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ELECTRONIC SUPPLEMENTARY INFORMATION

Circularly polarized luminescence reveals interaction between commercial stains and protein matrices used in paintings

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Materials and Methods

Materials

Proteins Three different proteinaceous painting media were selected for this study: dried egg white from chicken (Sigma Aldrich, for egg), casein powder (Zecchi, from cow milk) and rabbit skin glue (Zecchi, for animal tissue). Ovalbumin from chicken (OVA) was from Sigma Aldrich (purity > 97%). The materials were added to buffer solutions and sonicated in ultrasonic bath until total solubilization of the sample. The molar concentration of the protein will be indicated as C_P.

Fluorescent tags Sypro Ruby was the protein blot stain by Molecular Probes. Flamingo was purchased as a solution in 35-50% ethanol, 2.5-5%, methanol, 0.01-0.1% dimethyl sulfoxide and 35-50% water mixture (BioRad). For these two dyes the molar concentration of the dyes was evaluated using the molar absorption coefficients ε (463 nm) = 19×10^3 $M^{-1}cm^{-1}$ for Sypro Ruby and ε (508 nm) = $1 \bullet 10^5$ $M^{-1}cm^{-1}$ for Flamingo. AMCA (succinimydyl-7-amino-4-methylcoumarin-3-acetic acid) and FITC (fluorescein isothiocyanate) were purchased from Sigma-Aldrich as powders; AMCA-X (6-((7-Amino-4-methylcoumarin-3-acetyl)amino) hexanoic acid, N-succinimidyl ester) solid was purchased from Adipogen life Sciences. Working solutions of the above dyes were obtained by dissolving weighted amount of the solid in DMSO and kept in the dark at 4°C. The molar concentration of the dyes will be indicated as C_D.

Buffers Experiments were carried out at pH 7.2 in 50 mM Tris-HCl, 0.28 M NaCl (buffer 1) or 0.1 M Na_2CO_3 pH 9.0 (buffer 2). Solutions were prepared with milliQ water.

Embedding resin Hardrock 554 epoxy resin and hardner were purchased from Tecnocontrol, Italy.

Methods

Cross sections and OM observation

After curing of the epoxy resin the blocks were cut and polished to reveal the casein and polysaccharide gum layer in cross section. The cross sections were dry polished with successively finer grades of micromesh abrasive cloths (600, 800 and 1200 mesh). Felt was used for the final

polishing. The cross-sections were observed at different magnifications (from 5× to 20×) using an BX51M imaging binocular microscope and the images were acquired using a Olympus XC30 digital color camera, coupled to the microscope (provided with a mercury lamp USH-1030OL).

The staining test on cross-sections was carried out depositing few drops of the stain solution on the embedded protein and leaving it in the dark for 10 min. The cross-section was then washed with a solvent (hexane for SYPRO Ruby, Flamingo and DMSO for FITC, NHS-AMCA and NHS-AMCA-X) 3 times.

Fluorescence experiments

Protein solubilization was carried out using an Sonorex super 10 P (Bandelin) ultrasonic bath, by applying to suitable proteinaceous materials sample one cycle of 60' at 25°C.

Measurements of pH were made by a Metrohm 713 pH-meter equipped with a combined glass electrode.

Fluorescence measurements were carried out on a PerkinElmer LS55 spectrofluorometer. Fluorescence titrations were performed at λ_{exc} = 490 nm (Flamingo), λ_{exc} = 470 nm (Sypro), λ_{exc}=450 nm (FITC) and λ_{exc} = 350 nm (AMCA and AMCA-X). The titrations were done using a batch wise procedure: several samples were prepared containing the same amount of fluorescent dye (0.28 μM Flamingo, 6.4 μM Sypro Ruby, 0.55 μM FITC, 0.38 μM AMCA and 0.25 μM AMCA-X) and increasing amounts of protein.

Circularly polarized luminescence (CPL) experiments

All the circularly polarized luminescence (CPL) measurements were performed using our home-built CPL spectrofluoropolarimeter (see below for a brief description). The measurement were carried out on clear solutions containing 6.4 μM Sypro Ruby or 0.55 μM FITC and 245 μM protein concentration. The solutions were clear and both the proteins and the stains completed dissolved.

All the spectra were run at least two times each on freshly prepared solutions.

All the samples were excited using a 90° geometry. In order to avoid artifacts to linear anisotropies, the excitation light was linearly polarized along the direction of the detection direction. Moreover, in the case of Sypro Ruby, the proteins in the matrices have a mass from 20-40 KDa (CAS[S1] and EW[S2]) to 100 KDa (RSG[S3]) corresponding to a tumbling rate ranging from 10-20 nsec to 50 nsec (RSG, where CPL signals are never observed). Excited state life-time of stain **1** is around 500 nsec in water,[S4] so after being excited, the molecules have enough the time to randomly re-orientate

themselves losing excitation photoselection (if any). Artifacts due to overlap between CPL and ECD bands cannot occur since there are no ECD bands in the emission region (Fig. S11 and S12). Samples containing Sypro Ruby were excited using a blue LED source (λ_{exc}=470 nm) and are the average of 8 accumulations each. Samples containing FITC were excited using a UV fluorescent lamp (λ_{exc}=365 nm) and are the average of 4 accumulations each. The spectra were not corrected for the baseline.

Instrument description The instrument was built using the chassis, photoelastic modulator (PEM), lock-in amplifier and photomultiplier tube of a decommissioned Jasco J-500 C spectropolarimeter. The PEM, followed by a linear polarizer and a focusing lens, is placed in front of the sample holder. The light then passes through a Jasco CT-10 monochromator (1200 grooves/mm blazed at 500 nm) driven by a step motor controlled by an electronic prototyping platform. The same platform digitalizes the analog output signals (both direct and alternate current signals). The spectra are plotted in real time in an Excel spreadsheet. For a scheme of the set-up see ref [S5].

