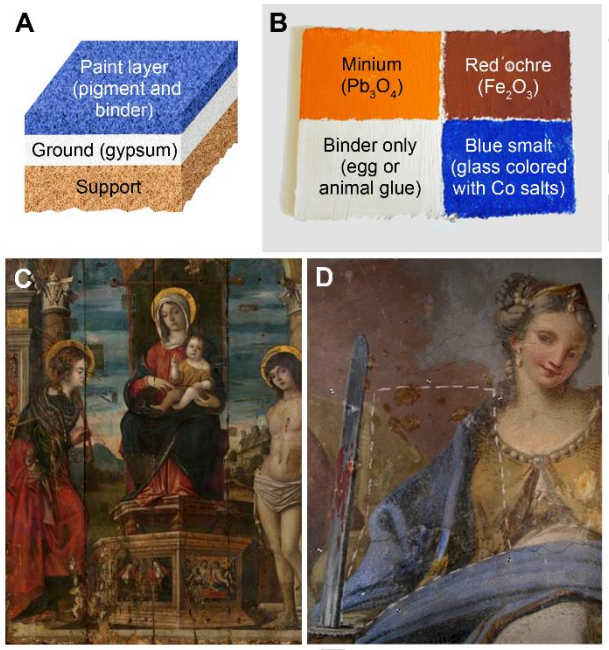


**Figure 5.** a) Structure of paint mockups; b) Paint mockup prepared using different pigments; c), d) Real paintings used to assess the applicability of the CL LFIA system.

In conclusion, portable devices that non-specialist scientists can use easily to diagnose cultural heritage items have been developed. We have compared different devices in terms of assay feasibility and sensitivity, pointing out the higher performances of the LFIA-CL system. To facilitate the use of this system we designed an *ad hoc* cartridge that allows to perform the CL analysis in a semi-automatic manner. Furthermore, the cartridge is versatile and it could also be adapted for other CL assays. Thus, this research represent a step towards the development of a new generation of simple analytical devices suitable for performing sensitive onsite analysis, that could find application in different fields, from clinical and environmental analyses to forensic investigations. The need for a sample may limit the use of these devices in cultural heritage analysis, where the collection of samples may be prohibited. Nevertheless, the amount of samples required is rather small and our research is currently focused on increasing sensitivity to further reduce the amount of sample required, testing new primary antibodies. Although we have obtained significant results for the detection of proteins, these represent only one example of the different types of analytes that can be found in works of art. Thus, future studies will investigate a number of possible analytes different from proteins. For example, we are currently developing an immune-based kit to detect microbial contaminations in situ in cultural heritage items.

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**Keywords:** Analytical Methods • Art conservation • Biosensors • Immunoassays • Point of Care Testing

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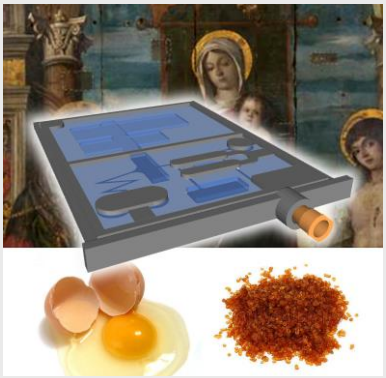
# COMMUNICATION

This research reports on the development of simple and sensitive miniaturized systems to detect proteins in artworks. These systems can support restoration campaigns at a limited cost. An innovative ready-to-use cartridge was developed ad hoc, allowing non-specialized personnel to perform these rapid tests. This follows the point-of-care testing principle, and should facilitate the widespread application of this new diagnostic tool.



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