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Abstract

This research is aimed to the study and application of an electrochemical immunosensor for the detection of ovalbumin (OVA) from egg white (or albumen) used as a binder in some works of art, such as some historical photographic prints and tempera paintings. The immunosensor takes advantage of the interesting biodetection capabilities offered by nanoelectrode ensembles (NEEs). The NEEs used to this aim are prepared by template deposition of gold nanoelectrodes within the pores of track-etched polycarbonate (PC) membranes. The affinity of polycarbonate for proteins is exploited to capture OVA from the aqueous extract obtained by incubation in phosphate buffer of a small sample fragment (< 1 mg). The captured protein is reacted selectively with anti-OVA antibody, labeled with glucose oxidase (GOx). In the case of positive response, the addition of the GOx

substrate (i.e. glucose) and a suitable redox mediator (a ferrocenyl derivative) reflects in the up rise of an electrocatalytic oxidation current, which depends on the OVA amount captured on the NEE, this amount correlating with OVA concentration in the extract. After optimization, the sensor is successfully applied to identify OVA in photographic prints dating back to the late 19th century, as well as in ancient tempera paintings from the 15th and 18th centuries.

1. Introduction

Egg white has been widely used by humans, probably since prehistory, as a water dispersible binder for the production and decoration of different kinds of manufactured goods including works of art, spanning from middle age tempera paintings to modern photographic prints [1,2]. These binding properties derive from the presence of the protein ovalbumin (OVA) which constitute 55% of the total egg white content [3]. In tempera paintings, egg white was used alone or, more commonly, mixed with egg yolk for the so-called "whole egg" tempera technique. Tempera was the most widely diffused painting technique in the Middle Age; however, it was also used by famous Renaissance painters such as Botticelli and Mantegna, up to modern painters e.g. Böcklin and De Chirico.

After the invention of photography in 1836, the first method to produce on a wide scale photographic prints on paper from a negative, was based on the so-called "albumen prints" or "albumen silver prints". The use of silver albumen for the production of a photosensitive emulsion on paper was introduced by Louis Blanquart-Evrard in 1846 [4,5]. An emulsion of egg white, beaten and decanted and added with a chloride salt (sodium chloride or ammonium chloride), was applied as a coating on a sheet of paper and let to dry. The sensitization to light was achieved by floating the coated paper in a solution of silver nitrate, so that photosensitive silver chloride crystals were

formed in the coating. The dried paper was placed in direct contact with a negative (typically a glass negative) and exposed to light, until the formation of the image was observed. This is a printing-out process so that the formation of the image can be monitored during the exposure to light. The image was fixed in a sodium thiosulfate bath, which helped preventing further darkening of the image. As an option, the obtained image could be stabilized by toning with a solution of gold salts or other toning agents, featuring a slight change of the print grey tones. In albumen prints, the image is printed directly by light rather than being developed from a "latent" image, as in the case of gelatin prints.

Printing photographs on albumen silver emulsion was the prevailing method to produce photographic positives from the mid-1800s up to the beginning of the 1900s, when gelatin fully substituted albumen in the production of commercial photographic paper [6]. It is worth stressing that, in the case of gelatin-silver halide paper, the photographic image can be obtained not only by the direct action of light (i.e. by direct blackening) as for albumen prints, but also by the chemical development of a latent image. Thanks to this discovery, printing photographs on gelatin silver paper started to become popular in the last decades of the 1800s, up to quickly becoming the dominant black & white photographic printing process from 1900 onwards.

With the purpose of identification and conservation, different analytical methods have been proposed to distinguish whether a work of art (e.g. a painting or a photograph) contains gelatine or albumin in the image layer. Since both binders are proteins, the interpretation of the results is not always straightforward and univocal, particularly for aged or deteriorated objects and works of art. Identification by ATR-FTIR is possible due to the characteristic peaks of the proteinaceous substances in the region between 1300 and 1600 cm^{-1}. However, in the case of degraded materials this technique does not provide univocal results, because the two binders give close similar spectra,

where the main difference is a stronger signal associated to the bending vibrations of the C-H groups at 1449 cm^{-1} in the gelatine spectrum, while it is more intense at 1391 cm^{-1} in albumen spectrum [6].

