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# Advanced label-free electrochemical immunosensor for a minimally invasive detection of proteins in paintings

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ABSTRACT

In recent decades, scientific methodologies applied in theCultural Heritage field have been growing, due to their pivotal role in guiding informed decisions concerning conservation strategies and daily maintenance. To achieve this goal, minimally/non-invasive quantitative and qualitative analyses are needed. However, the non-invasive and selective identification of proteinaceous binders and coatings in artworks represent an open issue in Cultural Heritage science.

Herein, a novel miniaturized system is introduced, which consists of a label-free electrochemical immunosensor integrated with biocompatible Gellan gel. This method is intended to selectively and minimally invasively identify ovalbumin (OVA) on-site in paintings. The label-free immunosensor is made up on screen-printed electrodes (SPEs) by functionalizing the working electrode (WE) with a primary antibody (anti-ovalbumin) for the specific recognition of OVA. The presence of OVA produces antigen-antibody reaction, which results in the development of a bulky immunocomplex on the WE. This complex is quantified using square wave voltammetry (SWV) and a reversible redox probe: the current measured is inversely proportional to the OVA concentrations. The developed immunosensors showed good analytical performances when applied directly to painted mock-ups, exhibiting a limit of detection (LOD) of 1.6 ng $mL^{-1}$, a limit of quantification (LOQ) equal to 16 ng $mL^{-1}$, a working range between 0.01 and 0.4 μg $mL^{-1}$ and selectivity for OVA over other protein components commonly present in painted artworks, including bovine serum albumin (BSA), collagen, and casein.

The outcomes highlighted the dependability of the immunosensor in detecting OVA and the efficacy of Gellan gel as a streamlined method for extracting the target protein while preventing residue accumulation on the painting surface. This advancement suggests the potential of Gellan gel-coupled immunosensor systems as viable diagnostic alternatives for artwork management and preservation.

## 1. Introduction

The investigation of cultural heritage objects often requires a comprehensive characterization of their execution technique and components. This is particularly crucial when conducting research related to attribution and dating or when assessing the conservation status [1,2]. At the same time, a paramount objective is to minimize or prevent damage to these objects by developing minimally or non-invasive analytical strategies.

The selective and minimally-invasive identification of organic substances, such as proteins, used as binders and coatings in paintings, remains a persistent challenge. This often involves striking a balance between selectivity and non-invasiveness. Chromatographic techniques are the most commonly used methods for characterizing organic components in paintings due to their well-established selectivity and the ability to detect multiple compounds in complex matrices [1–5]. However, these techniques are micro-destructive as they require the collection of a sample from the painted surface [5]. On the contrary, spectroscopy-based strategies can be employed for the minimally/non-invasive examination of organic components in

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paintings. Components in the artworks that are below their detection limits cannot be determined because non-invasive spectroscopic techniques often lack the sensitivity of chromatographic and immunochemical approaches [6–8]. In the last decades, analytical strategies based on immunochemical methods have been implemented, to provide highly sensitive approaches for the characterization of proteins [9]. In particular, immunochemical chemiluminescence (CL) imaging methods have been developed to identify and localize binders with high sensitivity, selectivity, and spatial resolution in painted stratigraphies [10–13]. Additionally, miniaturized CL biosensors have been proposed for *in-situ* protein recognition [14,15]. These methods have overcome the conventional limitations of spectroscopic investigations in terms of selectivity and sensitivity, as well as the disadvantages associated with chromatographic techniques concerning cost, time, and spatial information. Nevertheless, it is worth noting that immunochemical methods are micro-destructive since they need the collection of samples and/or the extraction of the protein for analysis.

To minimize invasiveness towards artworks, recent papers have proposed minimally invasive methods for the extraction of the analytes and the digestion steps for proteomic analysis. These methods utilize novel film in vinyl-acetate [16], fungal hydrophobics adhered on cellulose acetate sheets [17] or hydrogels loaded with trypsin and chymotrypsin [18,19]. However, in the last cases for example, after a first not-invasive step, the subsequent proteomic analyses still involve complex and time-consuming laboratory procedures. Indeed, despite the micro-invasive sampling and, if necessary, the *in-situ* hydrolysis, in these previous works the analyte must be treated for Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) analysis in the laboratory.

Thus, to overcome persistent drawbacks related to the investigation of artworks, the present study focuses on the development of a label-free electrochemical miniaturized immunosensor (LF-EC-MIS) coupled with a biocompatible Gellan gel for the minimally invasive, selective, cost-effective identification of ovalbumin, a protein commonly found as binder or coatings in paintings.

Label-free electrochemical immunosensors have been already developed and applied in health care, food and environmental monitoring fields [20–22], due to their significant advantages in terms of ease of use, sensitivity, low costs and affordable miniaturization. Notably, the label-free immunosensor enables the direct detection of the target protein without the use of labelled antibodies, which may compromise the affinity between the receptor and its target, decreasing reproducibility, sensitivity, and selectivity [23].

