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A simplified protocol for usage of new immuno-SERS probes for detection of casein, collagens and ovalbumin in cross-sections of artworks

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Abstract

Although it is now relatively simple to identify protein binders in works of art, their proper localization within the corresponding layer still represents significant analytical challenge. Until now, the identification of proteins has been allowed e.g. by peptide mass fingerprinting using mass spectrometric methods and their localizations have been realized using optical microscopy by application of fluorescent stain Sypro Ruby (SR) on polished cross-sections. In this work we propose a novel and simplified protocol for immuno-surface enhanced Raman scattering (immuno-SERS) using gold nanoparticles attached to biphenyl-4,4'-dithiol (BPDT) as the SERS-nanotag. These in laboratory easily obtainable labeled nanoparticles have been applied on multilayered mock up samples prepared as cross-sections. The layers contain egg, casein, and different animal glues binders (prepared in various ratios with linseed oil or a carbohydrate component) mixed with azurite, vermilion and chalk. On the model samples the sensitivity of the Sypro Ruby has been tested for comparison between the

reported protocol and the current procedure for the first time. Protein detection possibilities of the both methods are shown in this paper.

Introduction

The identification and simultaneous localization of protein binders contained in multi-layered samples taken from artworks is demanded by restorers and art historians. This complex knowledge is essential for the selection of a proper restoration process and for obtaining of more information about the author, provenance, painting workshops, and about development of painting techniques in past.

In the last decade a several methods have been applied on the simultaneous identification and localization of protein binders directly on the cross-sections of artworks. Usually it represents a combination of an analytical method with an optical microscopy. The analytical methods as matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS) [1-4], liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [4-6], and enzyme-linked immunosorbent assay (ELISA) [7-9] is used for identification of the proteins.

For the optical microscopy, it is mostly common to use of fluorescent stain Sypro Ruby [10] to localize the proteinaceous binders. Sypro Ruby is also used in biochemistry for detecting of proteins in gels with nanogram sensitivity [11]. However this stain is not protein specific and when the sample contains more layers with different proteinaceous binders, it is possible to assign the identified protein to the incorrect layer. The single methods enable simultaneously identify and localise proteins in the artworks samples are immuno-fluorescence microscopy (IFM) [12], and immuno-surface-enhanced Raman scattering (immuno-SERS) [13-15].

These two immunological techniques are particularly powerful analytical tools based on the highly specific antigen-antibody reaction. In the case of IFM, detection is achieved using a fluorescence microscope and the excitation of a fluorophore that is conjugated to a secondary antibody, while immuno-SERS is based on enhancement of Raman signal using metallic (e.g. gold, silver, Fe₃O₄@Au, gold/silver (Au@Ag) core-shell) nanoparticles (NPs) [16-19]. The signal may be amplified by many orders of magnitude [21] with enhancements in the range of 10⁸ to 10¹². Nevertheless, this level of SERS-enhancement has not been seen on the biological samples; just only, one or two orders of magnitude are possible [22]. The NPs which are functionalized by nanotags (e.g. rhodamine B, crystal violet, rhodamine 6G, Nile blue, biphenyl-4,4'-dithiol, 1,4-benzenedithiol) [15,22-24] provide Raman signal. Then they

may conjugate with polyclonal secondary antibody through either an amino or a carboxyl functional group [25]. This conjugation selectively reacts with the primary antibodies bounded to the target protein contained in the searched proteinaceous binder. Depending on the nanoparticles size used in immuno-SERS and their nanotags, the SERS spectra are obviously acquired at 785 nm [14-16], but sometimes also at 633 nm [25] while avoiding sample burning. In the resulting Raman spectra the signal of nanotag reports the presence of the prospected protein.

In this work, we present the preparation of the colloidal gold NPs attached to biphenyl-4,4'-dithiol (BPDT) [23] as a Raman active molecule in 785 nm laser activation. The methodology of sample preparation is originally based on the commercial protocol (Oxonica (Mountain View, CA, USA)) used by group of Prof. Arslanoglu [13] for thiol-modified SERS nanotags. The protocol was simplified and consequently tested on mock-up samples containing casein, collagens (animal glues) and egg binders by Raman spectroscopy. In addition, we describe modified and detailed protocol for the application of the prepared NPs on the cross-sections.

