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Coupling non invasive and fast sampling of proteins from work of art surfaces to surface plasmon resonance biosensing: Differential and simultaneous detection of egg components for cultural heritage diagnosis and conservation

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ABSTRACT

Despite the wide application of surface plasmon resonance (SPR) to a broad area of interests, from environment to food analysis, from drug discovery to diagnostics, its exploitation in cultural heritage conservation is still unexplored. Water-based highly viscous polymeric dispersions (HVPD) composed by partially hydrolyzed polyvinyl acetate (PVA), borax, and water, were recently developed and successfully applied for the selective removal of surface degradation patinas (i.e. protein materials, natural resins etc.) from paintings of historical and artistic interest. This approach is here coupled for the first time to a SPR biosensor to simultaneously recognize albumen, yolk, or their mixtures in HVPD extracts. Ovalbumin and immunoglobulin Y are selected as analytes for egg white and yolk recognition, respectively. The biosensor was first characterized on standard analytes within the range 0–400 mg L^{-1} and then on fresh and dried egg albumen and yolk down to $2 \cdot 10^4$ and $1 \cdot 10^5$ dilution factors, respectively. Once optimized, the biosensor was combined to the HVPD application on simulated and real art samples for the evaluation of hen egg presence in the extract, i.e. albumen, yolk, or their co-presence in the matrix. For a contemporary 'sacred icon', realized by the traditional egg tempera procedure described by Cennino Cennini, the biosensor successfully distinguished different uses of egg components for the realization of painted and gilded areas, i.e. yolk and albumen, respectively. Finally, a XVIII century italian painting whose the realization technique is unknown, was tested confirming its egg tempera-based realization technique.

1. Introduction

Biosensors based on Surface plasmon resonance (SPR) are applied to a wide panorama of analytical problems with excellent results in label free and real time molecular diagnostics in medicine, environmental and food analysis, drug discovery and anti doping (Homola, 2006; Mayer and Hafner, 2011; Minunni et al., 2008; Scarano et al., 2010; Shankaran et al., 2007; Willets and Van Duyne, 2007). However, despite their large diffusion in many fields of chemistry and biochemistry, the literature on the use of these devices in the field of cultural heritage conservation is still surprisingly scarce. In fact, the (bio)chemical characterization of artworks is nowadays an important information to set up effective and low invasive restoration interventions and authentication studies helped by protein fingerprinting (Marijnissen, 1985; Ramírez et al., 2005). Therefore, considering that SPR technique has been extensively and successfully applied to proteins and peptides identification (Nguyen et al., 2015), its application to art diagnosis, conservation, and restoration could be very promising. In this framework, we developed an immuno-based SPR biosensor able to simultaneously identify and distinguish different egg proteins sampled on artwork surfaces, addressing the need in distinguishing pure components from whole egg, i.e. pure albumen or yolk from their mixture. In fact, traditional egg-based tempera is usually prepared by using only yolk as binding medium for pigments, but other preparations are based on whole homogenized egg or pure albumen. Pure albumen is also applied as "glue" for the realization of gildings layers (Mayer, 1985; Mounier et al., 2011). Moreover albumen, extensively used as surface protective in past restorations, is prone to spontaneous degradation causing alteration of the color surface. This requires the selective removal of albumen layers as one of the first steps to be taken in account in the restoration of paintings. Up to now, a limited number of

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attempts in exploiting affinity-based biosensors for such as investigations on artworks have been reported in literature (Micheli et al., 2012; Bottari et al., 2014). Only recently, Ugo and coworkers reported an electrochemical biosensor based on gold microelectrodes with application to art samples for the detection of immunoglobulin Y (IgY) (Bottari et al., 2014), as indicative of egg use in paintings (Osticioli et al., 2008). However, if singly detected, IgY are not informative of different uses of egg in artworks. Furthermore, labeled techniques suffer from possible enzyme activity depletion or catalysis interference by matrix components (mainly pigments), as also demonstrated in ELISA-based immunoassays for egg detection in simulated temperas (Cartechini et al., 2010), even if sandwich-based strategies can successfully eliminate matrix effects thanks to the specificity of the secondary antibody used to reveal the target analyte. In this framework, the use of label free techniques is thus very attractive, and SPR-based biosensing represents a promising platform for analysis of proteins dedicated to cultural heritage. Other analytical approaches than biosensors are based on expensive and/or labeled techniques (Cartechini et al., 2010; Gambino et al., 2013; Mazurek et al., 2014), that have limited perspectives for a widespread diffusion. On the contrary, recent innovations in the field of SPR platforms (mainly based on Localized SPR) have already gained encouraging results as cheap and portable analytical platforms in this sense. Working in this direction, this paper reports the successful coupling of an innovative, low invasive, and highly versatile sampling method based on a Highly Viscous Polymeric Dispersion (HVPD) to the simultaneous identification of egg albumen and yolk by SPR. These HVPD have been recently optimized for the selective removal of surface degradation patinas from painted surfaces of historical and artistic interest (Angelova et al., 2011; Baglioni et al., 2015). These systems are composed by partially hydrolyzed polyvinyl acetate (PVA) covalently crosslinked with borax. Its intrinsic shear elastic modulus allows its complete removal after the cleaning action simply by peeling it off from the artwork surface, without leaving any instrumentally detectable residue onto the cleaned area (Natali et al., 2011). PVA-based HVPDs are thus a very interesting material to be coupled to optical biosensing to both investigate surface material from artworks and/or monitor the progress of a cleaning action. This approach allows to evaluate the composition of the extracted material and to provide information on the progression of the cleaning process in fast and label free mode, compared to traditional immuno-based assays such as ELISA. The biosensor was first developed and tested on standard analytes (ovalbumin and IgY) and on dried egg films. Then its detection ability was checked on HVPD extracts after its application on simulated and real art samples. The direct detection through primary antibodies (a-OVA and a-IgY) was reinforced by a secondary sandwich-based detection tailored for each analyte, exploited to solve uncertain direct responses on yolk. As proof of concept, the method was finally applied to a contemporary 'Sacred icon', first, and then to a XVIII century italian painting. SPR data confirmed the ability of the whole procedure in both detecting the egg presence and in distinguishing its different uses for the realization of painted and gilded areas. The method results non-invasive, fast and reproducible, giving specific information within 1 h from HVPD application to SPR results.