Recent studies have also explored the combination of Gellan gel (composed of deacylated Gellan gum and calcium acetate) and electrochemical biosensors for cleaning paper artworks, providing real-time monitoring of the pollutants and degradation products removal process [24,25]. This is because the gel is transparent, and rigid [26–28] and therefore compatible and suitable to be used in the cultural heritage field.

In this research, Gellan gel was employed as a minimally invasive sampler for ovalbumin (OVA), which is selectively detected using screen-printed electrodes (SPEs) functionalized with a primary and a secondary antibody. When every OVA is captured by the Gellan gel from the painted surface, an immunocomplex between OVA and its specific antibody is formed on the working electrode (WE), which is in contact with the gel. Subsequently, the immunocomplex is detected by measuring current changes using a reversible electroactive probe, such as $[Fe(CN)_6]^{3-/4-}$. In this approach, the concentration of the formed immunocomplex is inversely proportional to the recorded current when using Square Wave Voltammetry (SWV) as an electrochemical technique. Indeed, a higher OVA concentration results in increased immunocomplex formation, greater steric hindrance, and consequently, a lower diffusivity of the redox probe, leading to a decrease in the recorded current [29].

A brief description of the advantages and disadvantages of the most used diagnostic methods for proteinaceous binders with respect to the one proposed here are reported in Table 1.

The analytical protocol was initially assessed on standard paper samples with homogenous and simplified matrix. Subsequently, it was applied on aged painted specimens to evaluate their analytical performances in terms of sensitivity, reproducibility, and invasiveness towards the painted surfaces. Finally, the method was applied on the historical painting dated back to the beginning of XVII century.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals

Gellan gum is a KELCOGEL CG-LA product by CP Kelco (Atlanta Georgia, USA). Ovalbumin (OVA) for the preparation of standard solutions was from Sigma-Aldrich (St. Louis, USA). Calcium acetate ($(CH_3COO)_2Ca$), potassium chloride (KCl), anti-rabbit IgG antibody ($Ab_2$) produced in goat ($Ab_2$, secondary antibody, 0.68 mg $mL^{-1}$ stock concentration, 12–348), polyvinyl alcohol (PVA), Tween® 20 and potassium ferri- and ferrocyanide ($K_3[Fe(CN)]_6$, $K_4[Fe(CN)]_6$) were purchase by Sigma-Aldrich (St. Louis, USA). Anti-chicken ovalbumin antibody ($Ab_1$, primary antibody, 1.0 mg $mL^{-1}$ stock concentration, ab181688) was purchased by Abcam, Cambridge, UK. In the preparation of buffer solutions, distillated water was used (Millipore, Billerica, MA, USA). 0.05 M phosphate-buffered saline (PBS) with 0.01 M KCl (pH 7.4)

**Table 1**
Description of the most common diagnostic method for binding proteins in artworks.

| Method | Advantages | Disadvantages | Reference |
|---|---|---|---|
| Chemiluminescent immunochemical contact imaging | • portable analytical device<br>• applied on historical paintings<br>• high specificity<br>• no interferences<br>• *in-situ* analyses | • sample treatment<br>• extraction of protein | [14] |
| Paleoproteomic | • high sensitivity<br>• high specificity | • sampling<br>• sample treatment<br>• analyses on laboratory only | [1,30] |
| Fluorescent labeling combined with electrophoresis | • low expensive<br>• quite easy and fast | • sampling<br>• sample treatment<br>• low specificity<br>• interferences | [31] |
| Electrochemical immunosensor based on DMSNs/Au | • high sensitivity<br>• high specificity<br>• *in-situ* analyses<br>• miniaturized system | • complex preparation of nanotags<br>• sampling<br>• sample treatment | [32] |
| Microfluidic device integrated with electrochemical sensing platform | • simultaneous detection binders<br>• portable system<br>• in-situ analyses | • Time consuming preparation of electrochemical nanotags<br>• invasive sampling<br>• sample treatment | [33] |
| Gellan gel combined with label-free electrochemical immunosensor | • High sensitivity<br>• High specificity<br>• No interferences<br>• Minimally-invasive sampling<br>• *In-situ* analyses<br>• Miniaturized system | • preparation of the SPEs (personnel skill) | present work |

was used for electrodes pretreatment, while 15 mM PBS (pH 7.4) was used for electrodes washings. 0.05 M Carbonate buffer (pH 9.6) was used for the preparation of $Ab_1$ solution.

### 2.1.2. Synthesis of the Gellan gel

The Gellan gel was prepared following the procedure reported in according with Micheli et al. (2018) [25]. Calcium acetate powder $(CH_3COO)_2Ca$, 0.04 % w/w) and Gellan gum (2 % w/w) were dissolved in MilliQ water and then heated in a microwave at 600 W (Mars Microwave, CEM Corporation, Matthews, NC, USA) until the samples were boiling. Then it was transferred to a Petri dish, and it was left to cool at room temperature. When it was solid, squares of gel (2x2x0.5 cm) were cut.