Experimental

Reagents and material

Anti-collagen antibody (rabbit polyclonal to collagen; 10 μ l /ml) was purchased from AbCam (Cambridge, UK), rabbit anti-bovine casein was obtained from Abbiotec (San Diego, USA; 1:200 – 1:1000). Anti-chicken ovalbumin antibody (1:1000) and polyclonal anti-rabbit IgG antibody (produced in goat) were purchased from Millipore (Merck Millipore, Darmstadt, Germany). The polyclonal anti-rabbit IgG antibodies were cross-linked with sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Sigma Aldrich). Antibody dilutions were prepared in a 0.1% newborn calf serum (NCS, HyClone, Thermo Fisher Scientific)/1X phosphate buffered saline solution (PBS, diluted from 10X, Fisher BioReagents).

Procedure

Scheme 1 shows the schematic diagram of the procedure. Explanations of each step are as following:

Preparation of gold nanoparticles and mixing with BPDT as a label

Gold nanoparticles with the average size of about 20 nm were prepared according to the procedure described in literature by method of chemical reduction [26-28]. In this method, the 100 ml solution of 0.01% $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Aldrich) was stirred and heated up to boil. Then, 2.5 ml citrate sodium (Merck) 1% was added to the solution, keep boiling for 10 min, until the deep red colour change observed. The solution was left at room temperature in order to cool down gradually. For labelling of NPs by the Raman active tag, the biphenyl-4,4'-dithiol (BPDT, Sigma-Aldrich) was selected. 5 μl of 0.05 M BPDT dissolved in absolute ethanol was carefully added to 2 ml of NPs solution and gently shook. The solution of labelled gold nanoparticles was kept in the refrigerator and it was stable for approximately one month (Figure 1, A)

Characterisation of the gold colloid

Transmission electron microscopy (TEM)

Transmission electron microscopy images were obtained using EFTEM Jeol 2200 FS microscope. The accelerated voltage 200 kV and Schottky cathode with tungsten tip coated with zirconium oxide. Sample was prepared by drop casting of sample suspension on 200 mesh Lacey carbon TEM grid.

Dynamic light scattering measurement

The dynamic light scattering (DLS) was performed using Zetasizer Nano ZS (Malvern, England). The measurement was performed at room temperature (20 °C) using glass cuvette.

UV-Vis

UV-Vis absorption spectra of the gold colloidal solution were obtained with a UV-Unicam 5625 spectrometer. The UV-Vis spectra were used to elucidate the relative size of the particles in the solutions by comparing the location of the maximum peak wavelength in the spectra. The wavenumber range was set between 200 nm to 800 nm. The spectral bandwidth of 2 nm was used to scan.

Pretreatment of the secondary antibody

The SERS-nanotagged secondary antibodies were prepared according to the paper [13]. Briefly, 22 μl of secondary antibody was let to react with 13 μl of sulfo-SMCC for 45 min at room temperature. Sulfo-SMCC acts as a cross-linker reagent to connect the secondary

antibody to the labelled gold nanoparticles. The unreacted cross-linker was removed using Zeba® Desalt Spin columns (Pierce) according to the manufacturer's information – the columns were equilibrated by 300 µl of 50 mM phosphate buffer (pH 7.2) for 3–4 times and for each washing the columns were centrifuged at 1500 RCF for 1 min. The same conditions were applied for the purification of SERS nanotags (Figure 1, B).

Conjugation of labelled gold nanoparticles with secondary antibody

8 µl of the purified activated secondary antibody was added to 4 eppendorfs with 1.5 ml of labelled gold NPs and let to react for 3 hours at room temperature in dark. Consequently, the volume was divided to halves in 8 eppendorfs and 100 µl of blocker casein in PBS (Thermo Fisher Scientific) (it makes the centrifugation smoother and the NPs are not adhered on the eppendorf's walls). Then, 10 µl of 10 µg/ml of sodium 2-mercaptoethanesulfonate (as a stabilizer of NPs in aqueous phase) (MESA, Sigma-Aldrich) was added to each microtube. After 45 min of reaction, the solution was centrifuged at 1000 RFC for 12 min. The excessive amount of a light pink solution above the pellet was taken off to obtain approx. 80 µl of deep purple solution of labelled nanotags. The resulting solution was the SERS nanotag and could be stored in a fridge for one day when they could be used again for next analyses (Figure 1, C).

