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Localization of proteins in paint cross-sections by scanning electrochemical microscopy as an alternative immunochemical detection technique

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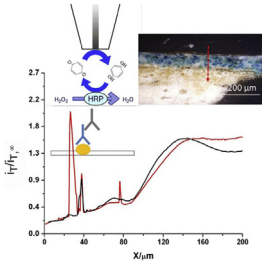
HIGHLIGHTS

- Advanced immuno-electrochemical detection of proteins in paint samples by SECM.
- Analysis performed directly on cross-section with high spatial resolution.
- Identification of HRP catalytic activity for a selective location of analyte.
- Satisfactory results were obtained for aged real samples.
- The way forward for an extensive application of SECM in conservation science is shown.

GRAPHICAL ABSTRACT



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ABSTRACT

The qualitative identification of proteinaceous substances, as well as their location within a complex paint stratigraphy, is one of the most challenging issues in the characterization of painting materials. Nevertheless, information on paint components represent a crucial task for studies concerning both the ancient painting techniques adopted and the state of conservation, being fundamental investigations for the selection of appropriate conservation actions. The present research was aimed at developing a new detection approach for the immunochemical localization of ovalbumin in paint cross-sections based on the use of scanning electrochemical microscopy (SECM). The immunochemical analyses were performed using an anti-ovalbumin primary antibody and a secondary antibody labelled with horseradish peroxidase (HRP). SECM measurements were performed in feedback mode using benzoquinone (BQ)/hydroquinone (H_2Q) redox couple. In presence of hydrogen peroxide (H_2O_2), HRP catalyzes the re-oxidation of H_2Q to BQ and the increment of BQ concentration in correspondence of the target protein was detected by SECM through the electrochemical reduction of the regenerated BQ at the microelectrode. Indeed, the localization of ovalbumin was possible thanks to a clear discrimination of SECM currents, achieved by the comparison of the measurements recorded before and after H_2O_2 administration, based on the HRP on/off approach. The method was evaluated both on samples from standard mocks-up and on a historical sample, collected from a Renaissance wood painting. The obtained results were promising, foreseeing a wider application of SECM on cultural heritage researches.

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1. Introduction

A polychrome sample is usually formed by overlaid paint layers of different thickness (10–100 μm) applied over a support (canvas, wood, mortar) and composed of mixtures of organic and inorganic materials. In particular, a ground layer is often spread all over the support in order to reduce its porosity. One or several paint layers composed of a mixture of pigments or colorants and an organic binder may be applied directly on the preparatory layer or under a priming layer named "imprimitura" whose aim is to further reduce the porosity. A varnish is generally applied on the paint layers, aiming at protecting them and optimizing the chromatic effects.

Each layer is characterized by heterogeneous mixtures of organic and inorganic materials, whose complexity is generally engendered by a variable composition, according to the traditional painting technique adopted, the support used, the historical period and geographic context. The characterization and localization of paint components have the outmost importance both to study ancient painting techniques and to understand degradation phenomena, the latter being a preliminary operation when planning appropriate conservation measures.

However, the qualitative identification of organic compounds, used since ancient times as binders, varnishes and adhesives and in particular, the characterization of proteins (which are one of the classes of organic substances most widely used as painting materials) [1], still represents a challenging task. This may be ascribable to their limited amount and distribution in the complex multi-layered paint structure. In addition, difficulties in the detection of such components are also emphasised by their sensitivity to degradation with ageing.

Chromatographic techniques probably represent the most employed methods for the analysis of organic components in paintings, including proteins, thanks to their high selectivity [2–4]. Proteomic methods based either on MALDI-TOF mass spectrometry or chromatography-mass spectrometry have been employed for the characterization and quantification of proteins [5–8]. Nevertheless, such investigations require the extraction of the analytes from the sample, with quite complicated and long extraction procedures, losing information concerning stratigraphic localization of the target protein. Alternatively, FTIR microscopy can be considered an essential tool in the micro-destructive analysis of small samples. Indeed, the application of this techniques – in mapping or imaging modes – allows to perform stratigraphic analyses producing chemical maps of the identified compounds [9,10]. However, a crucial drawback of FTIR microscopy is the lack of specificity, thus proteinaceous materials can be detected but specific proteins cannot be identified. Moreover, the spatial resolution is limited by the theoretical diffraction limit of about 10 μm, even if, in particular conditions, it is possible to analyse areas as small as $6.25 \times 6.25\ \mu m^2$ [11].