2. Materials and methods

2.1. Reagents and buffers

N-Hydroxysuccinimide (NHS) was from Fluka (Milan, Italy); 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) was from Merck-Calbiochem (Darmstadt, Germany); 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sodium hydroxide, hydrochloric acid, Tween 20, ethylenediaminetetraacetic acid (EDTA), ethanolamine hydrochloride (EA), chicken ovalbumin (OVA) and sodium tetraborate decahydrate (borax, $>99.5\%$) were all from Sigma Aldrich (Milan, Italy). Skim milk powder (analytical grade) was purchased from Oxoid Ltd. (Hampshire, UK) and used without any further purification. 80PVAc was supplied by the Kuraray Co. Europe, Ltd. (Hattersheim am Main, Germany) as a random copolymer, PVA-424 h (MW=47 300, 80% hydrolyzed), and was used as received. Water was purified by a Millipore Elix3 ($R \geq 15\ M\Omega \times cm$). Other chemicals were purchased from standard commercial sources at analytical grade. Running buffer used in Biacore X™ experiments was the HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4), both for protein dilution and binding. All salts used for buffers were analytical grade and were purchased from Merck (Darmstadt, Germany). Deionized water (Millipore) was used throughout the preparations and buffer solutions were finally filtered (0.22 μm) before use. Purified chicken IgY, whole molecule, and the relative polyclonal antibody ((anti-IgY), goat) were from GenScript (Twin Helix Srl, Milan, Italy). Purified polyclonal (anti-OVA)lbumin (rabbit, 1.0 mg mL^{-1}) were from Rockland Inc. (Tebu-bio, Milan, Italy). Secondary antibodies used for the sandwich strategy were rabbit (anti-OVA) from Sigma Aldrich (Milan, Italy), goat (anti-IgY) from Abcam (Cambridge, UK).

2.2. SPR instrumentation and biochip preparation

SPR measurements were performed on Biacore X™, by using carboxymethylated dextran CM5 biochips (General Electric Healthcare Bio-Sciences AB; Uppsala, Sweden). Antibodies were immobilized on CM5 biochips by amino-coupling reaction as follows: at a constant flow rate of 5 μL min^{-1}, the dextran layer was activated with a water solution of NHS/EDAC (50 mM/200 mM) for 7 min Afterwards, 200 mg L^{-1} of each antibody in 10 mM sodium acetate solution at proper pH was flowed separately on the active surface of a channel, and the binding monitored in real time for 15 min The biochip surface was finally saturated by 1 M EA pH 8.5 for 20 min and the biochip was let under flow until equilibration of the baseline. All experiments were performed at least three times at a temperature of 25.00 ± 0.01 °C to determine the reproducibility of the biosensor. After each measuring cycle, the biosensor surface was regenerated by an injection of 100 mM NaOH aqueous solution for 30 s Dual channel biochips worked with the two measuring channels opened in series, each one carrying one immobilized antibody ((anti-OVA) or (anti-IgY)) for simultaneous detection. For this reason, preliminary experiments aimed to check the absence of unspecific binding of analytes on CM5 biochip treated to work as negative reference were carried out (see Supporting information and Fig. S1).

2.3. Sandwich on standard analytes solutions

Secondary (anti-OVA) and (anti-IgY) antibodies (different from the primary ones) were used to bind the analytes by a different epitope after their binding with the primary antibodies. The secondary antibodies were serially injected following the binding process in real time, and a specific amplification of each signal was obtained. The concentration of the secondary antibodies was optimized and consisted in 10 mg L^{-1} for both. It resulted enough to amplify the direct signals of standard analytes in a dynamic fashion, up to the highest concentration tested (400 mg L^{-1}). Also the effect of the injection order (aOVA/aIgY or aIgY/aOVA) on the overall amplified signals was evaluated, showing that the injection of secondary (anti-IgY) at first allows to avoid some unspecific binding of the secondary (anti-IgY) on the secondary (anti-OVA),

when the latter is injected at first on the two channel in series (Fig. S2). Negative controls were performed to verify that no unspecific binding of the secondary antibodies occur on the primary ones in absence of the relative standard analytes (data not shown). To recover the initial baseline after the secondary binding, a double step of regeneration was performed: 0.005% SDS for 120 s followed by 0.1 M NaOH for 60 s

2.4. Samples preparation

Standard OVA and IgY were dissolved in HBS-EP buffer at a starting concentration and serially diluted for calibration experiments by SPR. Fresh eggs were gently provided from free-range, corn-fed hens to be as close in composition as possible to those found in artists' materials. Eggs were carefully opened and gently poured in Petri dishes. Albumen was separated with a 5 mL syringe without needle. The yolk was washed by gently spraying MilliQ and discarding the washing solution three times. Finally, the intact yolk was rolled on paper and part of its content was extracted by piercing the encasing film with a syringe with needle. Albumen and yolk were transferred in plastic tubes and stored at 4 °C until use. HVPDs were prepared according to the procedure already reported in the literature (Natali et al., 2011).