Application of SERS nanotags on cross-sections

The fragments taken from mock up samples were prepared as cross-sections; they were embedded in polyester resin (Technovit) and polished to see the stratigraphy of colour layers. On the surface of cross-sections, a drop of 40 µl of a solution of 10 mg bovine serum albumin (BSA, Jackson immune research) in Soya milk (Alpro Soya milk, Carrefour) was applied for 30 min at room temperature to block a non-specific places on the colour layers. Then the samples were washed by a freshly prepared solution containing 15 mg BSA in 1.5 ml of TRIS-HCl. For washing step, it was better to apply a drop of the solution on the cross-section and carefully wiped using paper towel without the direct contact between the sample and paper. The washing procedure was repeated for two or three times to not to see the white film of milk on the surface. On the still wet cross-section, 40 µl of the primary antibodies diluted by solution of 5% NCS in 1X PBS (anti-casein 1:200 or 1:1000, anti-collagen 10 µg/1 ml, anti-ovalbumin 1:1000) was added on the surface of the polished sample. The antibodies were incubated with proteins in the samples at 37 °C for 3 hours in a small glass bowl containing wet cellulose pad as prevention against desiccation. Then, the drops were wiped

by the paper towels and the samples were washed by 1X PBS (similarly to washing with TRIS-HCl) for two times. Consequently, 10 µl of the SERS nanotags was applied on every sample and let to incubate at 37 °C for one hour. Finally the solutions were washed with 1X PBS several times and dried using paper towels (at least for four times) till no seeing the purple edges of the drops on the cross-sections and let dry before Raman spectroscopy measurements (Figure 1, D).

Fluorescent staining by Sypro Ruby

10 µl of Sypro Ruby Molecular Probes (Eugene, OR, USA) was applied on the surface of polished cross-sections for 30–60 s and then were gently dried by paper tissue. The samples were let to react for 5 min in dark and consequently the optical analyses were performed with a Zeiss Axioplan 2 polarizing microscope with an AxioCam MRc5 camera attached to an AxioVision Digital Asset Management System at 50x magnification using green filter. The positive reaction with proteins is indicated by orange/reddish colour.

Paint samples and cross-sections preparation

The mock-up samples were prepared using three pigments (azurite – $\text{Cu}_3(\text{CO}_3)_2(\text{OH})_2$ (Kremer Pigmente), chalk – CaCO_3 (Janssen Chimica, Belgium), vermilion – HgS (s.a. UCB N.V., Belgium) and six types of binders containing proteins (casein binder, four different animal glues – beef glue, fish glue, rabbit glue, gelatine, whole egg and egg yolk binder, egg white binder and oil binder with a traces of proteins – all animal glues and egg white). The binders were prepared according the recipes in [29] and then in different ratios between proteins and other organic components of the binders. All together 65 layers were prepared on wooden plates; on one plate 5 to 6 layers were stacked and every layer was isolated using 10% solution of Paraloid B72 (Rohm+Haas/Dow) in ethanol. The content of proteins in the final and dry colour layers are shown in Tables I–VII.

Preparation of casein binder

10 ml (5.2 g) of casein (Royal Institute for Cultural Heritage – KIK/IRPA storage) was dissolved in 25 ml of water with an addition of 1.37 g of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, Belgolabo) in 20 ml of water. Consequently, 5 ml of polymerised linseed oil (BLOCKX, Belgium) and other 10 ml of water were added. This binder prepared in this way was considered as a binder with ratio 2:1. In the others casein binders the ratios has changed to 1:1, 1:5 and 1:10 according the ratio between casein:oil (v/v).

Animal glues

The animal glues (3.03 g of beef glue (KIK/IRPA storage), 3.08 g of fish glue (Bordeace, F. Gillet), 3.00 g of rabbit glue (KIK/IRPA storage), were dissolved in 50 ml of hot water. Gelatine (Totin) in the weight of 3.03 g was dissolved in 80 ml of hot water.

Whole egg binder

The binder was prepared by mixing of whole egg together with polymerised linseed oil in following ratios 3:1, 2:1, 1:1, and 1:2 (v/v).