An alternative is offered by immunochemical methods, for example by enzyme-linked immunosorbent assay (ELISA)[7–9], although it requires relatively large amounts of sample, is time consuming and not easily suitable for decentralized analysis. Here, we present for the first time an electrochemical OVA-immunosensor based on arrays (or ensembles) of gold nanoelectrodes (NEEs) for the univocal identification of ovalbumin, typically employed as binder in albumen photographic prints or tempera paintings. The sensor is based on the properties of NEEs prepared by electroless deposition of gold within the nanopores of track-etched polycarbonate (PC) membranes used as templates [10,11]. The PC surface, which the gold nanowire electrodes are embedded in, act not only as the insulator between the nanoelectrodes, but also promotes the immobilization of proteins on the PC portion of the sensor. These characteristics have been exploited for developing NEE-based immunosensors for the detection of disease markers such as the tumour marker HER2 [12] and anti-transglutaminase for celiac disease diagnostics [13], but also for detecting immunoglobulin IgY from egg yolk in tempera paintings [14] and foodstuff [15]. According to this sensing strategy, the captured protein is later reacted with a specific antibody, labelled with a redox enzyme. The addition of the enzyme substrate and a redox mediator, which shuttles electrons between the nanoelectrodes and the active site of the enzyme label, reflects in an electrocatalytic amplification of the faradic current that scales proportionally to the amount of captured analyte protein. Note that this detection methodology is characterized by very low detection limits, since it exploits the high signal/noise ratios that are typical of voltammetric responses of NEEs [16], thus allowing to detect small concentration of the target analyte in small volume/mass samples [17].

2. Experimental

2.1. Materials

NEEs were prepared as described in [10,11], (ferrocenylmethyl)trimethylammonium hexafluorophosphate (FA^+) was synthesized in house. Phosphate buffer PBS, TWEEN 20, and glucose were from Sigma Aldrich. The primary antibody (Ab) was affinity purified rabbit anti-chicken Ovalbumin, from Immunology Consultants Laboratory Inc., diluted 1:10 in 10 mM PBS as suggest by the producer; the secondary antibody was goat-anti Rabbit IgG H&L (Glucose Oxidase) from Abcam, diluted 1:50 in 10 mM PBS as suggested by the producer. All the reagents used in the procedure were analytical grade. Soymilk was purchased from a supermarket in Venice.

Measurements were carried out in a typical three electrodes cell with NEEs as working electrodes, Ag/AgCl (KCl sat) as reference electrode and a Pt wire as counter electrode. Autolab PGstat 101 (Metrohm, The Netherlands) controlled with the software NOVA 1.1 was used for the electrochemical measurements.

2.2 Preparation of the ovalbumin immunosensor and analytical protocol

In the immunosensor, the target protein, ovalbumin (OVA, from egg white or from the studied sample) is directly immobilized on the NEE electrochemical platform, where it is captured mainly by the polycarbonate part of the NEEs. For the analysis on photographic and painting samples the target analyte was extracted from a very small amount of sample (it is specified for each case, but usually less than 1 mg) by incubation in few microliters of 10 mM PBS solution, pH 7, for 1 hour under ultrasonication.

A blocking step is performed with soymilk to avoid nonspecific interactions and then, a primary anti-OVA antibody is employed to recognize the protein. A polyclonal anti-OVA primary antibody was chosen to promote the recognition of the protein also in complex or degraded samples. Following, a secondary antibody anti-anti-OVA labelled with glucose oxidase is incubated to detect

the antigen-antibody complex. A scheme of the immunosensor is presented in Fig. 1. To develop the typical electrocatalytic cycle between the enzyme glucose oxidase and the nanoelectrodes, the redox mediator FA^+, a ferrocene derivative with $E_{1/2}$ = 0.43 V vs Ag/AgCl (sat KCl), was chosen [17,18].

Figure 1 close to here

In details, the preparation of the immunosensor with the NEE platform (geometric area of 0.022 cm^2) consists of the following steps:

- five microliters of the sample extraction solution are deposited on the surface of a NEE and incubated for 30 minutes at room temperature, in a humidity chamber made by a sealed Petri's plate with wet filter paper on the bottom. Proteins, like OVA, bound on the carboxylic groups present on the PC surface; after the NEE is rinsed four times with a rinsing solution (1% TWEEN 20 in 10 mM PBS);
- a blocking step is carried out upon incubation at room temperature in 5% soymilk solution for 30 min on an orbital shaker plate, followed by rinsing four times with the rinsing solution;
- the incubation of the primary Ab, is carried out by dropping of 5 µL of Anti-OVA solution for 30 min, followed by rinsing four times with the rinsing solution;
- the incubation of the GOx-labelled secondary Ab is done by dropping 5 µL of secondary Ab solution for 30 min, followed by rinsing four times with the rinsing solution.