Fluorescence data treatment

As for data treatment, the model used is based on the (reasonable) assumption that, under the diluted dye conditions used, the intensity of the emitted light is proportional to the molar concentration, i.e. relationships analogous to the Beer's law do apply. In the fluorescence titrations we measure the light emission changes upon the addition of proteins. The association of fluorescent tags/dyes (D) with proteins (P) may be represented by the following equation:

$$P + D \leftrightarrows PD$$

where the related association constant is defined as $K_b = [PD]/([P]\times[D])$. The association constants were determined by the fluorescence spectral changes using the equation (S.9) that is derived as described below.

$$K_b = \frac{[PD]}{[P][D]} \quad (S.1)$$

$$C_P = [P]+[PD] \quad (S.2)$$

$$C_D = [D]+[PD] \quad (S.3)$$

where C_P and C_D are total analytical concentrations. If D and PD only emit light, then:

$F = F_D + F_{PD} = \varphi_D [D] + \varphi_{PD} [PD]$ (S.4)

If we now define

$$\Delta F = F - \varphi_D C_D \quad \text{(S.5)}$$

$$\Delta\varphi = \varphi_{PD} - \varphi_D \quad \text{(S.6)}$$

equation (S.4) becomes

$\Delta F = \Delta\varphi[PD]$ (S.7)

Introduction of equations (S.2) and (S.3) into (S.1) yields

$$K_b = \frac{[PD]}{(C_P - [PD])(C_D - [PD])} \quad \text{(S.8)}$$

Substituting [PD] obtained from equation (S.7) into equation (S.8) and rearranging, one obtains

$$\frac{C_P C_D}{\Delta F} + \frac{\Delta F}{\Delta\varphi^2} = \frac{1}{K_b \Delta\varphi} + \frac{C_P + C_D}{\Delta\varphi} \quad \text{(S.9)}$$

Such equation enables K_b and $\Delta\varphi$ to be obtained by an iterative procedure. That is, disregarding the $\Delta F/\Delta\varphi^2$ term on first approximation, $\Delta\varphi$ can be calculated from the reciprocal of the slope of the straight line interpolating the data of the $C_P C_D/\Delta F$ vs. (C_P+C_D). Then, introduction of this $\Delta\varphi$ value into equation (S.9) enables the $(C_P C_D/\Delta F + \Delta F/\Delta\varphi^2)$ term to be evaluated and new values of K_b and $\Delta\varphi$ to be obtained. The procedure is repeated until convergence is reached. Equation (S.9) is a modification of the Hildebrand and Benesi Equation[S6] and is valid also if the conditions of protein excess are not fulfilled.

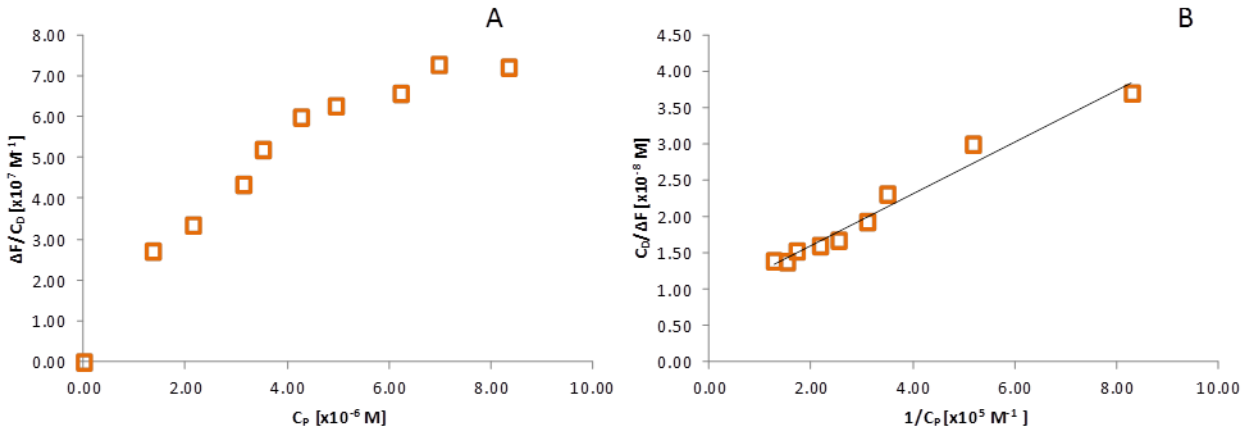

Figure S1: Fluorescence titrations of Sypro Ruby with OVA (A) and relevant analysis according to equation (S.9) of the text (B). $C_D = 6.4\times10^{-7}$, $\lambda_{ex} = 470$ nm, $\lambda_{em} = 603$ nm; T = 298 K, pH = 7.2. $K_b = 2.5\times10^5\ M^{-1}$.