Egg yolk binder

3 parts of separated egg yolk taken using syringe without any traces of egg white were mixed with 1.5 part of linseed oil and 1 part of water – ratio 2:1 (v/v), yolk:oil). The other yolk binders were prepared in following ratios: 1.5:1, 1:1, and 1:2.

Egg white binder

8 ml (8.00 g) of beaten egg white (Carrefour) was mixed with 5 ml (6.43 g) of honey (Carrefour) and 1 ml (1.07 g) of solution of Arabic gum (15.66 g of Arabic gum in 40 ml of water) (Gummi arabicum, Carl Roth). This binder was prepared in ratio 8:1, egg white:Arabic gum (v/v). The other egg white binders were prepared in ratios: 4:1, 1:1, and 1:10.

Oil binder

This mixture was specially prepared with azurite. Firstly, azurite (0.58–0.94 g) was mixed together with 0.09–0.14 g) of 6% solutions of animal glues, 3.8% gelatine or beaten egg white and let to dry. Then, the azurite coated by proteins was mixed with linseed polymerised oil in approx. ratio 1:1 (m/m).

Instrumentation

The SERS spectra were collected with a Raman Renishaw inVia multiple laser dispersive Raman spectrometer with a Peltier-cooled (203 K), near-infrared enhanced, deep depletion CCD detector (576 × 384 pixels) using a high power diode laser (Toptica Photonics XTRA, Graefelfing (Munich), Germany) operating at 785 nm in combination with a 1200 l/mm grating. The samples were analysed using the 50x objective in a direct-coupled Leica DMLM microscope with enclosure and over spectrum range 400–1800 cm⁻¹ at a 1 cm⁻¹ resolution. Integration times of 30 seconds, with laser power 5 %, and 2 accumulations were employed.

At least three points from every studied layer were measured. The points were selected on the basis of the morphology of the surface – the highest places (on the same level with the embedding resin) of the layers were preferred, because it meant that the material was not washed out sometime during the sample preparation. Spectra were acquired using the Wire 2 Raman software and were subsequently baseline corrected when necessary.

Results and discussion

At first the gold colloid of labelled nanoparticles was characterised by UV-Vis spectrophotometry and transmission electron microscopy (TEM). The maximum absorbance wavelength in the UV-VIS spectrum (Figure 2) is 520 nm. Figure 3 represents the TEM image for the synthesized and conjugated gold nanoparticles with BPDT. As can be seen in Figure 3, the size distribution of nanoparticles is approximately 20 nm and they are monodispersed. This was also confirmed by the measurement of dynamic light scattering giving the particle size distribution with maxima in the range of 20-25 nm and relatively narrow distribution (Figure 4).

The selection of Raman active tag for binding to metallic nanoparticles is subjected to several main criteria. One is to obtain enough strong signals in resulting spectra and the next one is the distinct signals from most of inorganic pigments that could be found in the artwork samples. As it is shown in the spectra (Fig. 5), the BPDT signals could be substituted by azurite peaks because of their similarity. However, the characteristic peaks values 1080 cm^{-1} , 1280 cm^{-1} , and 1080 cm^{-1} can confirm the presence of AuNPs with BPDT, and thus they can help to avoid false negative response that can be mostly caused by improper final washing.

The BPDT attached to AuNPs provides significant peaks at 1585 cm^{-1} , 1080 cm^{-1} , 1280 cm^{-1} , 1010 cm^{-1} , 1200 cm^{-1} , 545 cm^{-1} , and 407 cm^{-1} in Raman spectra (Fig. 5). These signals were used for confirmation of presence of searched protein (casein, ovalbumin, and collagen) in the mock up sample using the specific reaction between primary and secondary antibodies that were conjugated to the labelled AuNPs. All the immuno-SERS results in Tables I–VII were obtained after at least three point measurements in a layer. In the case of positive response, to ensure that it is not false positive response, the layer that did not contain the searched protein was examined (also for at least 3 times).

In this research, the detection possibilities of immuno-SERS on mock up samples containing mixtures of proteins with mostly oil or saccharides and three inorganic pigments (azurite, chalk, vermilion) were tested. The content of proteins and oil, respectively their ratios, are shown in Tables I–VII. The content of organic components was calculated as they are

represented in dry mixtures with pigments and on the base of their content in the used proteinaceous materials. It was anticipated that casein and collagen materials contain 100 %, liquid egg white contains 10 %, egg yolk 16 %, and whole egg contains 12.5 % of proteins.