2.5. Application protocol of HVPD

Fifty mg HVPD were applied on the surface of interest and carefully pressed to assure a homogeneous adhesion of the material to the surface. The final covered surface was standardized to a circular diameter of 1 cm by the use of a plastic support surrounding the HVPD matrix. Once applied, the material was let in contact with the surface for the desired time window (1–30 min), after which the HVPD was removed by tweezers with flat tips. The material was then transferred in a 1.5 mL plastic tube and 200 µL of HBS-EP buffer added. The best HVPD/buffer (weight/volume) ratio was optimized to retain the binding conditions for extracted proteins after incubation of HVPD (pH 9.0) in buffer (pH 7.4). To this aim, different ratios (1:1, 1:4, 1:10 HVPD weight/HBS-EP volume) were prepared and checked both for the final pH after incubation and for the binding efficiency of the extracts by SPR analysis. In fact, the biorecognition between protein extracts and the specific receptors immobilized on the biochip strictly depends on the pH of the binding solution. Therefore, a wrong pH of the solution affects the SPR signal. Final pH values were pH 7.5 for 1:10 ratio, pH 7.8 for 1:4 ratio, and pH 9.0 for 1:1 ratio. Accordingly, SPR data displayed negligible variation of the binding ability for the first two procedures (1:10 and 1:4), whereas no binding for the last one (1:1). To minimize real sample dilution, the 1:4 ratio as the best HVPD weight/HBS-EP volume ratio, i.e. 50 mg HVPD suspended in 200 µL of HBS-EP buffer. All the reported experiments were thus performed by following this procedure. Finally, the tube was let under circular rotation for 30 min at room temperature and then volumes (20 µL) of each sample were tested on the SPR biosensor. The overall procedure is sketched in Fig. 1.

2.6. Simulated and real samples preparation

Before testing the HVPD/SPR method on real artwork samples, the procedure was tested and optimized on glass slides on which albumen, yolk, or homogenized whole egg were brushed and let dry for five days at room temperature in a capped (not sealed) Petri dishes to avoid dust deposition. In particular, a slide surface was divided into eight areas and casted with 1 µL each samples, i.e. albumen, yolk, and homogenized whole egg (each in triplicate). One portion of the slide was left as control surface (blank: no matrix), and another one was spread with 1.5 mg mL^{-1} skim milk in HBS-EP and used as negative control. Afterwards, 50 mg of HVPD were applied and processed as indicated above.

The method was then tested on the surface of a contemporary painting, 'Sacred icon', containing some gilded area (where egg albumen was used as glue to stick gold leaves) and some regions realized through the traditional egg tempera technique (whole egg used as binding medium). All the tests were carried out as before, but HVPD aliquots were applied on different regions (1 cm^2 each)

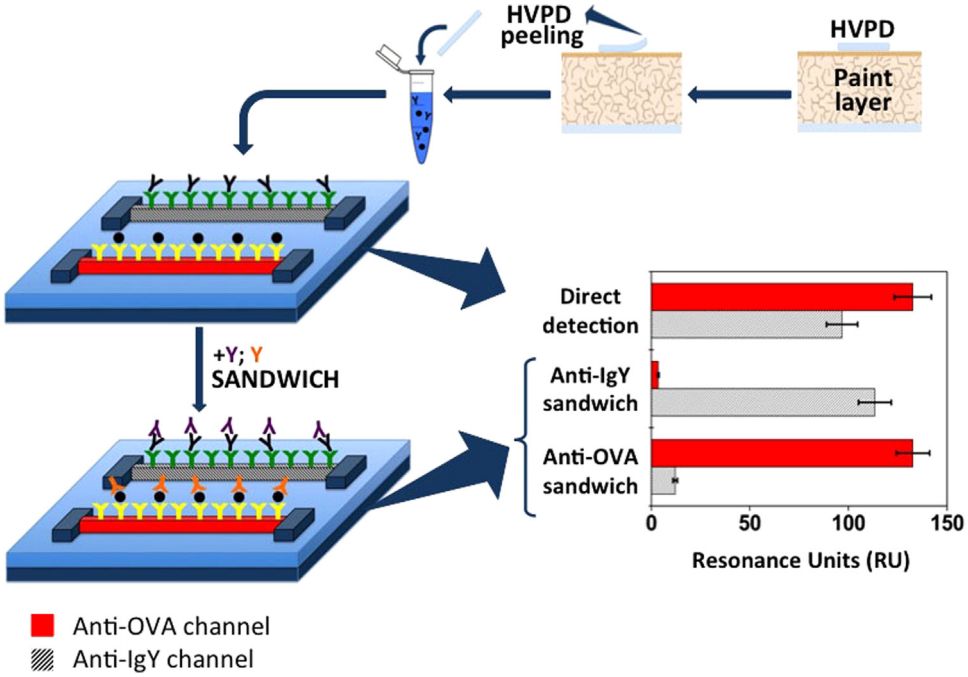

Fig. 1. The HVPD is let adhere on the artwork surface, then peeled off and dipped into a buffer solution to extract the protein material. The surnatant is directly analyzed by SPR giving simultaneous results on OVA and/or IgY presence, eventually amplified by a dedicated sandwich strategy to obtain further information on sample composition. The bar plot shows the simultaneous direct response of standard OVA and IgY on both channels (top), and the specific secondary signals elicited for the two analytes by the sandwich-based assay (bottom).

selected on a gilded area (5 samples) and on a dark brown painted area (5 samples); each area was sampled only once varying the contact time from 1 up to 30 min; after this time the HVPDs were peeled from the surface and processed as before. Proximal surface locations were tested where the surface distribution of the egg's proteins was supposed to be homogeneous. Finally, the HVPD/SPR method was tested on the surface of a XVIII century italian painting, of which we ignored the realization technique, while using as negative control a standard painted surface known not to contain egg proteins.