### 2.1.3. Production of screen-printed electrodes (SPEs) for immunoassay

SPEs were produced in the Laboratory of Analytical Chemistry of the University of Roma "Tor Vergata" with a 245 DEK (High performance multi-purpose precision screen printer, Weymouth-UK) screen-printing machine. They are comprised of a working and a counter electrode deposited using graphite-based inks (Elettrodag 421) from Acheson (Milan, Italy) and a reference electrode made with silver ink (Acheson Elettrodag 4038 SS). The electrochemical cell has been defined using an insulating layer (Argon Carbonflex 25.101S). The diameter of the working electrode was 0.3 cm resulting in a geometric area of 0.07 $cm^2$ [34].

Initially, the SPEs were pre-treated before their use, with the addition of 80 μL of PBS (0.05 M) plus KCl (0.1 M, pH 7.4), to reduce impurities on the surface of the working electrode. Then an anodic potential of +1.7 V was applied for 180 s (chronoamperometry measurement). After that, an overnight at 4 °C functionalization of the SPEs was carried out using 6 μL of 1:1470 (v/v) anti-rabbit IgG as secondary antibody (produced in goat and used in $Na_2CO_3$ 50 mM, pH 9.6). Modified SPEs were rinsed with 45 μL of PBS (15 mM, pH 7.4) and then incubated with 6 μL of a blocking solution with 1 % PVA in $Na_2CO_3$ (50 mM, pH 9.6) for 15 min [35–37]. Afterwords, SPEs were washed again with 45 μL of PBS and coated with 6 μL of anti-chicken ovalbumin primary antibody 1:7500 (v/v) in $Na_2CO_3$ (50 mM, pH 9.6) for 30 min at 4 °C in a dark box. After one washing with 45 μL of PBS (15 mM, pH 7.4), SPEs were incubated again with 6 μL of the blocking solution (PVA 1 % in $Na_2CO_3$) for 15 min and washed with 45 μL of PBS.

### 2.1.4. Testing samples

Whatman® paper squares were used as reference specimens. 2.5 × 2.5 cm Whatman® paper samples were soaked in a 1 mL OVA standard solution at different concentrations (0.00, 0.01, 0.10, 1.00, 1.50, and 2.00 mg $mL^{-1}$). The specimens were then dried in the oven at 30 °C for 1 h. Paper samples were weight before and after the addition of OVA solution, in order to determine, by weight, the amount of OVA on the paper pieces. Specimens were prepared using the same procedure using different protein solutions (casein, collagen, bovine serum albumin) with a concentration of 0.1 μg $mL^{-1}$ to assess the selectivity of the method.

Natural aged paint reconstruction were used to assess the method on painting materials. The painting samples were prepared in according to ancient painting treatises described by Cennino Cennini in "Il libro dell'Arte, The book of Art" [38]. Briefly, a gypsum ground layer was obtained from a mixture of gypsum (190 g) and rabbit glue (10 g dissolved in 150 mL of hot water). Then, after drying, painted layers obtained mixing inorganic pigments with rabbit glue or egg or casein as binders were applied on the ground layer. The structures of the paint reconstructions (also called specimens or mock-ups) are reported in Table 2 together with the weight ratios between the components of each layer (the ratios depend on the nature of the materials and are optimal to obtain a mixture suitable for the application of the layer).

The method was validated on an historical oil painting dated back to XVII century. The painting was characterised by several layers (Table SI1). In detail, two protective layers were detected, a thinner one (4–7 μm, layer 2) and a superimposed one (12–16 μm, layer 3), characterized as terpenic resin (Table SI1).

**Table 2**
Description of the mockup's layers.

| Samples | Layers | |
|---|---|---|
| B1 | Layer 1 (painting layer) | Lead white/oil 1:1 (w/w) |
| | Layer 0 (ground layer) | Gypsum/rabbit glue 19:1 (w/w) |
| B2 | Layer 1 (painting layer) | Lead white/egg 1:1 (w/w) |
| | Layer 0 (ground layer) | Gypsum/rabbit glue 19:1 (w/w) |
| B3 | Layer 1 (painting layer) | Lead white/glue 1:1 (w/w) |
| | Layer 0 (ground layer) | Gypsum/rabbit glue 19:1 (w/w) |
| 2L2 | Layer 2 (painting layer) | lead white/egg 5:2 (w/w) |
| | Layer 1 (painting layer) | red ochre/casein 4:3 (w/w) |
| | Layer 0 (ground layer) | Gypsum/rabbit glue 19:1 (w/w) |

## 2.2. Methods

### 2.2.1. Immunochemical assay

Gellan gel was applied, pressing gently, to the sample (paper specimens or mock-up) for 5 min. After the application, the surface of the gel in contact with the sample was positioned in contact with the electrode for 10 min (Fig. 1). This two-steps procedure allows minimizing the contact time between the gel and the sample, reducing invasiveness, and maximizing the interaction between the gel (with the extracted protein) and the electrode to support the formation of the immune complex.