Casein tempera

For the detection of casein in its mixtures with pigments, the anti-casein primary antibodies were used. The results for application of immuno-SERS and Sypro Ruby on casein tempera are shown in Table I. Immuno-SERS confirmed the presence of casein in all samples mixed with chalk (CaCO_3) (up to 1/77, content of proteins/oil in dry layers expressed in %) and in vermilion (HgS) up to (2/31). In case of copper based pigment azurite ($\text{Cu}_3(\text{CO}_3)_2(\text{OH})_2$), the detection limit decreased to 6/11.

Sypro Ruby showed even lower sensitivity than SERS, because it detected casein with azurite at only 7/6. The explanation of the lower sensitivity of the stain could be in the presence of copper cations that bind in protein structures and the higher content of negatively charged phosphate groups in casein, because SR noncovalently interacts with basic amino acid residues [30].

Egg white binder

The results for application of immuno-SERS and Sypro Ruby on egg white binder are shown in Table II. Immuno-SERS exhibited its high sensitivity for egg white, respectively for ovalbumin because of usage of anti-ovalbumin antibody. The only exception where immuno-SERS was not successful was egg white mixed with azurite containing 1 % of proteins. The first mixture of egg white and azurite that was positively detected contains 3 % of egg proteins. However, egg white contains approximately 54 % of ovalbumin, thus the detection limit decreases to about 1.5 % of ovalbumin.

Sypro Ruby detected proteins in all samples (Fig. 6) and only weak influence of azurite (similarly to vermilion) that manifests by lower intensity of SR was observed.

Egg yolk tempera

The results for application of immuno-SERS (anti-ovalbumin antibody) and Sypro Ruby on egg yolk tempera are shown in Table III. Egg yolk tempera could not be detected by SERS because it does not contain ovalbumin that occurs only in egg white. These layers served as a reference layers to avoid false positive responses.

Positive reaction of SR was not observed in layers containing chalk where the orange fluorescence was overlapped by green autofluorescence (Fig. 6) and azurite. In the case of layers with azurite, the negative observations were caused not by the high content of oil component (e.g. 37 %), but by the copper interactions with proteins. As we can see in Table I, the high amount of oil did not represent significant limitation for the stain. SR was able to detect caseins in layers containing chalk with much higher content of lipids (e.g. 65 %).

Whole egg tempera

Within these results in Table IV is clearly seen the decrease of immuno-SERS ability to detect ovalbumin (after using anti-ovalbumin antibody) in the all mock up samples of egg tempera. Surprisingly, the protein binder was detected in all samples with vermilion, but in samples containing chalk up to 9/37 and in azurite layer up to 6/20. In comparison to egg white binder (Table II) that does not contain oil component, the immuno-SERS sensitivity significantly decreases.

SR was not so influenced by the presence of oil (oil coming from linseed oil addition and lipids from egg yolk) and it found proteins in all mock up samples with chalk and vermilion but only in one sample with azurite (6/20). The presence of azurite has more significant influence on protein detection than the oil.

Animal glues

The results for beef glue and rabbit glue are shown in Table V, and for fish glue and gelatine in Table IV. Primary antibody against collagens did not react with fish glue and then only with beef glue when it was mixed together with azurite (4/0). Unlike to the fish glue, the mammal's collagens contain suitable epitopes (antigenic determinants) for the used anti-collagen antibody. From this result implies the importance of selection of the appropriately anti-body.

Sypro Ruby localised collagens in all samples with the exception of gelatine with azurite that was presented in the lowest concentration (3/0) of the all used animal glues.

Oil binder

The most challenging mixtures for immuno-SERS and SR were the mock up samples containing azurite together with linseed oil and only the traces of proteins (Table VII). The

content of proteins in dried layers was in the tenths to 1 % and the content of oil was up 44 to 55 %. SERS did not localize any of the animal glues (beef glue, fish glue, gelatine, rabbit glue) by usage of anti-collagen antibody or egg white by anti-ovalbumin antibody. The reasons why the analyses were not successful could be the following: a) too low concentration of proteins, b) too high concentration of linseed oil, c) proteins complexed with copper ions.