3. Results and discussion

3.1. Standard ovalbumin and IgY calibration on the dual-based biosensor

A preliminary pre-concentration test to identify the pH values at which each antibody is positively charged was carried out. This is the best condition to obtain an effective receptor immobilization elicited by strong attraction between receptor and negatively charged dextran matrix. The optimal pH values found were 4.5 ((anti-OVA)) and 5.0 ((anti-IgY)), selected for the further immobilizations. To investigate the dynamic response of the two channels by direct assay, a concentration range 0.1–400 mg L^{-1} of both analytes in HBS-EP buffer was assayed, and both displayed a dynamic range extended up to 400 mg L^{-1} (Fig. 2).

SPR signals obtained for IgY (180 kDa) were higher than those elicited by the same dilutions of OVA (45 kDa) but this was expected from the ratios $MW_{analyte}/MW_{ligand}$ sensibly different between the two couples (0.3 for aOVA/OVA, 1.2 for IgY/aIgY). The averaged reproducibilities, expressed as percentage coefficients of variation (CV%) resulted 5% for OVA and 4% for IgY, respectively. In both cases an efficient regeneration with 0.1 M NaOH for 30 s (flow 5 μL min^{-1}) allowed the simultaneous regeneration of both the antibodies and the successful reuse of the biosensor surface for tens of cycles (up to 50) without affecting the antibodies activity (data not shown). Since each analyte was injected on both channels during experiments, the absence of cross-interaction was verified by observing each analyte response on the non specific antibody immobilized on the other channel, i.e. OVA on (anti-IgY) and IgY on (anti-OVA) respectively. In both cases negligible cross binding was observed (Fig. S2), allowing thus also the simultaneous analysis of the two analytes co-present in solution. This was the prerequisite for the application of the system to real matrices. To improve the biosensor performances in terms of signal amplification (by mass enhancement) and to provide a more efficient diagnostic tool in case of protein identification in very complex matrices such as artworks extracts, a sandwich assay was also developed.

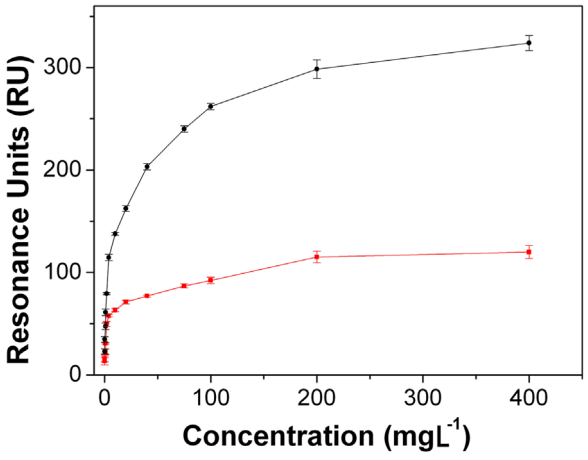

Fig. 2. Dynamic range of standard OVA and IgY on the dual-based biosensor in HBS-EP buffer by direct detection within the concentration range 0.1–400 mg L^{-1}.

3.2. Optimization of the sandwich strategy for albumen/yolk discrimination in fresh egg samples

To evaluate the biosensor response in complex matrices, fresh untreated albumen and yolk were tested after serial dilutions in HBS-EP buffer both by direct and sandwich-based assay. Matrix effects were evaluated by comparing their behaviors with those obtained with standard analytes solutions. The first evidence was that the higher complexity of yolk composition respect to albumen has direct reflection on biosensor behavior, i.e. albumen binds specifically to the (anti-OVA) receptor with negligible binding on (anti-IgY) one, whereas yolk shows comparable binding signals on both receptors (Fig. S4); this indicates some, possibly unspecific, contribution of yolk components other than IgY. When dealing with real samples, the presence of a significant response on both channels could lead to unclear results in the discrimination between pure yolk and albumen/yolk mixtures, compromising the simple and fast simultaneous detection of both matrices, which is one of the aims of the method. To overcome this problem, the sandwich strategy set up on standard analytes was therefore exploited on fresh egg samples. The results clearly showed that the unspecific binding of yolk components on (anti-OVA) channel is not ascribable to IgY binding, since no secondary binding is recorded on this channel (Fig. 3).

On the other hand, the strong enhancement of the SPR signal on IgY channel demonstrates that the sandwich strategy greatly helps to obtain specific and reliable responses on whole untreated complex matrices by-passing pre-treatments. Moreover, the sandwich approach leads to significant improvement of the SPR signal, an useful feature when dealing with low-concentration samples and/or minimum availability of precious samples as in the case of artworks. The effectiveness of this approach was verified also on albumen, and overall results on both matrices are reported in Fig. S5 for serial dilutions of untreated matrices.