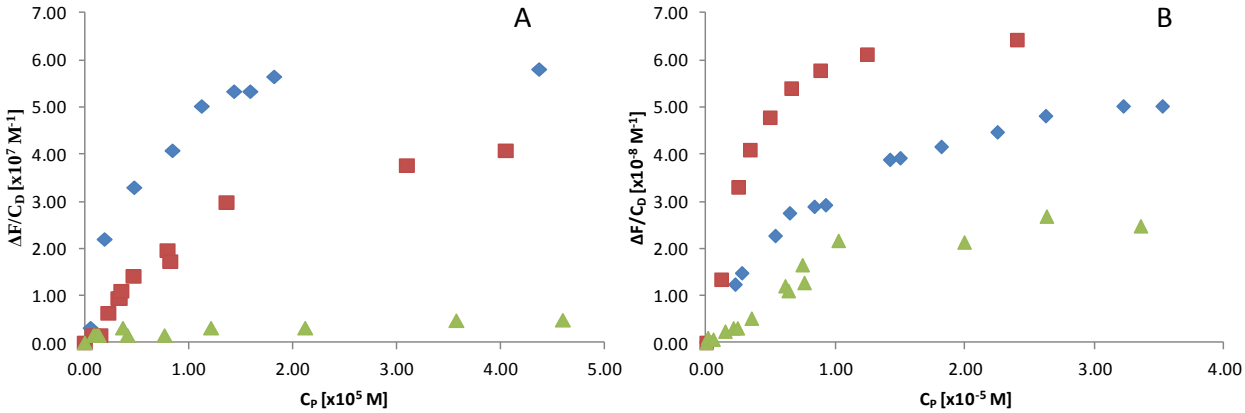

Figure S2: Fluorescence titrations of Sypro Ruby (A) and Flamingo (B) with casein (blue diamonds), egg white (red squares) and rabbit skin glue (green triangles), T = 298 K, pH = 7.2. (A) $C_1 = 6.37\text{x}10^{-7}$ $\lambda_{ex} = 470$nm, $\lambda_{em} = 603$nm; (B) $C_2 = 2.91\text{x}10^{-7}$ $\lambda_{ex} = 490$nm, $\lambda_{em} = 535$nm.

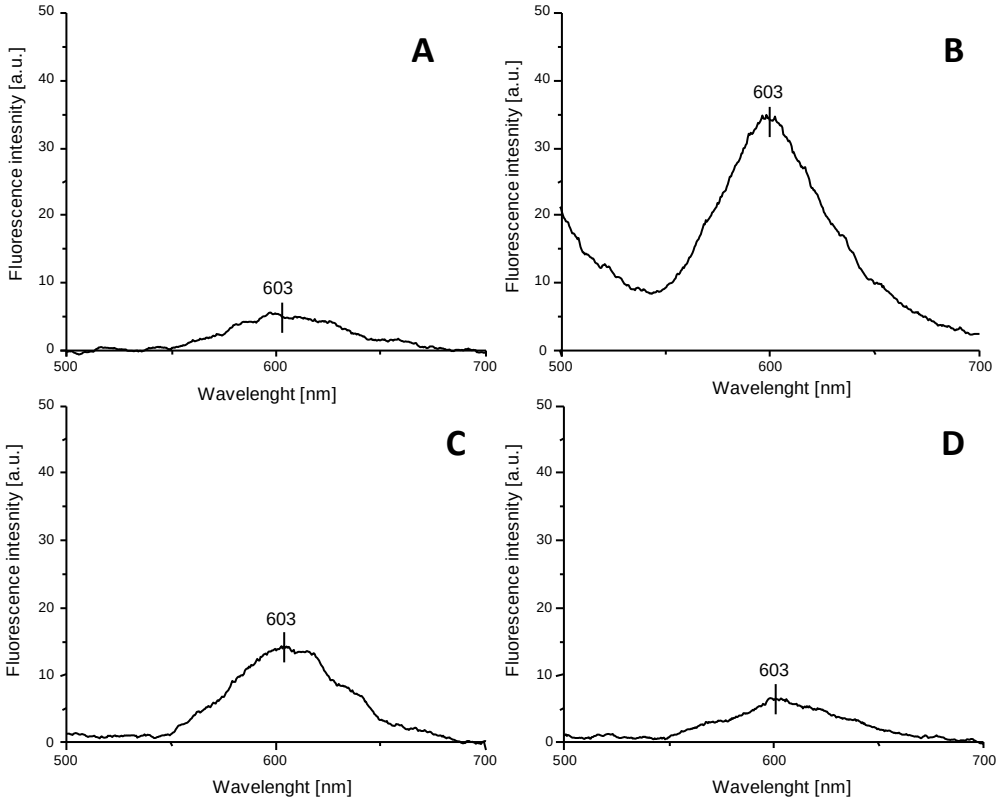

Figura S3: Fluorescence spectra of Sypro Ruby alone (A), with casein C_P = 141 µM (B), with egg white C_P = 121 µM (C) and with animal glue C_P = 41 µM (D). C_D= 6.4 µM, pH = 7.2, λ_{ex} = 470nm, λ_{em} = 603nm, T= 25°C.