Sypro Ruby showed highest sensitivity (similarly to the analyses of samples containing egg white binder in Table II) to egg white. The layer exhibited weak orange coloration around the azurite grains. The other samples containing collagens provided negative response. On the other hand, sample with egg white contained approximately two times higher concentration of proteins (1/52, protein/oil) in comparison to the animal glues (beef glue: 0.6/50, fish glue: 0.5/46, gelatine: 0.2/44, rabbit glue: 0.6/55). The additional reason could be the presence of glycoproteins in egg white to which SR preferably noncovalently binds [30].

Conclusions

In this work we prepared new AuNPs labelled with BPDT and conjugated with polyclonal anti-rabbit IgG antibody. After finding the suitable conditions for protein binders detection and their localization in multi-layered mock up samples, the simplified protocol for their application was created.

The labelled AuNPs were applied on 65 mock up samples containing egg proteins, casein and different collagen (beef glue, fish glue, rabbit glue, gelatine) binders prepared in different ratios of proteins and oil or proteins and saccharides mixed together with azurite, vermilion and chalk.

As a pigment that complicates the identification/localization of proteins is azurite that also mostly causes problems with protein identification by other analytical methods like GC-MS and MALDI-TOF MS [31,32]. In almost all cases of samples where azurite was mixed with low amount of proteins and with majority of oil binder, the proteins were not detectable when they were presented only in traces to 1 % (m/m). Moreover, it was proved that the presence of oil complicates the analyses; probably because of hydrophobic protection of the analysed layers.

The research showed the selection of the primary antibodies was very critical for the correct identification of the protein binders, because the anti-collagen antibody did not interact with collagen from fish glue having dissimilar amino acid sequence than the mammal's collagens.

The advantages of immuno-SERS the high selectivity and the possibility of localization of protein binders in correct layers in compare with fluorescent stain Sypro Ruby protocol. However, the main challenge of this technique is loosing of samples that prohibits reuse them for other analyses and moreover, only one type of protein can be searched in any experiment, because no mixture of primary antibodies can be used simultaneously.

Although SR is not a specific indicator of particular proteins, it is an appropriate complementary method of SERS; because it generally shows greater sensitivity to protein in the mock up samples. Nevertheless even this method faces a number of problems, e.g. a) azurite makes the localization of proteins more difficult than vermilion and chalk; b) The nonspecific fluorescent stain cannot detect collagens in mixtures with oil binder and azurite and also casein binder. It shows lover sensitivity for SR probably because of the higher content of phosphate groups in casein structure; c) The next problem is green fluorescence of chalk layers containing egg yolk that disables to observe the stain reaction.

In general, immuno-SERS is a promising analytical method that enables simultaneous identification and localization of proteinaceous binders in multi-layered systems prepared as cross-sections. The additional advantages of this technique are the availability of Raman spectrometer in the chemical laboratories, low running costs and the high sensitivity.

Conflicts of interest

There are no conflicts of interest to declare.

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Figures

Figure 1. Schematic process of NPs preparation and their application on samples. A - labelling of the NPs with a Raman active tag (biphenyl-4,4'-dithiol, BPDT), B - cross-linking of secondary antibody with sulfo-SMCC, C - conjugation of labelled NPs with cross-linked secondary antibody, D - application of SERS nanotags on cross-sections and measurement of Raman spectra.

Figure 2. UV-Vis spectrum of gold nanoparticles colloid with the absorbance maximum at 520 nm.

Figure 3. Transmission electron microscopy of gold nanoparticles labelled with BPDT. The size of nanoparticles is approximately 20 nm and the nanoparticles are monodispersed.

Figure 4. The particle sized distribution measured by dynamic light scattering.

Figure 5. Comparison of Raman spectra of AuNPs attached to BPDT, positive response (confirmed presence of BPDT signals – blue stripes) of sample containing azurite and rabbit glue and negative response of sample containing azurite and rabbit glue.

Figure 6. Protein localization by fluorescent stain Sypro Ruby. In sample B are visible the most intensive orange layers containing proteins. In sample D the green fluorescence of layer containing chalk and egg yolk is dominant and it not to allow to observe the positive reaction in other layers.