3.3. HVPD/SPR combined procedure on simulated surfaces

Once verified the good functioning of the biosensor on standard analytes and fresh diluted yolk and albumen, we moved towards the application of HVPD aliquots to dried layers of yolk,

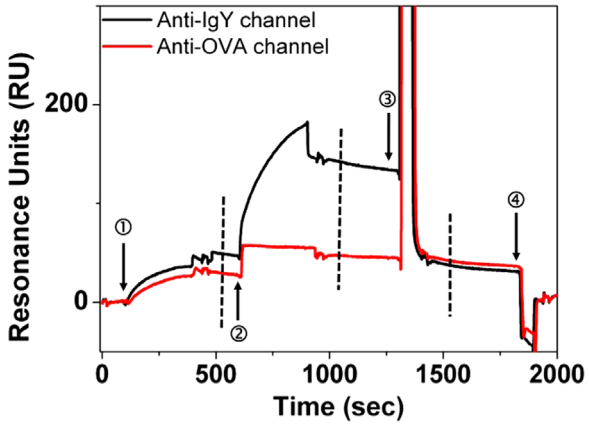

Fig. 3. SPR sensorgrams recorded on the dual channel biosensor for diluted fresh yolk in HBS-EP buffer (point 1) followed by the injection of the secondary (anti-IgY) (point 2) to achieve specific amplification of the SPR signal. SPR signals are recorded in correspondence of dashed lines, at the end of each injection. Points 3 and 4 are relative to the two regeneration steps required for the sandwich strategy.

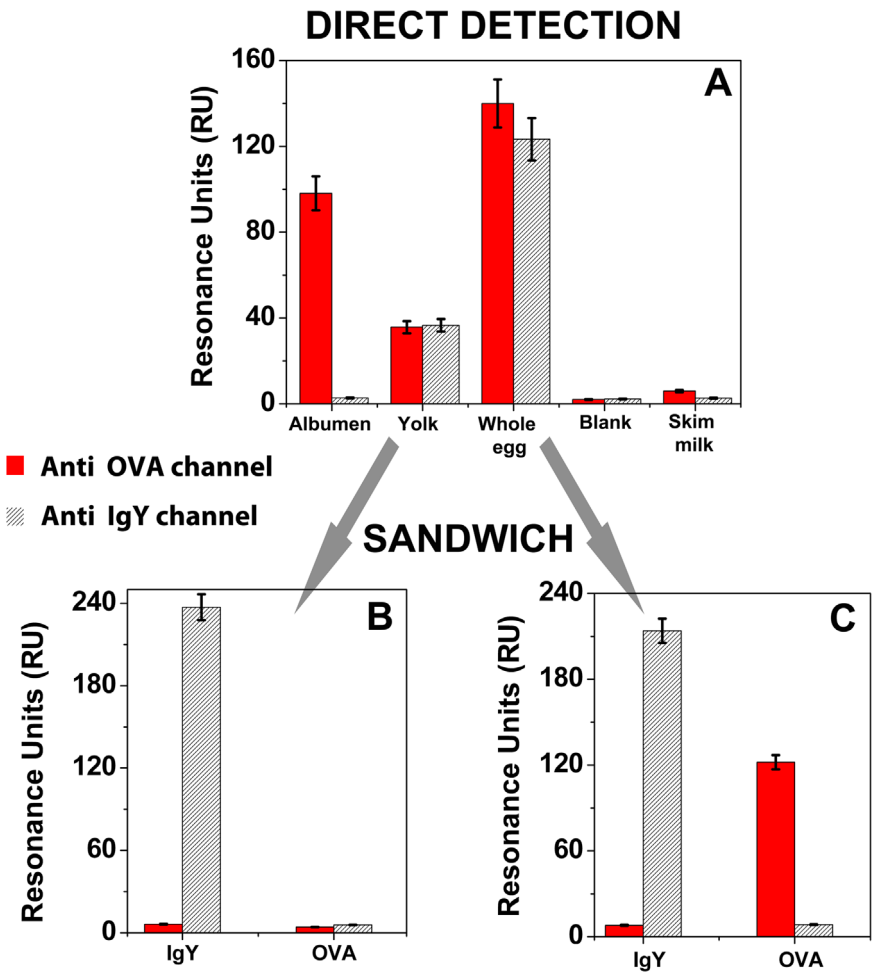

Fig. 4. SPR results obtained by direct detection on simulated samples and processed by the developed method (A). On the bottom, B and C plots report biosensor responses relative to the secondary signals obtained by sandwich to distinguish pure yolk (B) from albumen/yolk mixture (C).

albumen, or homogenized whole egg, following the procedure reported in the experimental section.

As reported in Fig. 4(A), pure albumen showed a high and selective signal (89.1 ± 3.9 RU) towards the relative (anti-OVA) receptor by direct assay, giving no unspecific signal on the (anti-IgY) channel (specific/unspecific ratio 36.33). On the contrary, pure yolk confirmed the presence of an unspecific signal on (anti-OVA) channel (35.7 ± 1.4 RU), comparable to that obtained on the specific one ((anti-IgY) channel, 36.6 ± 1.5 RU), as already evidenced on fresh yolk. Moreover, the direct signal referred to yolk on (anti-IgY) channel seems quite low considering that in this case it was sampled without any dilution of the raw matrix, as previously performed on fresh yolk. By applying the sandwich strategy, also in this case we obtained a clear identification of pure yolk sample (Fig. 4(B)) from albumen/yolk mixture (Fig. 4(C)). These results confirmed the ability of the dual channel biosensor in distinguishing pure matrices (albumen or yolk) from mixtures of them. Moreover, the control surface treated with HVPD and proteins from bovine milk gave both no signals.