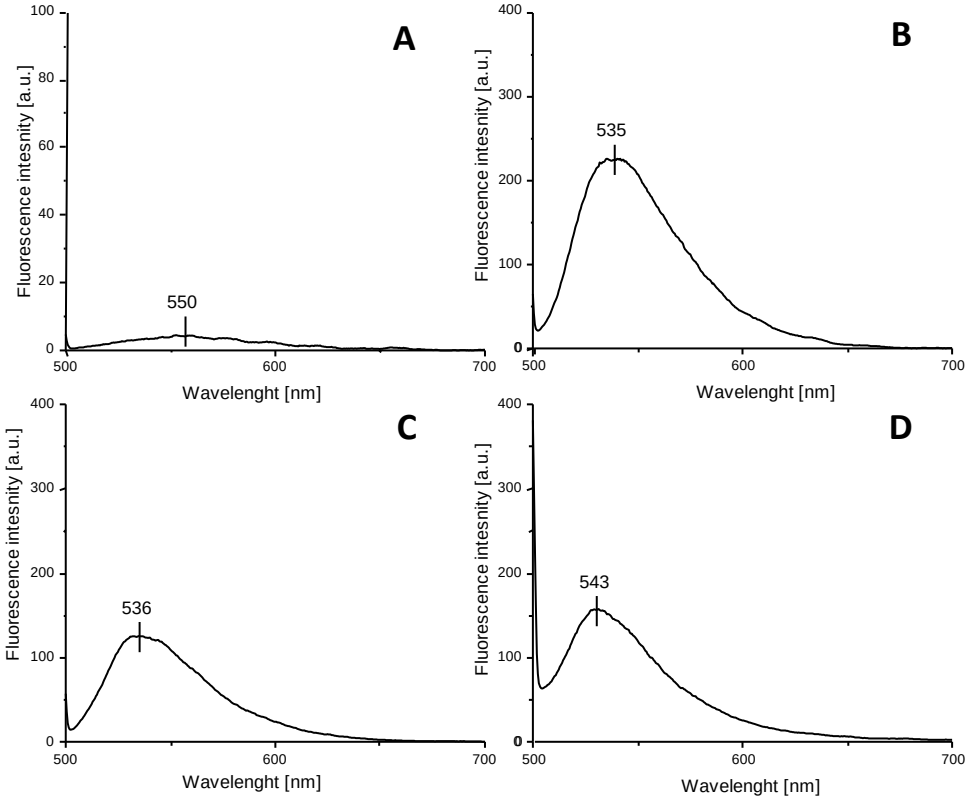

Figura S4: Fluorescence spectra of Flamingo alone (A), with casein C_P = 141 µM (B), with egg white C_P = 121 µM (C) and with animal glue C_P = 41 µM (D). C_D= 0.28 µM, pH = 7.2, λ_{ex} = 490nm, λ_{em} = 535 nm, T= 25°C.

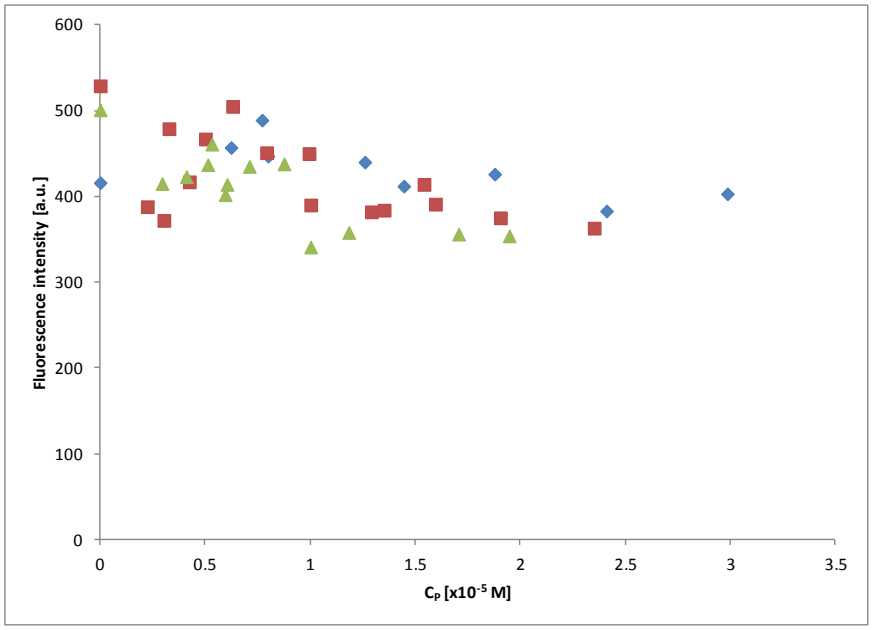

Figure S5: Fluorescence titrations of FITC with casein (blue diamonds), egg white (red squares) and rabbit skin glue (green triangles). $C_D = 5.5\times10^{-7}$, $\lambda_{ex} = 450$ nm, $\lambda_{em} = 518$ nm; T = 298 K, pH = 9.0.

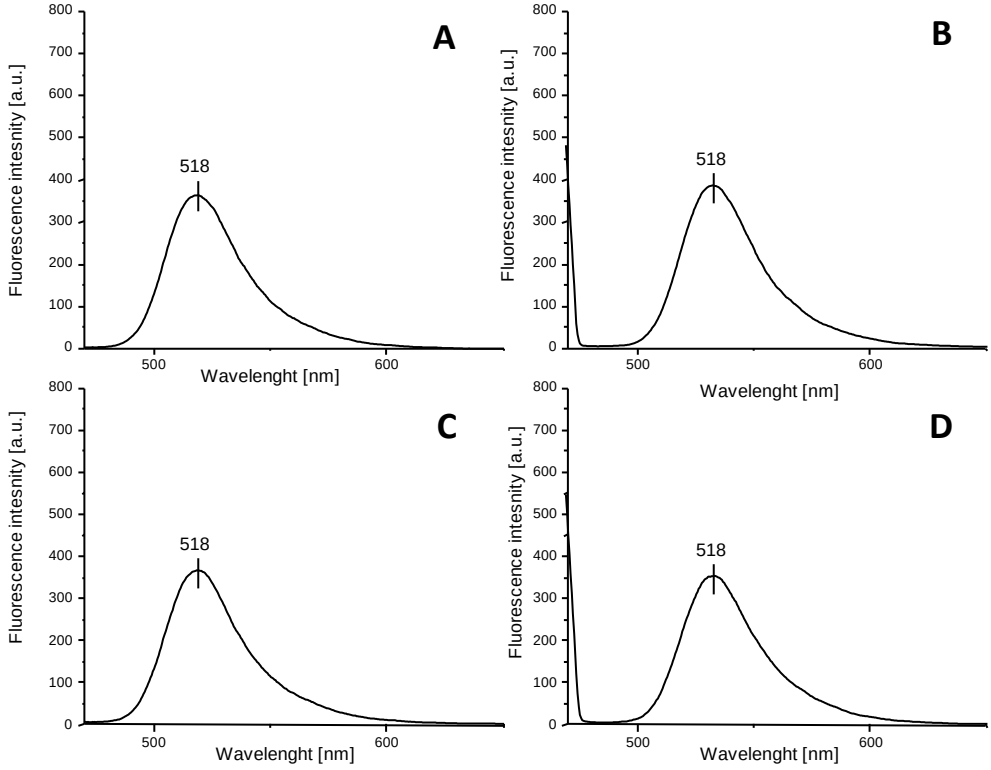

Figura S6: Fluorescence spectra of FITC alone (A), with casein $C_P = 141$ µM (B), with egg white $C_P = 121$ µM (C) and with animal glue $C_P = 41$ µM (D). $C_D = 0.55$ µM, pH = 9.0, $\lambda_{ex} = 450$nm, $\lambda_{em} = 518$ nm, T= 25°C.