Afterwards, the electrode was removed and rinsed once with PBS (15 mM, pH 7.4) and 0.1 % Tween to remove eventual gel residues and twice with 15 mM PBS to remove tween residues. Finally, 80 μL of the redox couple potassium ferro-ferricyanide solution (3 mM + KCl 10 mM) were used to perform square wave voltammetry (SWV) investigation, applying −0.1 V as starting potential, 0.4 V as ending potential and 0.003 V as step (Fig. 1). All the electrochemical measurements were performed by a μAutolab III potentiostat/galvanostat (Metrohm Autolab, Utrecht, The Netherlands).

### 2.2.2. Assessment of the performance of the immunochemical assay

The performance of the immunochemical assay was assessed by creating calibration curves. This was achieved by employing aqueous ovalbumin solutions at different concentrations (ranging from 0 to 10 μg $mL^{-1}$ directly applied on the SPE functionalized), and then plotting the concentration values on a semi-logarithmic scale against the anodic current recorded using SWV. The standard curves were fitted using a 3-logistic function with 3 parameters:

$$y = \frac{(a)}{1 + (x|x_0)^b} \qquad \text{eq.x}$$

For each concentration, three replicates were performed, and the current values were normalized as follows:

$$I\% = \left(\frac{I - I_{min}}{I_{max} - I_{min}}\right) \times 100 \qquad \text{eq.y}$$

Where $I$ corresponds to the faradaic current value relative to the different protein concentration, $I_{max}$ corresponds to the maximum registered during the analysis and $I_{min}$ corresponds to the minimum value registered during the analysis. The normalization allowed to remove the intrinsic errors due to the electrode fabrication (*i.e.*, printing), considering only the current variation induced by the sample.

After the performance assessment of the immunosensor, the sensitivity of the complete protocol involving gel and electrochemical detection was evaluated. This evaluation was conducted by analyzing paper samples prepared with OVA solutions at different concentrations (refer to paragraph 2.1.4) following the procedure outlined in paragraph 2.2.1. The data were fitted using the same function as before and

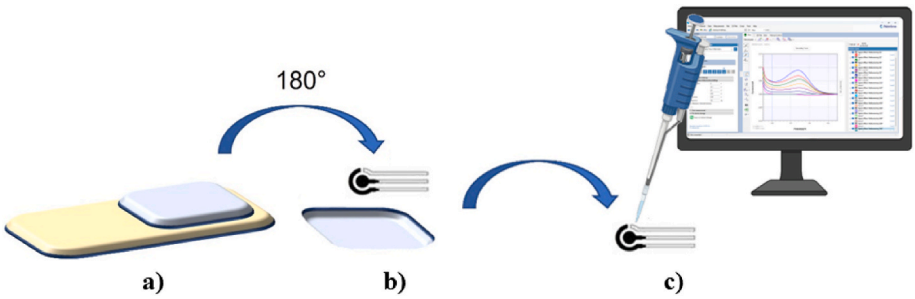


**Fig. 1.** Schematic representation of OVA immunosensor functioning. In a) the application of the gel on the sample for 5 min, in b) the application of the electrode on the surface of the gel (previously in contact with the sample) and in c) the detection of the current signal.

normalized.

To assess the selectivity of the method, the same protocol applied for pure OVA solutions was employed on pure solutions of BSA, collagen, and casein (as described in paragraph 2.1.4).

*2.2.3. Characterization of residues on the test systems*

To evaluate the presence of gel residues on the painting, we conducted optical observations using a Leica® MZ6 stereomicroscope connected to a Canon® PowerShot 550 digital camera and an Olympus BX51 M optical microscope (Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP70 digital camera. FTIR spectra were performed also on the mock-ups before and after the application of the gel to assess the presence of residues of gel on the test systems with a Thermo Scientific Nicolet™ iN10 MX imaging microscope (Thermo Fisher Scientific, Waltham, MA, USA) with a mercury-cadmium-telluride (MCT) detector cooled by liquid nitrogen. The analyses were performed using a slide-on ATR objective, equipped with a conical germanium crystal (4000-675 $cm^{-1}$) at a spectral resolution of 4 $cm^{-1}$.

*2.2.4. Validation of the protocol on the historical painting*

To assess the method on a real case study, an historical painting dated back to the beginning of XVII century was submitteted to the immunochemical investigation. The LF-EC-MIS was employed applying the Gellan gel on the painting surface, during a restoration campaign. Addittionally, small fragment was sampled and the cross-section prepared and analyzed through micro-FTIR using the set up and the conditions reported in Ref. [39] and a non-competitive indirect immunochemical protocol combined with a chemiluminescence detection according to the method reported in Refs. [11,13].