The electrochemical detection of the analyte is carried out in the voltammetric cell containing 0.1 M PBS solution at pH 7 as supporting electrolyte, and 0.1 mM FA^+ as redox mediator. The cyclic voltammetry (CV) is recorded at a scan rate of 10 mV s^{-1}, initial potential 0.1 V, vertex potential 0.8 V. The enzyme substrate glucose 0.1 M is added to the electrolyte solution and the CV is recorded under the above indicated conditions.

2.3 Photographic samples

The photographic items, kindly provided by private collections, were pictures from the end of 19th century. In this work we present the results obtained from the analysis of the photographs shown in Fig. 2. On the bases of historical information, the first (Photo-1, see Fig 2a) was expected to contain white egg; while the second one (Photo-2, see Fig 2b), had the sensitive layer composed by gelatine and silver. This later sample should be taken as negative test.

Figure 2 close to here

Preparation of photographic samples: Two synthetic laboratory samples of albumin based-photosensitive coatings were prepared in the laboratory following the procedure described in the Introduction [4]: in the first sample (lab-sample A) the complete procedure was followed, while in the second one (lab-sample B) silver nitrate was not added, in order to evaluate a possible interference of silver in the immunosensing procedure.

The extraction procedure of OVA from the samples was carried out by incubating very small amounts of the sample with few microliters of 10 mM PBS solution for 1 hour under ultrasonication. The extraction procedure from the studied samples was the following: 5.3 mg of lab-sample A incubated in 50 µL of PBS; 17.5 mg of lab-sample B incubated in 150 µL of PBS; 3.5 mg taken from a peripheral zone of Photo-1 incubated in 50 µL PBS; 17.1 mg from a peripheral zone of Photo-2 incubated in 150 µL PBS.

2.4 Painting samples

Paintings samples were provided by restorer Giovanna Niero (Noale, Italy). The works were a canvas of unknown Italian artist from a Venetian villa, approximately dated back in the 18th century (see Fig. 3 a); and a painting of a Virgin with child, attributed to Mantegna, now in phase of study, from the 15th century (see Fig. 3 b). According to art historians, all these paintings are supposed to

be prepared using egg-tempera. However, further investigation is necessary to verify the presence or absence of egg white, as a marker of the whole-egg tempera technique. For this reason, analysis with the OVA-immunosensor was performed to all the samples.

Figure 3 close to here

Painting samples preparation:

Sample (named) Canvas: 1.5 mg of the painting layer was collected and diluted in 30 µL of PBS; Sample (named) Virgin: three different micro-samples were taken from different areas of painting; in this case, micro samples were diluted in 11 µL of PBS, enough to perform the determination of both OVA and IgY immunosensing.

To have more complete information about the composition of the painting ligand and to fully understand the tempera technique exploited by the artists, the paintings samples were also analysed with an IgY electrochemical immunosensor previously developed in our laboratory [14]. The mains results obtained with IgY immunosensor are shown in the Supporting information file.

3. Results and discussion

3.1 Electrochemical characterization of the immunosensor

Based on previous studies, FA^+ was used as suitable redox mediator to shuttle electrons from NEE to the glucose oxidase label bound to the secondary anti-antiOVA. The CV (dashed-line) in Fig. 4 was recorded at the bare NEE and shows the reversible voltammetric behaviour of the mediator, with $E_{1/2}$ = + 0.43 V [18]. When the same NEE was incubated with OVA solution followed by anti-OVA primary Ab and with GOx-labelled anti-anti-OVA secondary Ab (see Experimental for detailed procedure), a comparable CV pattern (dotted-line, in Fig 4) was recorded, which showed only a quite small decrease of the anodic peak current. This decrease can be ascribed to the procedure of capturing of the analyte OVA, followed by treatment with soymilk as blocking agent and the primary

and secondary antibodies. This electrochemical response supports the hypothesis that the biomolecules preferably bind on the PC part of the electrode surface, leaving the gold nanodisks surface free for the electron exchange electrode-mediator. A completely different response was observed when the enzyme substrate (glucose) was added to the solution. As shown by the solid-line CV in Fig. 4, the voltammetric pattern presents a sigmoidal shape in the presence of 0.1 M glucose, with a dramatic increase of the anodic current that reaches a plateau, and the concomitant disappearance of the cathodic current. The limiting anodic current resulted proportional to the amount of analyte (OVA) immobilized on the electrode surface (see Supplementary Information for more details).