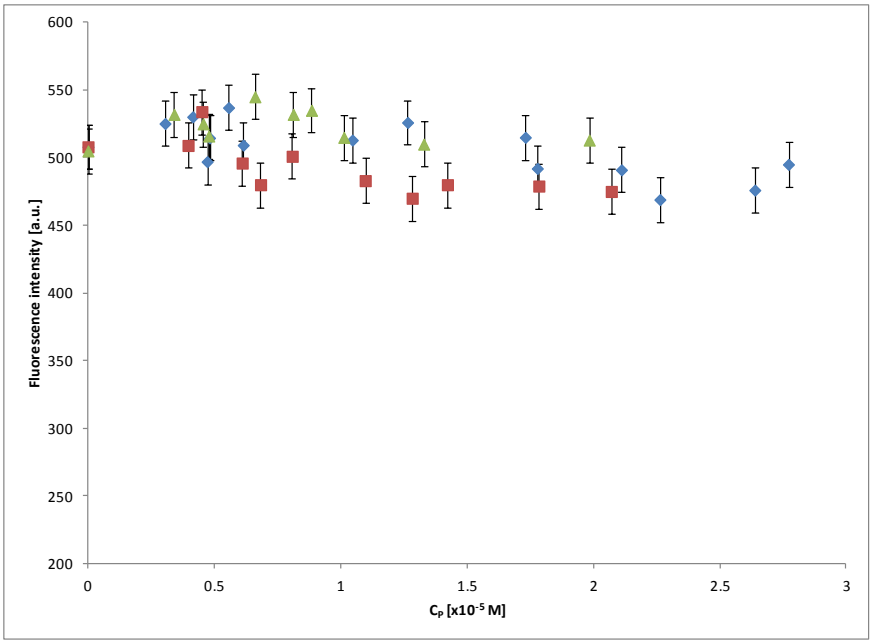

Figure S7: Fluorescence titrations of AMCA with casein (blue diamonds), egg white (red squares) and rabbit skin glue (green triangles). $C_D = 4.0\times10^{-7}$, λ_{ex} = 350 nm, λ_{em} = 449 nm; T = 298 K, pH = 9.0.

The small decrease of the fluorescence intensity of AMCA falls within the standard deviation calculated on 10 replicates.

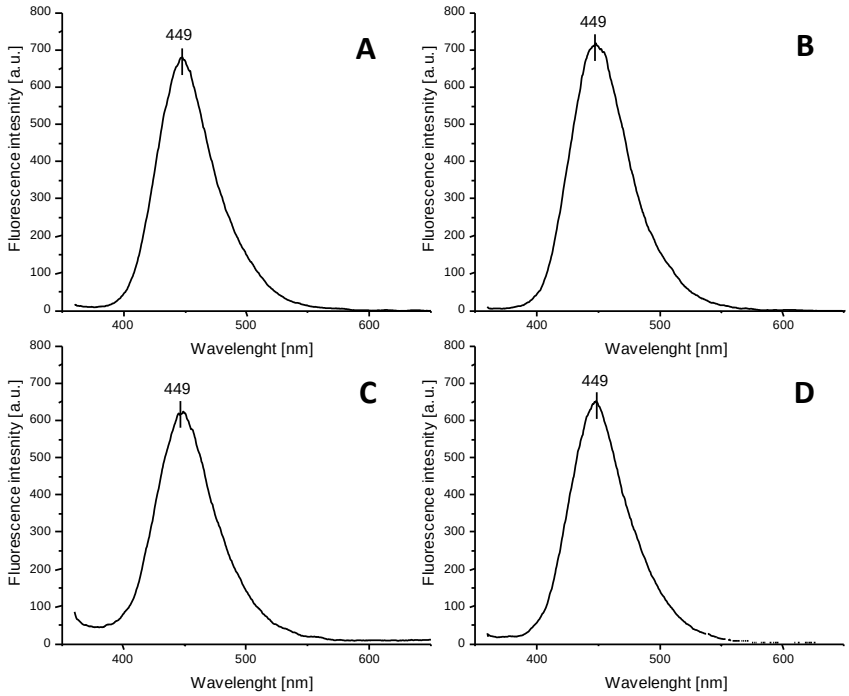

Figura S8: Fluorescence spectra of AMCA alone (A), with casein C_P = 141 µM (B), with egg white C_P = 121 µM (C) and with animal glue C_P = 41 µM (D). C_D= 0.38 µM, pH = 9.0, λ_{ex} = 350nm, λ_{em} = 449 nm, T= 25°C.