## 3. Results and discussion

### *3.1. Set-up of OVA label-free electrochemical immunosensor*

Several factors were considered in attempt to develop a robust and sensitive immune sensor, including: (i) the concentration of the primary antibody ($Ab_1$), (ii) possible cross reactivity of the primary antibody ($Ab_1$) and (iii) the stability of the immunosensor in storage condition (4 °C).

WEs were initially functionalized with precoating (1:1470 (v/v) anti-IgG $Ab_2$) (Fig. SI2) in carbonate buffer pH 9.6 and incubated at 4 °C overnight with anti-OVA $Ab_1$ at different dilutions (1:2500 (v/v), 1:5000 (v/v) and 1:7500 (v/v)) in carbonate buffer pH 9.6 following protocols reported by Cancelliere et al. [40] (Fig. SI3).

Precoating improves the interaction between anti-OVA $Ab_1$ and the analyte by facilitating the organized arrangement of the $Ab_1$ through its interaction with the heavy chain at the precoating. This ensures the constant availability of the primary antibody's variable region for antigen binding, resulting in an overall enhancement in binding efficiency [35].

The best results in terms of repeatability (with a relative standard deviation, RSD% equal to 4 %) and current drop (greater covering effect corresponding to a minimal current signal) were obtained with 1:7500 (v/v) as the dilution for $Ab_1$ (Fig. SI3). Once defined the antibodies dilutions, the performance of the immunochemical assay was evaluated by generating calibration curves by using OVA solutions at different concentration directly spotted on the functionalized SPE (Fig. 2).

The increment of OVA concentration led to a decrease of the electrochemical signal, as expected. Therefore, in the presence of the antigen-antibody immunocomplex that covers the surface of the SPE, the electrode area available for the redox reaction of the ferrocyanide decreased, leading to a reduced electrochemical signal when compared to that obtained in the absence of immunochemical complexes. On the contrary, if the protein is not probed by the sensor the intensity of the recorded current remains at its maximum value. Therefore, the current signal decreases as the protein content on the electrode increases. The maximum achievable current signal for each electrode (100 % of current) corresponds to the blank signal obtained from the electrode that was pre-treated with antibodies but without the analyte (only PBS, no OVA - blank). This signal was utilized to normalize the signals on the curve.

The electrochemical signals presented a good reproducibility, and their intensities were well inversely correlated with the amount of target. According to the curves, the limits of detection (LOD) of the immunochemical procedure (estimated as the amount of target giving an electrochemical signal corresponding to the background plus three times its standard deviation) was about 1.6 ng $mL^{-1}$ (limit of quantification -LOQ-equal to 16 ng $mL^{-1}$, calculated as the target's electrochemical signal strength, plus nine times its standard deviation, relative to the background.) and a working range between 0.01 and 0.4 µg $mL^{-1}$

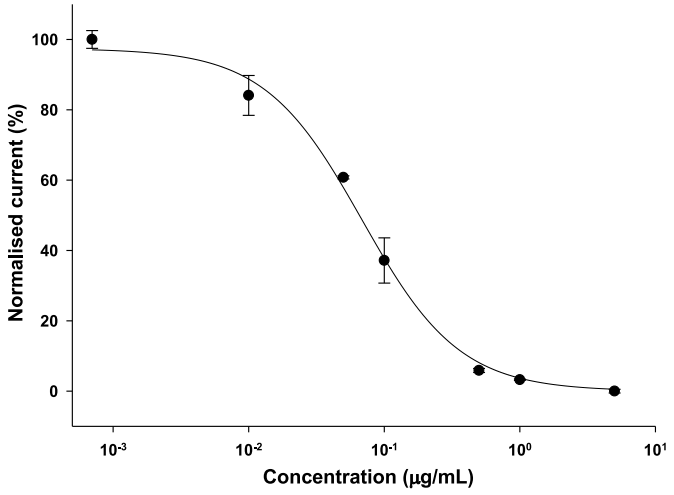


**Fig. 2.** Current *vs* Log [Ovalbumin]. 1:7500 v/v Ab-OVA in CB with Ab-Rabbit 1:1470 (v/v) as pre-coating. Measurements carried out in SWV with 80 µL of 3 mM $K_3Fe(CN)_6$ + 10 mM KCl. From the fitting: a = 94.43; b = 1.21; x0 = 0.068.

(linear section between 10 and 80 % of the curve) of OVA in buffer.

The low value of the detection limit confirmed the proper performances of SPEs in terms of selectivity and sensitivity needed for the suitable electrochemical detection of OVA in painted samples.