Indeed, in the last decades scientific researches were mainly addressed to the development of new analytical methods for the characterisation of such compounds, providing detailed information on both their chemical composition and location in a heterogeneous multilayered system. To this aim, the potential and advantages of immunological techniques were recently deeply investigated, to propose alternative methods for the identification and localization of proteins in paint samples and cross-sections. Immunological approaches are well-known and widely used in bioanalytical chemistry [12,13] thanks to the high selectivity of antibodies, which allow the identification of proteinaceous materials. In particular, to detect the presence of the analyte (antigen), it is a common procedure to employ a secondary labeled anti-species antibody, which, depending on the biological source, can selectively recognize the primary one (specific for the target protein). Different markers (enzymes, fluorochromes, nano-particles of gold plus SERS reporter) and detection techniques (chemiluminescence, fluorescence and Raman imaging) have been combined in order to localize proteins within paint stratigraphies.

Imaging approaches based on fluorescence detection usually present significant interferences due to intense autofluorescence produced by sample materials (such as pigments and/or binding media), and by light scattering phenomena at the sample surface, which could hamper the use of immunofluorescence techniques [14–16]. Indeed, several research works were devoted to the development of higly sensitive immunochemical methods for the localization of proteins based on chemiluminescence (CL) imaging detection, which is not affected by interferences from sample components, thanks to the absence of any excitation source. In particular, CL imaging has already proved to be more sensitive than colorimetric and fluorescence detection techniques, allowing the localization of proteins in paint stratigraphies with a spatial resolution of the order of micrometers, by using single and multiplexed immunoassays [17–19]. Nevertheless, this method – as the fluorescence detection – is not able to provide information on different substances, which constitute (alone or in mixtures) the paint layers. Recently immuno-SERS based methods have been developed, allowing to selectively map the protein and the other Raman active substances at the same time [20,21].

Within this scenario, the present research work was aimed at investigating electrochemical detection as an alternative and powerful method for the identification proteins in artistic samples. The herein proposed approach is based on the combination of immunoassay procedures with the scanning electrochemical microscopy (SECM).

SECM is based on electrochemical detection, and it can be considered as a complementary tool in the investigation of complex substrates. This technique allows to obtain information on the electrochemical process in which the target molecules are involved, providing chemical and topographic information about the investigated surfaces. The typical spatial resolution achieved by this technique, according to geometry and size of the UME used (usually ranging between 5 and 25 μm), is suitable for the characterization of thin paint layers.

To the author's best knowledge no application of SECM for paint stratigraphies investigations in the cultural heritage field has been reported so far. Indeed, the present work was also aimed at opening the way for a wider application of SECM to analyse the organic composition of paint samples.

Among various scanning probe microscopy (SPM) techniques, SECM is based on the use of an ultramicroelectrode (UME) probe for scanning the investigated substrate, where the recorded signal is the current produced by an electrochemical reaction occurring at the tip. SECM may be considered a powerful and flexible tool for the characterization of a wide variety of surfaces [22]. This electrochemical technique has been used in several fields, including studies on metal corrosion, organic and inorganic film permeability, and metabolites absorption in cells [23–26]. In addition, SECM has been proposed as alternative method for local surface modification, drawing micro- and pre-defined patterns of reactive spots onto conducting substrates that may be subsequently functionalized [27,28]. An interesting application regarding the use of SECM for the characterization of enzyme-modified wood surfaces, aimed at mapping the distribution of the enzymatic activity along the tree annual rings has been also reported [29].

Nevertheless, only few applications of the electrochemical microscopy have been reported in the field of conservation science for the characterization of heritage materials, such as ancient copper-based alloys [30].

The use of electrochemical immunosensing device based on the combination of SECM with immunochemical methods was