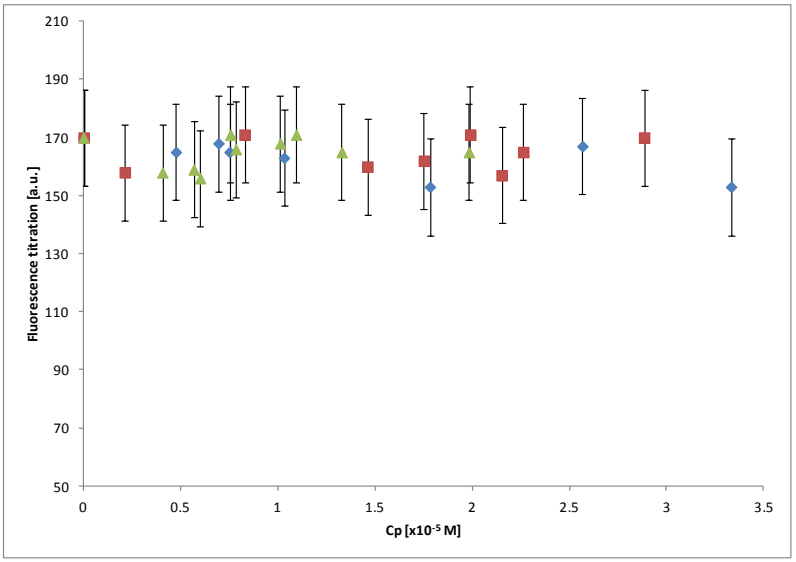

Figure S9: Fluorescence titrations of AMCA-X with casein (blue diamonds), egg white (red squares) and rabbit skin glue (green triangles). $C_D = 2.5\times10^{-7}$, λ_{ex} = 350 nm, λ_{em} = 449 nm; T = 298 K, pH = 9.0.

The small decrease of the fluorescence intensity of AMCA-X falls within the standard deviation calculated on 10 replicates.

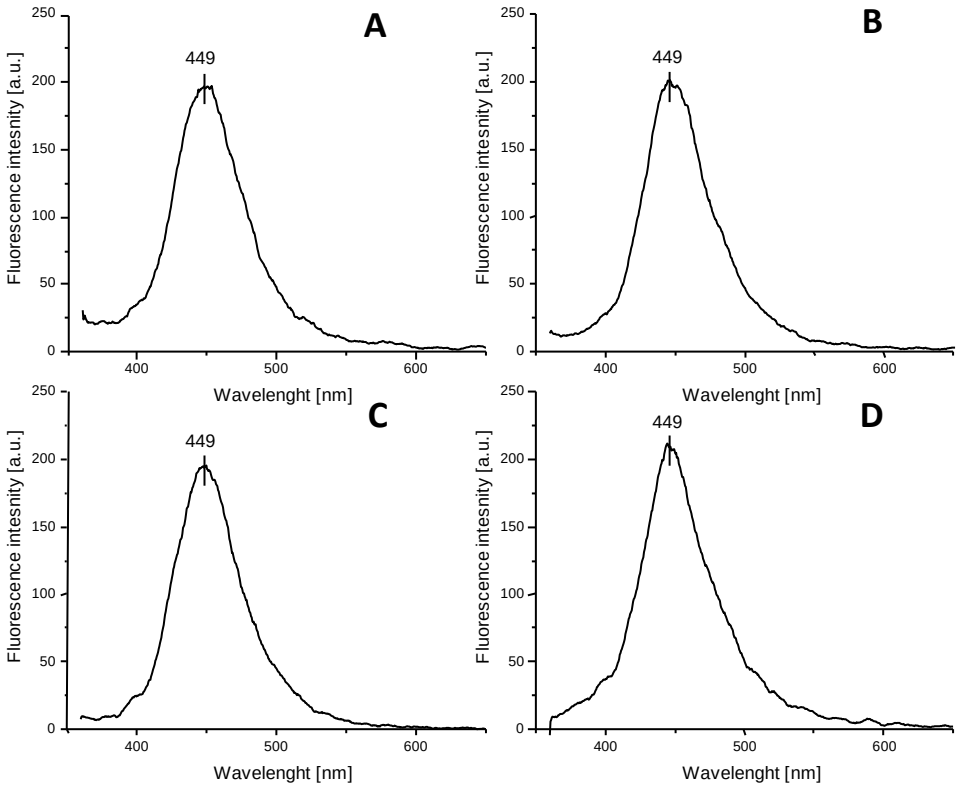

Figura S10: Fluorescence spectra of AMCA-X alone (A), with casein C_P = 141 µM (B), with egg white C_P = 121 µM (C) and with animal glue C_P = 41 µM (D). C_D= 0.25 µM, pH = 9.0, λ_{ex} = 350nm, λ_{em} = 449 nm, T= 25 °C.

Electronic circular dichroism spectra

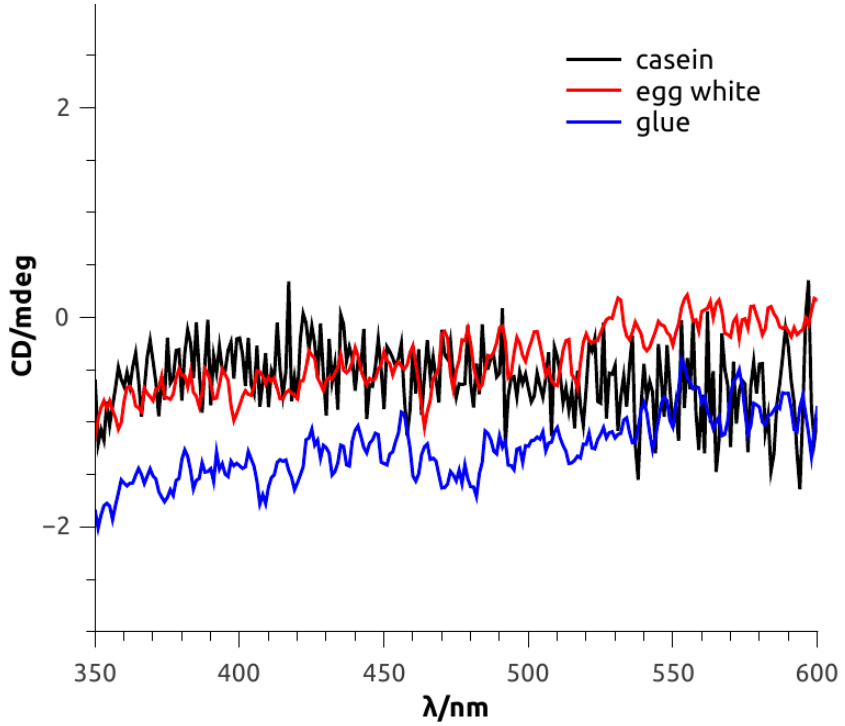

Figure S11: CD spectra of Sypro Ruby with the three proteins studied (casein, egg white and rabbit skin glue). As explained in the text, no significant signal was detected.