As reported in the previous paragraph, the gel sampling is minimally invasive since just a limited amount of ovalbumin spotted on a solid substrate is sampled [41,42]. We evaluated the electrochemical immunosensor sensitivity creating a calibration curve measuring the electrochemical signal of the SPE subsequent to its interaction with the gel surface that was used to sample OVA from paper samples treated with solutions at different concentrations, following the assay protocol described in section 2.2.2. Even if the gel is minimally invasive, the limit of detection (LOD) for the assay, calculated as amount of ovalbumin in the paper samples normalized for the weight of the paper was approximately 10 ng $g^{-1}$ (with RDS of 8 %, Fig. 3). These results demonstrated notable sensitivity and reproducibility.

The selectivity of the system was assessed by testing the biosensor using the same protocols described in paragraph 2.1.4 in presence of solutions at the same concentration of different proteins (casein, collagen, bovine serum albumin) directly applied on the SPE to induce the formation of the immunocomplex reaction. These are other proteins commonly used as binders in paintings [9,43–45]. The identification of protein-based material is important information for art historians as it permits to fully understand the manufacturing process and in turn, the technique used by an artist to identify the best restoration and conservation procedures. The outcomes unveiled that the immunosensors were minimally impacted by cross-reactivity with alternative proteins or nonspecific interactions involving assay reagents and the electrodes (Fig. 4). Indeed, while in the presence of OVA, a drastic drop in current was recorded (~40 % of current), in the presence of other non-specific proteins, percentages of current close to the maximum, thus equal or close to 100 % (i.e. no immunocomplex formation), were obtained.

The effectiveness can be attributed to the selectivity of the immunochemical reaction, along with the efficacy of the blocking solution treatment and the washing steps integrated into the protocol (described in paragraph 2.2.1). These findings (Fig. 4) corroborated the immunosensor specificity for the designated protein (OVA), rather than for other proteins.

To ensure the use of the biosensor during on-site diagnostic campaigns, it is essential to have pre-functionalized SPEs, readily available for immediate use. To address this requirement, the stability of the pre-functionalized SPEs was assessed.

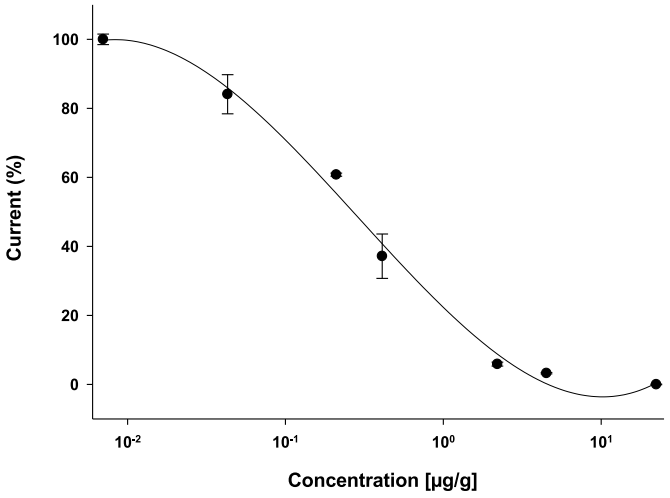


**Fig. 3.** Normalized current (%) *vs* OVA concentrations of OVA in the treated paper. 1:7500 v/v Ab-OVA in CB with Ab-Rabbit 1:1470 (v/v) as pre-coating, contact between electrode and gel after protein extraction from the paper. Measurements carried out in SWV with 80 µL of 3 mM $K_3Fe(CN)_6$ + 10 mM KCl. From the fitting: a = 98.2; b = 1.195; $x_0$ = 0.281.

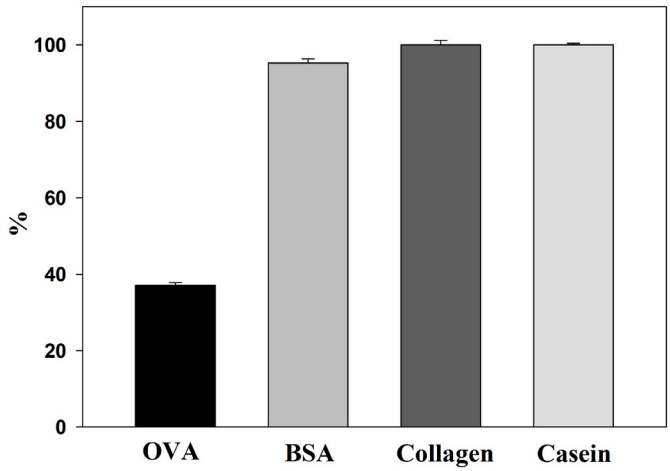


**Fig. 4.** Histograms represent the normalized current values (%) measured using the protocols described in 2.2.1 on solution of different proteins (0.1 µg $mL^{-1}$) in order to evaluate the cross-reactivity of the immunosensor: the higher is the signal, the lower is the protein binding.

The immunosensors, after their preparation as described in paragraph 2.1.3, were stored in a humid chamber in the dark at 4 °C until their use, and periodically (1, 14, 30 and 60 days) directly tested using paper samples added with the OVA solution at concentration of 0.1 µg $mL^{-1}$. After 60 days it was possible to observe a minimal reduction in the efficiency of the system (about 37 % less compared to the current detected at day 0) (data not shown).