The direct assay of albumen/yolk mixture revealed the presence of both matrices (140.0 ± 5.6 RU on (anti-OVA) channel, 123.3 ± 4.9 RU on (anti-IgY) channel), with higher signal intensities with respect to those obtained by cleaning the pure matrices. This can be described as a cooperative extraction effect attributable to albumen in promoting the HVPD extraction of dried yolk. This evidence was further confirmed by a separate experiment in which the HVPD sampling was repeated several times on the same surface (dried albumen or yolk) by applying a new HVPD aliquot each cleaning cycle. The gradual disappearance of the SPR signals over treatments confirmed that albumen layer can be removed at 80% at the first cleaning cycle, whereas yolk requires five cycles to obtain about the same result (Fig. S6). This finding, achieved thanks to the combined approach here reported, represents a further advantage in the perspective of tailoring effective and conservative cleaning treatments for artistic surfaces. In particular, the method demonstrates its usefulness in monitoring the gradual removal of old albumen layer from artwork surfaces, leading to the perspective of innovative methods allowing controlled cleaning of surfaces without damaging the underlying layers, e.g. yolk-based tempera.

3.4. Real artwork samples

The efficacy of this innovative method was finally tested on real works of art: 1) a contemporary “sacred icon”, a wood panel naturally aged for 12 years and characterized by the presence of both gilded and painted areas and, 2) a XVIII century italian painting. Unlikely from the latter, of which no information are available about the technique used, the realization of the “sacred icon” was known and is based on the traditional procedure described in XV century by Cennino Cennini in “Il libro dell’Arte, The book of Art”. In particular, white egg was used as glue to fix the gold leaves to the wood surface, whereas the painted area was realized with the classic egg tempera technique, i.e. pigments were dispersed into a mixture of water and egg yolk that has the function of binder. On its surface, two different series of tests were

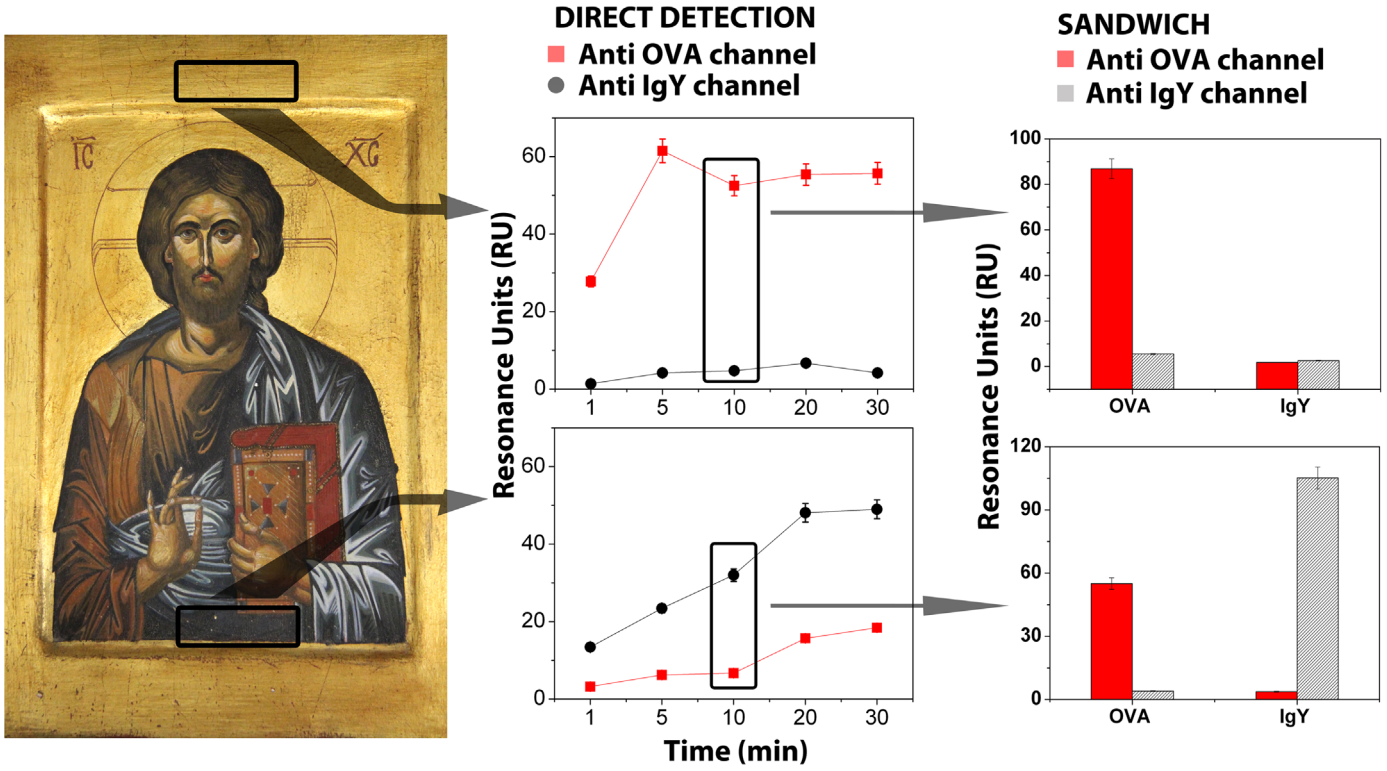

Fig. 5. 'Sacred icon' tested by the optimized HVPD/SPR method (left). On the centre, SPR data relative to HVPD extracts from the gilded (rectangular section on the top) and painted areas (rectangular section on the bottom) obtained by direct detection. On the right, through the sandwich strategy both results were confirmed displaying albumen in gilding and both albumen/yolk in paint.

carried out: five equivalent aliquots of HVPD (50 mg each) were applied both onto the gilded and the painted areas ranging the contact time from 1 to 30 min (1, 5, 10, 20 and 30 min), then processed as usual (see experimental section). The experiment was aimed to evaluate the correlation between the time of contact of HVPD on the surface to be sampled and SPR results, keeping in mind that very short application times are strongly desired to ensure safe and non-invasive treatment of the surfaces. Fig. 5 (left) shows the "sacred icon" with the two open black rectangles indicating the areas on both gilded and painted regions, where the sampling tests were carried out.