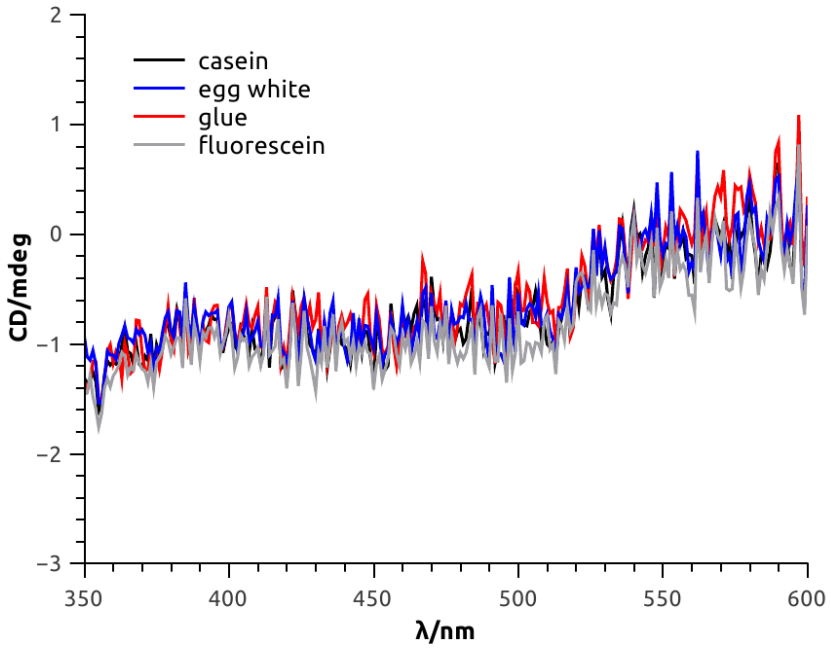

Figure S12: ECD spectra of FITC without protein (grey line) and with the three proteins studied (casein, egg white and rabbit skin glue). As explained in the text, no significant signal was detected.