### 3.2. *Application of the electrochemical immunosensor on painted samples*

To assess the applicability of the developed immunosensor on painted samples, the device was employed on standard mock-ups.

Painted samples, prepared by using egg, glue and oil as binders as reported in par. 2.1.4, were submitted to the analysis to validate the system and verify its selectivity.

Particular attention was devoted to the possible presence of spurious electrochemically active materials on the painting surface, such as dust and/or pigment particles, which may be entrapped into the gel and interact with the electrodes, inducing not specific variation of the current signal. To avoid this matrix effect, after sampling, the same gel was analyzed with both the immunochemical sensor described in paragraph 2.1.3 and with a SPE which was not functionalized with the $Ab_1$. In this way, it was possible to measure the current induced by the spurious materials and to subtract it from the signal registered with the immunochemical sensor. To compare data obtained from different painted mock-ups and assess the variation in OVA content within the treated samples, the current values were interpolated using the calibration curve corresponding to OVA standard solutions. The OVA concentration values obtained from different samples are reported in Table 3; results established the performances of the developed approach. As shown, first, OVA is not detectable if not present in the samples. The high current values, obtained for samples containing siccative oil and rabbit glue

**Table 3**
The table shows the estimated concentration values of OVA detected on the different mock-ups with different binders and corrected with a blank (n.d. = not detectable).

| Sample | OVA concentration (ng $mL^{-1}$ ± σ) |
|---|---|
| 2L2 (egg) | 20.5 ± 0.8 |
| B1 (oil) | n.d. |
| B2 (egg) | 15 ± 2 |
| B3 (rabbit glue) | n.d. |

binders, were not significantly different from the blank values, confirming the absence of OVA.

Furthermore, the concentration found is higher in the mock-up with higher amount of OVA binder that is 2L2 (the pigment-binder ratio of 2L2 is 5:2 (w/w) while of B2 is 19:1 (w/w)), as expected. Comparable results were obtained using a commercial ELISA spectrophotometric kit method (Table SI2).

To evaluate the effect of the gel application on the painted surfaces, observation with optical microscopy and analyses with FTIR microscopy were performed before and after the gel application. The assessment was performed on different mock-ups with different binders (egg, glue, oil, casein) and different pigments (gypsum, lead white, red ochre, azurite). The morphological investigation did not reveal any significant modification of the painted surface. Moreover, micro-IR spectra collected after the treatments presented spectral profiles unvaried if compared with those obtained before the gel application, thus no bands related to the presence of gels residues on the surface were detected. As an illustrative example, data related to sample 2L2, are reported in Fig. 5 (for the other samples, see Fig. SI4, SI5, SI6).

The characteristic band of lead white (at 1400 $cm^{-1}$, ascribable to carbonate) and of the proteinaceous binder (at 1654 $cm^{-1}$, 1540 $cm^{-1}$ 2854 and 2923 $cm^{-1}$ of amide I, amide II and CH respectively) are clearly identifiable in both the spectra recorded. Notably, no IR bands ascribable to water or sugar associated with the Gellan gel were observed (refer to Table 4).

To evaluate the performance of the method, the assay was used for the detection of ovalbumin in a varnish layer from a Renaissance oil painting.

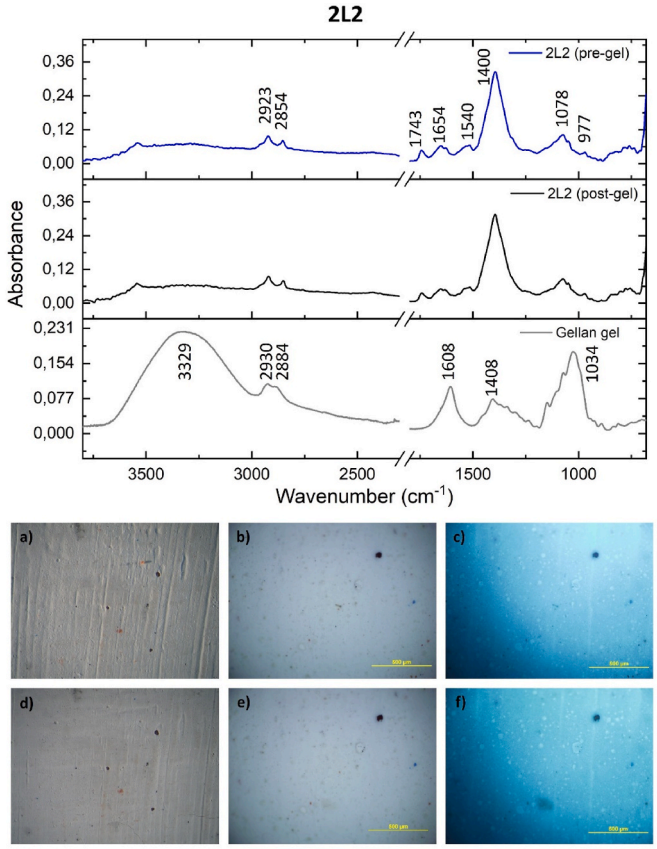


**Fig. 5.** micro-FTIR-ATR spectra of sample 2L2 before and after gel application and comparison with reference gel spectrum. The data was plotted using Origin software. Stereomicroscope (a,d) and optical microscope images in dark field (b, e) and UV light (c, f) of the painting mockups before and after gel application.