Figure 4 close to here

This behaviour agrees with the following electrocatalytic cycle [19,20] mediated by the redox probe FA^+:

$$GOx(FAD) + \text{D-glucose} \rightarrow GOx(FADH_2) + \text{D-glucono 1,5-lactone} \quad (1)$$

$$FA^+ \leftrightarrows FA^{2+} + e^- \quad (2)$$

$$GOx(FADH_2) + 2\ FA^{2+} \leftrightarrows GOx(FAD) + 2\ FA^+ \quad (3)$$

In reaction (1), the flavin-adenine dinucleotide (FAD) cofactor of glucose oxidase reacts enzymatically with the glucose substrate. The oxidized form of the enzyme is then regenerated by reaction (3), where the oxidised form of the redox mediator generated on the electrode surface by the electrochemical oxidation of FA^+ (reaction (2)), reoxidizes the $FADH_2$ cofactor. Under these conditions, the anodic peak current increases since FA^+ is continuously regenerated by reaction (2) while reaction (3) consumes chemically the FA^{2+} as soon as it is produced at the nanoelectrodes/solution interface by the electrochemical process (2), thus providing the disappearing of the cathodic current counterpart. Note that the anodic current value depends on

the amount of enzyme immobilized in proximity of the nanoelectrodes, this parameter being directly dependent on the amount of captured analyte (i.e. OVA).

3.2 Determination of ovalbumin in photographic artworks

The OVA immunosensor was applied to the determination of ovalbumin in the extract from the photographic historical Photo-1, as described in the Experimental section. Fig. 5 shows the CVs recorded at the immunosensor prepared by incubating the extract from Photo-1 and completing the procedure of preparation of the immunosensor (dotted-line), and after the addition of the relevant substrate glucose (solid-line).

Figure 5 close to here

A comparison between the two CVs from Fig. 5 indicates that the addition of glucose reflects in a change in the voltammetric pattern of FA^+ leading to an electrocatalytic pattern with features comparable with those described in the previous paragraph. In particular, results shown in Fig. 5 indicate a current increase of 0.27 μA calculated as $I_{net} = I_{ECa} - I_{pa}$, where I_{pa} and I_{ECa} are the anodic current measured at 0.48 V before and after the addition of the substrate, respectively. This current increment indicates the presence of OVA in the sample.

The application of the same immunosensing procedure to the synthetic photographic lab-sample A and lab-sample B was carried out to verify the influence of silver in the measurement, and to confirm the results obtained for the historical samples. The immunosensor response from the extracts of photographic lab-samples A and lab-sample B are shown in Fig. 6a and b.

Figure 6 close to here

The CVs recorded after the addition of 0.1 M glucose in solution reflects in the presence of an electrocatalytic current at NEEs incubated with the both the extracts. This behaviour indicates that the presence of the silver salt does not affect the response of the immunosensor. Current

increment values calculated for these samples are 0.26 µA and 0.27 µA, respectively, and are comparable to the one recorded for the historical photographic Photo-1 (namely, 0.27 µA). As a negative test, Figure 7 shows the CV patterns recorded on the OVA-immunosensor, incubated with the extracts from the sample Photo-2, which is a gelatine photographic print. Dotted-line refers to the absence of the substrate glucose, whereas solid-line to the addition of glucose. The two CV patterns are comparable, apart a slight decrease in the cathodic current of the redox probe observed after the addition of glucose. It is worth stressing that the presence of enzyme label substrate (i.e glucose) does not reflect in any electrocatalytic effect, for this sample, confirming the reliability of the proposed method.

Figure 7 close to here

All the above evidences indicate that the detection of an electrocatalytic current after incubating the NEE with the extract of a photographic print emulsion followed by reaction with GOx labelled anti-anti-OVA and glucose addition can be taken as a clear diagnostic criterion to identify the presence of albumin in the emulsion of historical photographic prints. Noticeably, the presence of silver in the image layer does not interfere with the analysis.