Additional CPL spectra

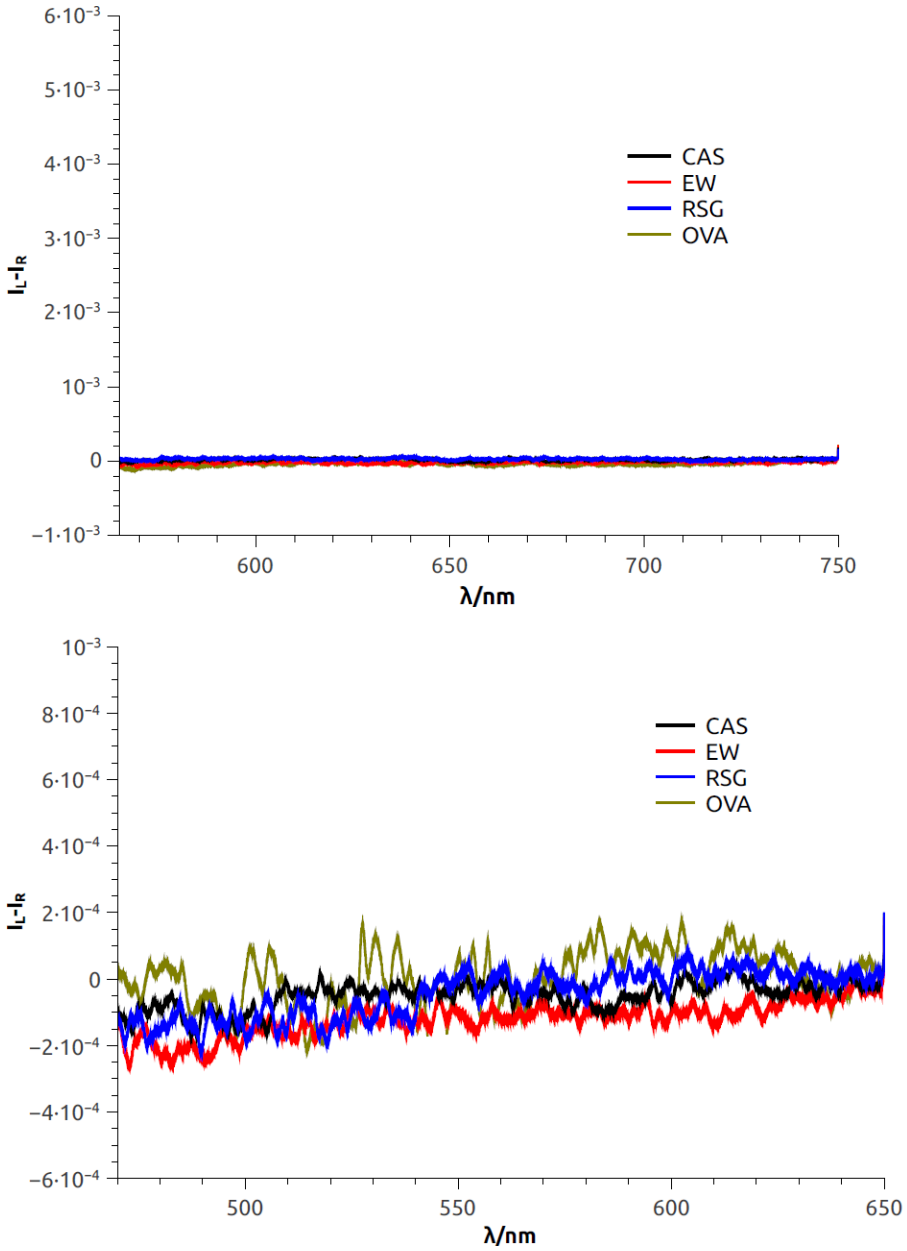

Figure S13: CPL spectra of the three investigated proteinaceous matrices and model OVA, without fluorescence stains, measured in the same conditions used to measure the spectra reported in Fig. 2 and Fig. 3 in the text. The same horizontal and vertical scales used in Fig. 2 and Fig. 3 respectively are used here.

In order to further test the reliability of our CPL data, we repeated the experiment reported by Brittain and Richardson. We measured the CPL spectrum of fluorescein dissolved in (*S*)-α-methylbenzylamine, obtaining a signal similar to that one reported in ref [S7] with a g_{lum} factor around $+3 \cdot 10^{-3}$, in agreement with the data in ref [S7]. Note that in ref [S7], the enantiomer compound (*R*)-α-methylbenzylamine was used and therefore the sign of the band is opposed.

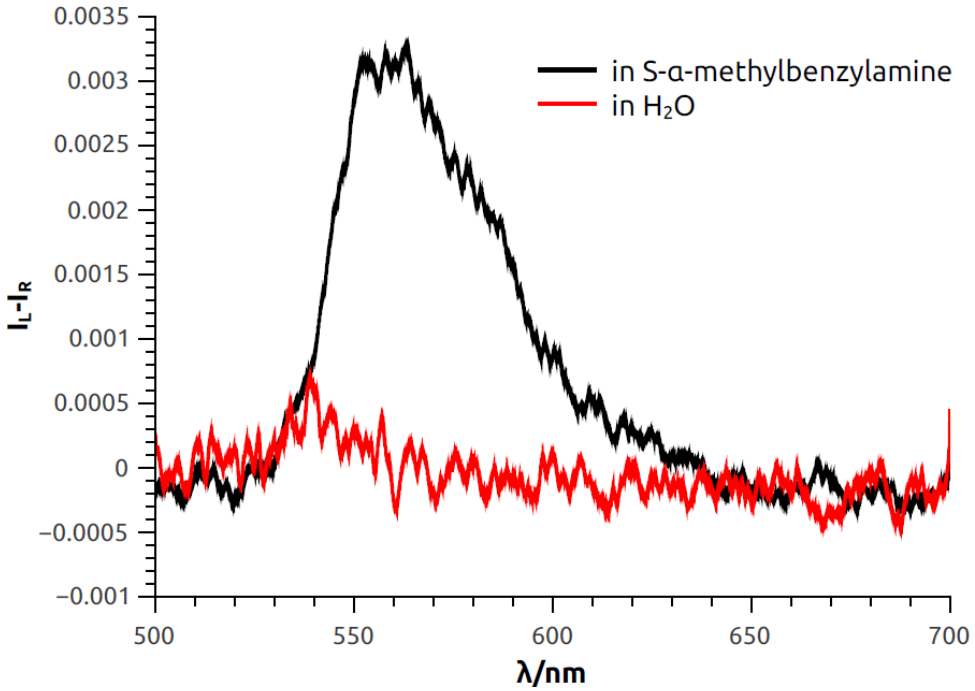

Figure S14. CPL spectrum of fluorescein dissolved in (*S*)-α-methylbenzylamine ($2 \cdot 10^{-3}$ M). The spectrum of fluorescein in water (same concentration) is reported for comparison.

Stains on cross sections

The effects of the stains investigated here upon interaction with protein-based painting matrices is clearly visible in **Figure 5**. **Figure 5** (right) displays a fluorescence microscopy image of a cross-section of a **CAS** matrix stained with SYPRO Ruby (**1**) responsible for the red luminescence. As a comparison, on the left, we show a cross section of a non-proteinaceous material (plant gum,) exposed to the same treatment with **1**. In this case no fluorescence is visible.

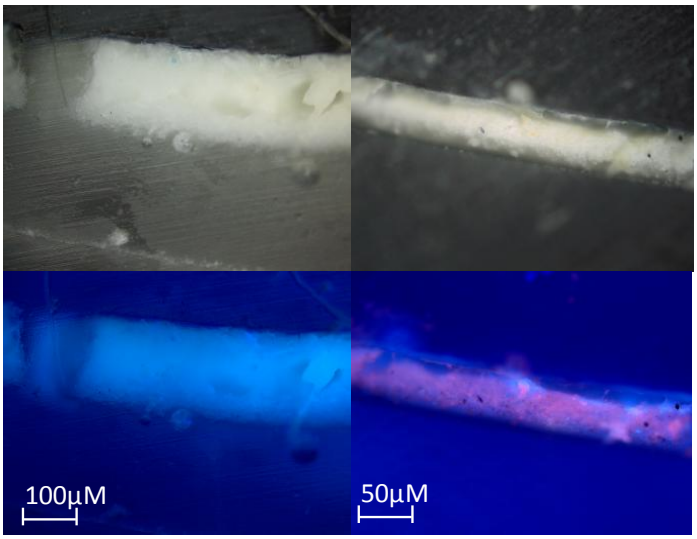

Figure S15: fluorescence microscopy image of a cross-section of a CAS matrix (right) and a plant gum (left), both stained with SYPRO Ruby, under visible (top) and UV (λ_{exc}=365 nm, bottom) light. The bluish luminescence is the intrinsic luminescence of the matrices.

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