**Table 4**
Peaks identified in the sample 2L2 and pure Gellan gel, and bands attribution.

| Sample | Wavenumbers ($cm^{-1}$) | Attribution |
|---|---|---|
| 2L2 | 1078 | $\nu$ (Si–O), quartz |
| | 1743 | $\nu$ (C═O) |
| | 1654 | Amide I, proteinaceous binder |
| | 1540 | Amide II, proteinaceous binder |
| | 1396 | $\nu$ $(CO_3)^{2-}$ (Lead white) |
| | 2854 | $\nu$ $(CH_2)$ |
| | 2923 | $\nu$ $(CH_3)$ |
| Gellan gel | 1034 | $\nu$ (C–O–C) |
| | 1408 | $\nu$ $(CO_3)^{2-}$ |
| | 1608 | $\nu$ (OH) due to water |
| | 2884 | $\nu$ $(CH_2)$ |
| | 2930 | $\nu$ $(CH_3)$ |
| | 3329 | $\nu$ (OH) |

Prior to the immuno-electrochemical measurement, a small fragment was sampled from the painting, and a cross-section was subjected to stratigraphic analysis. The sample revealed a complex stratigraphy, with two varnish layers (Fig. SI1), the external one 12–16 μm thick and an internal thinner one in the order of 4–7 μm.

The uppermost layer displayed a strong bluish fluorescence and FTIR analyses revealed the presence of a terpenic resin (Table SI1, Fig. SI1, Fig. SI7). Traces of proteinaceous materials were detected in layer 2 (Fig. SI7) together with contributions from the other layer since the spatial resolution of the technique is higher with respect to the thickness of the layer. Since it is known that ovalbumin was employed as external coating according to ancient recipes [13], the cross-section was examined using chemiluminescence (CL) immunochemical microscopy imaging. A clear CL signal emerged from layer 2 confirming the presence of OVA (Fig. SI1).

Gellan gel was directly applied on-site on the painting surface after a pre-cleaning treatment aimed at removing the external aged varnish and subsequently transferred to the lab for electro-immunochemical analysis. The results confirmed the presence of OVA in the sampled layer, corroborating the feasibility, specificity and sensitivity of the proposed method. As for mock-ups samples, the current values were interpolated using the calibration curve corresponding to OVA standard solutions and a concentration of 0.056 ± 0.002 ng $mL^{-1}$ was obtained.

## 4. Conclusions

The research provided a minimally invasive method for the selective identification of ovalbumin in paintings.

This achievement was made possible by the integration of Gellan gel with electro-immunochemical detection. The method preserves the integrity of artworks and combines the selectivity of immunological reaction with the sensitivity of the electrochemical detection. The developed device is portable, miniaturized, and user-friendly. The system is designed for an easy on-site application by restorers. Indeed, restorers are familiar with the preparation of gels, already used for cleaning purposes. Moreover, SPEs can be prepared as kits and stored in a refrigerator, facilitating on-site applications and the potential industrial production of these biosensors.

The immunosensor can be used to identify the type of proteinaceous coating applied or to determine the proteinaceous binder in unvarnished areas, such as on the borders under the frame. Moreover, during a restauration campaign the external coating is usually removed and substituted, thus the identification of the binder of painting layer can be performed after the cleaning procedure and prior to the application of the new varnish, as in the reported case study.

This research represents a step towards the development of a new generation of point-of-care analytical devices for qualitative and quantitative on-site analyses. The promising preliminary results suggest the possibility to further develop the biosensor for the simultaneous detection of different binders.

Furthermore, the selective identification of proteins with a minimal invasiveness and high selectivity may be extremely interesting in other application fields, such as forensic investigations.


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## CRediT authorship contribution statement

**L. Gatti:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **G. Sciutto:** Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization. **R. Cancelliere:** Writing – original draft, Data curation. **L. Severini:** Investigation, Data curation. **C. Lisarelli:** Investigation, Formal analysis, Data curation. **C. Mazzuca:** Writing – review & editing, Validation, Conceptualization. **S. Prati:** Writing – review & editing. **R. Mazzeo:** Writing – review & editing. **L. Micheli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Data curation, Conceptualization.

## Declaration of competing interest

X- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.127167.

## Data availability

No data was used for the research described in the article.

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