As reported by the bar plot relative to direct detection, the presence of albumen on gilding and the presence of both albumen and yolk on painted area were confirmed. Moreover, SPR analysis indicates that already after a contact time of 5 min the total amount of extracted OVA reached an asymptotic value and no further increase of the signal was observed for longer time. On the painted area, the HVPD extraction ability is slower, in accordance with previous observations on dried yolk sampling rate by HVPD. SPR signals of both albumen and yolk increased up to 20 min of contact time. To confirm these results, obtained by direct detection, the sandwich was applied to samples relative to a contact time of 10 min As shown by the bar plots of Fig. 5 (right), the secondary detection clearly confirmed the presence of the sole albumen on gilding, whereas the painted area was characterized by the co-presence of yolk and albumen since the quantitative separation of yolk from albumen requires many delicate steps, so that residues of albumen in yolk-based tempera are possible. On the contrary, the isolation of clean albumen avoiding yolk presence is much more easily achievable, therefore clean SPR signals ascribable only to OVA are reliable.

To finalize our findings, we tested also a XVIII century italian painting, of which we ignored the realization technique. To assess the result, we also analyzed as negative control a standard painted surface not containing egg proteins. The results obtained are reported in Fig. 6 and successfully confirm the presence of egg components in the paint used for its realization, clearly identified as almost pure yolk. The biosensor behavior is in accord with all the previous results, showing a direct signal of yolk with an unspecific signal on the (anti-OVA) channel, solved by the sandwich-based strategy. This last result is consolidated from the absence of any SPR response from the negative control (Fig. S7), allowing to conclude that the sampled surface of the artwork is likely realized by the use of yolk-based tempera.

4. Conclusion

We report for the first time the successful combination of innovative cleaning/sampling methods for cultural heritage conservation to SPR biosensing for the identification of hen egg proteins becoming from artwork surfaces. As proof of principle, whole egg and its components (i.e. albumen and yolk) can be extracted by PVA-based highly viscous polymeric dispersion (HVPD) in a fast (1–30 min) and non-invasive method, allowing the identification of egg biomarkers in label free manner by SPR biosensing. The method is immuno-based and works both by direct and sandwich format, giving the possibility to evaluate for the first time the co-presence of both the matrices in the same sample. Moreover, the method is easily expandable to a higher number of specific receptors on the same biochip, in microarray format i.e. SPR imaging (SPRi), to cover the simultaneous recognition of other matrices of interest. Some work in this direction is ongoing on animal glues, milk derivates, and plant resins. Antibodies can be flanked by synthetic receptors of new generation such as molecularly imprinted polymers (MIPs) (Bossi et al., 2007; Poma et al., 2010; Whitcombe et al., 2011) and aptamers (Blind and Blank, 2015; Sun and Zu, 2015), specifically developed to bind intact epitopes in

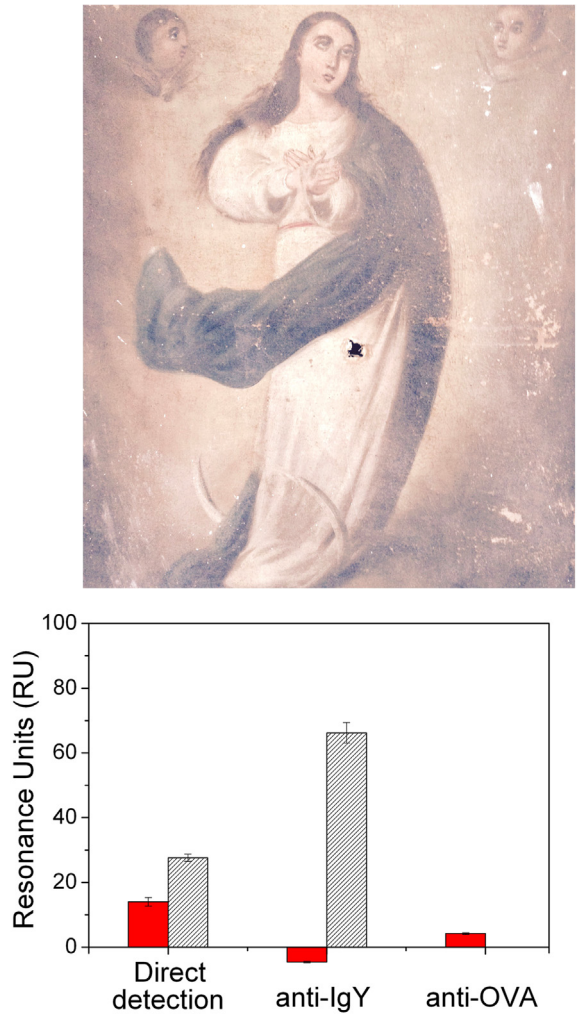

Fig. 6. XVIII century italian painting (top) and SPR data obtained (bottom) after 10 min time contact of HVPD, both relative to direct and sandwich-based signals.

integral and degraded proteins, by tuning the binding recognition towards epitopes resistant to degradation. Furthermore, the method demonstrated its potential in monitoring of the cleaning action, thus giving an unique and extremely informative tool to evaluate the progressive removal of different protein layers from artwork surfaces during restoration. This feature opens the route towards new methods allowing the fine control of the cleaning procedure, with improvement of the quality of this critical step. There is still room to go deeper in quantitative aspects, even if here the focus was the identification of different materials present on artwork surfaces rather than their quantification. This work represents a first step towards new approaches for cultural heritage diagnosis and conservation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.04.093.