Tables and captions

Table I. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing casein binder mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Casein binder | Azurite | | | Vermilion | | | Chalk | | |
|---------------|-----------------|------|----|-----------------|------|-----|-----------------|------|-----|
| | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR |
| 2:1 | 7/6 | + | + | 8/7 | + | + | 13/11 | + | +++ |
| 1:1 | 6/11 | + | - | 11/22 | + | +++ | 12/22 | + | + |
| 1:5 | 2/33 | - | - | 2/31 | + | + | 4/65 | + | + |
| 1:10 | 1/48 | - | - | 1/41 | - | + | 1/77 | + | + |

Table II. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing egg white binder mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Egg white binder | Azurite | | | Vermilion | | | Chalk | | |
|------------------|-----------------|------|----|-----------------|------|----|-----------------|------|-----|
| | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR |
| 8:1 | 2/0 | + | - | 3/0 | + | + | 4/0 | + | ++ |
| 4:1 | 3/0 | + | + | 3/0 | + | + | 5/0 | + | +++ |
| 1:1 | 1/0 | + | + | 2/0 | + | + | 3/0 | + | +++ |
| 1:10 | 0.4/0 | - | + | 0.4/0 | + | + | 0.6/0 | + | + |

Table III. Results of application of Sypro Ruby on mock up samples containing yolk binder mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Yolk binder | Azurite | | Vermilion | | Chalk | |
|--------------|-----------------|----|-----------------|-----|-----------------|----|
| | Protein:Oil (%) | SR | Protein:Oil (%) | SR | Protein:Oil (%) | SR |
| 2:1 | 4/23 | - | 4/18 | +++ | 6/32 | - |
| 1.5:1 | 3/19 | - | 3/20 | +++ | 5/36 | - |
| 1:1 | 3/28 | - | 3/29 | ++ | 5/47 | - |
| 1:2 | 3/37 | - | 2/30 | + | 3/45 | - |

Table IV. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing whole egg binder mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Whole egg binder | Azurite | | | Vermilion | | | Chalk | | |
|------------------|-----------------|------|----|-----------------|------|-----|-----------------|------|-----|
| | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR | Protein:Oil (%) | SERS | SR |
| 3:1 | 6/20 | + | + | 5/18 | + | +++ | 8/29 | + | ++ |
| 2:1 | 6/25 | - | - | 4/18 | + | ++ | 9/37 | + | +++ |
| 1:1 | 6/35 | - | - | 6/36 | + | +++ | 12/59 | - | ++ |
| 1:2 | 6/51 | - | - | 3/36 | + | ++ | 11/69 | - | ++ |

Table V. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing rabbit and beef glue mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Binder | Rabbit glue | | | Beef glue | | |
|------------------------|--------------------|------------------|--------------|------------------|------------------|--------------|
| Pigment | Azurite | Vermilion | Chalk | Azurite | Vermilion | Chalk |
| Protein:Oil (%) | 9/0 | 3/0 | 6/0 | 4/0 | 2/0 | 8/0 |
| SERS | + | + | + | - | + | + |
| SR | + | + | +++ | + | + | +++ |

Table VI. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing fish glue and gelatine mixed together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Binder | Fish glue | | | Gelatine | | |
|------------------------|------------------|------------------|--------------|-----------------|------------------|--------------|
| Pigment | Azurite | Vermilion | Chalk | Azurite | Vermilion | Chalk |
| Protein:Oil (%) | 5/0 | 2/0 | 7/0 | 3/0 | 2/0 | 3/0 |
| SERS | - | - | - | + | + | + |
| SR | + | + | + | - | + | +++ |

Table VII. Results of application of immuno-SERS and Sypro Ruby on mock up samples containing traces of beef glue, fish glue, gelatine, rabbit glue and egg white in linseed oil together with azurite, chalk and vermilion. The intensity of SR fluorescence: - none, + weak, ++ middle, +++ strong.

| Binder | Beef glue | Fish glue | Gelatine | Rabbit glue | Egg white |
|------------------------|------------------|------------------|-----------------|--------------------|------------------|
| Protein:Oil (%) | 0.6/50 | 0.5/46 | 0.2/44 | 0.6/55 | 1.0/52 |
| SERS | - | - | - | - | - |
| SR | - | - | - | - | + |