To compare the results obtained with OVA-immunosensor with a commonly used analytical method, historical samples Photo-1 and Photo-2 were analysed with ATR-FTIR, to double check the presence of albumen or gelatine. The ATR-FTIR spectra of both the samples show the typical absorption bands characteristic of a protein: the signals of amide groups are detectable at 1625 (Amide I, C=O and CN stretching), 1537 (Amide II) and 3277 cm^{-1} (N-H stretching). Moreover, the signal of the CH_3 amide groups is detectable at 1024 cm^{-1}. The peaks at 2928 and 2869 cm^{-1} are due to the asymmetric and symmetric stretching vibration of the aliphatic CH_2 bonds, respectively, while the peaks at 1444 and 1388 cm^{-1} are attributable to the CH_2 bending and rocking vibrations. These absorption peaks are shared for both the proteinaceous binders investigated [21]. It is possible to

better identify albumen (Fig. 8 red full line) thanks to the strong characteristic signal at 1391 cm^{-1} [21–23], while gelatin is detectable thanks to the strong peak at 1446 cm^{-1} and to the presence of the peak at 1330 cm^{-1} (Amide III) that is, on the other hand, absent in the albumen spectra [21,24,25]. Despite these slight differences, this technique is not as reliable as the proposed immunosensor, especially in case of degraded or complex samples where the peaks cannot be clearly distinguished.

Figure 8 close to here

3.3 Determination of ovalbumin in tempera painting samples

Fig. 9a shows the CVs relevant to the application of OVA-immunosensor to the sample Canvas, where it is possible to observe the redox voltammetric pattern of the mediator (Fig 9a, dashed-line) and the presence of the electrocatalytic current increment with the disappearance of the cathodic peak after the addition of the substrate (Fig. 9a, solid-line). This behaviour reveals the presence of the ovalbumin in this sample.

The same samples Canvas were analysed with the IgY immunosensing procedure proposed in [14] (see S.I. for more information). The typical results are presented in Fig. 9b. The dotted line CV recorded at a NEE modified with the sample Canvas extract, followed by BSA blocking and Anti-IgY HRP is characterized by well resolved redox peaks, which are attributed to the two electron–one proton reduction of MB to the leucoform (LB) and its relevant reoxidation. The CV recorded at the same NEE, when 0.5 mM H_2O_2 is added to the solution, showed an electrocatalytic pattern: a dramatic increase of the reduction current is observed, the reoxidation peak disappears and the CV pattern becomes sigmoidal shaped (full line in Fig. 9b). These results point to the presence of IgY, which is the main protein of egg yolk. Combining the results obtained with both immunosensors, it

is clear that the painter used both egg white and egg yolk for the production of this artwork, exploiting the whole-egg tempera technique.

Figure 9 close to here

The OVA immunosensor was applied to the three samples from the painting Virgin with child. The voltammograms shown in Fig. 10 a indicate the absence of the electrocatalytic cycle, evidenced by the presence of both the anodic and cathodic peaks after the addition of the substrate (0.1 M glucose) to the solution. The result of the albumin analysis is therefore negative. The application of the IgY immunosensor to these samples, following the procedure described above, gave the result shown in Fig 10b. In this case the electrocatalytic cycle is clearly observed indicating the presence of egg yolk in the tempera.

The results obtained with the OVA immunosensor (Fig 10a) and IgY immunosensor (Fig. 10b) indicate that in the painting Virgin with child the artist did not use the whole egg tempera technique to realise the painting, but only the egg yolk.

Figure 10 close to here

4. Conclusion

In this work we exploited the large polycarbonate surface of template NEEs to immobilize the protein ovalbumin to develop an immunosensor for the identification of its presence in egg tempera. We were able to identify and distinguish the protein both in historical and contemporary photograph samples in a reliable and univocal way, more than with the classical analytical techniques used until now, such as FTIR-ATR. The absence of the electrocatalytic signal in the negative samples supports the reliability of the OVA-electrochemical immunosensor.

Positive results were also obtained when the immunosensor was applied to egg-tempera samples. The method was useful to identify the technique used by the painters: in the case of the Venetian canvas, the artist used whole egg to produce the painting. In the case of the Virgin and Child, it was possible to observe the absence of the marker protein of egg white, suggesting an egg-yolk tempera.

Coupling the immunosensing procedures (OVA and IgY) to the collected samples provided reliable, fast and complete information about the composition of the binder, demonstrating the suitability of the proposed electrochemical immunosensors to the field of diagnostic for cultural heritage.

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