References

Angelova, L., Terech, P., Natali, I., Dei, L., Carretti, E., Weiss, R.G., 2011. Cosolvent gel-like materials from partially hydrolyzed poly(vinyl acetate)s and borax. Langmuir 27, 11671–11682.
Baglioni, P., Carretti, E., Chelazzi, D., 2015. Nanomaterials in art conservation. Nat. Nanotechnol. 10, 287–290.
Blind, M., Blank, M., 2015. Aptamer selection technology and recent advances. Mol. TherapyNucleic Acids 4, e223.
Bossi, A., Bonini, F., Turner, A., Piletsky, S., 2007. Molecularly imprinted polymers for the recognition of proteins: the state of the art. Biosens. Bioelectron. 22, 1131–1137.
Bottari, F., Oliveri, P., Ugo, P., 2014. Electrochemical immunosensor based on ensemble of nanoelectrodes for immunoglobulin IgY detection: application to identify hen's egg yolk in tempera paintings. Biosens. Bioelectron. 52, 403–410.
Cartechini, L., Vagnini, M., Palmieri, M., Pitzurra, L., Mello, T., Mazurek, J., Chiari, G., 2010. Immunodetection of proteins in ancient paint media. Acc. Chem. Res. 43, 867–976.
Gambino, M., Cappitelli, F., Cattò, C., Carpen, A., Principi, P., Ghezzi, L., Bonaduce, I., Galano, E., Pucci, P., Birolo, L., Villa, F., Forlani, F., 2013. A simple and reliable methodology to detect egg white in art samples. J. Biosci. 38 (2), 397–408.
Homola, J., 2006. Surface Plasmon Resonance based Sensors. Springer, Berlin.
Mayer, R., 1985. The Artist's Handbook of Materials and Techniques, 4th ed. Viking Penguin Inc, New York.
Mayer, K.M., Hafner, J.H., 2011. Localized surface plasmon resonance sensors. Chem. Rev. 111, 3828–3857.
Marijnissen, R.H., 1985. Paintings Genuine, Fraud, Fake: Modern Methods of Examining Paintings. Elsevier Librico, Brussels, Belgium.
Mazurek, J., Svoboda, M., Maish, J., Kawahara, K., Fukakusa, S., Nakazawa, T., Taniguchi, Y., 2014. Characterization of binding media in egyptian romano portraits using enzyme-linked immunosorbant assay and mass spectrometry. E-Preservation. Science 11, 76–83.
Micheli, L., Mazzuca, C., Palleschi, A., Palleschi, G., 2012. Combining a hydrogel and an electrochemical biosensor to determine the extent of degradation of paper artworks. Anal. Bioanal. Chem. 403, 1485–1489.
Minunni, M., Scarano, S., Mascini, M., 2008. Affinity-based biosensors as promising tools for gene doping detection. Trends Biotechnol. 26, 236–243.
Mounier, A., Dayet, L., Daniel, F., Belin, C., 2011. UV Fluorescence of the binding media used in gilding of mediaeval mural paintings. ArcheoSciences 35, 19–28.
Natali, I., Carretti, E., Angelova, L., Baglioni, P., Weiss, R.G., Dei, L., 2011. Structural and mechanical properties of "peelable" organoaqueous dispersions with partially hydrolyzed poly(vinyl acetate)-borate networks: applications to cleaning painted surfaces. Langmuir 27, 13226–13235.
Nguyen, H., Park, J., Kang, S., Kim, M., 2015. Surface plasmon resonance: a versatile technique for biosensor applications. Sensors 15, 10481–10510.
Osticioli, I., Nevin, A., Anglos, D., Burnstock, A., Cather, S., Becucci, M., Fotakis, K., Castellucci, E., 2008. Micro-Raman and fluorescence spectroscopy for the assessment of the effects of the exposure to light on films of egg white and egg yolk. J. Raman Spectrosc. 39, 307–313.
Poma, A., Turner, A.P., Piletsky, S.A., 2010. Advances in the manufacture of MIP nanoparticles. Trends Biotechnol. 28, 629–637.
Ramírez, J., Santana, M., Galindo-Castro, I., Gonzalez, A., 2005. The role of biotechnology in art preservation. Trends Biotechnol. 23, 584–588.
Scarano, S., Mascini, M., Turner, A.P.F., Minunni, M., 2010. Surface plasmon resonance imaging for affinity-based biosensors. Biosens. Bioelectron. 25, 957–966.
Shankaran, D., Gobi, K., Miura, N., 2007. Recent advancements in surface plasmon resonance immunosensors for detection of small molecules of biomedical, food and environmental interest. Sens. Actuators B: Chem. 121, 158–177.
Sun, H., Zu, Y., 2015. A highlight of recent advances in aptamer technology and its application. Molecules 20, 11959–11980.
Whitcombe, M.J., Chianella, I., Larcombe, L., Piletsky, S.A., Noble, J., Porter, R., Horgan, A., 2011. The rational development of molecularly imprinted polymer-based sensors for protein detection. Chem. Soc. Rev. 40, 1547–1571.
Willets, K.A., Van Duyne, R.P., 2007. Localized surface plasmon resonance spectroscopy and sensing. Annu. Rev. Phys. Chem. 58, 267–297.