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ORIGINAL PAPER

Fluorescence labeling of gelatin and methylcellulose: monitoring their penetration behavior into paper

Eva Hummert · Ute Henniges · Antje Potthast

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Abstract For the conservation of artworks on paper, powdery paint layers on the paper matrix are stabilized with dilute solutions of adhesives (0.25–1 %), commonly gelatin or methylcellulose, which are applied as aerosols. This technique allows non-contact application. The distribution of the adhesives must be carefully controlled: they have to be delivered to unstable paint layers in the right quantity to avoid visual alterations of the artwork during the stabilization treatment. To visualize the distribution of aerosol-misted adhesives in porous substrates, gelatin and methylcellulose were labeled with fluorescent dyes, purified from excess label, and applied on sample specimen featuring powdery pigment layers on handmade rag paper. As blank comparisons, sample papers without pigment layers were included to verify whether aerosol-misted adhesives are a suitable method to stabilize fragile papers. Penetration of the adhesive-label-conjugates was observed at thin sections of the samples by fluorescence microscopy. The fluorescence labeling of gelatin with Texas Red™ allowed an excellent visualization of aerosol-misted adhesive (0.5–1 %) in all sample types. Methylcellulose (Methocel™ A4C) labeled with Texas Red™ C2-dichlorotriazine enabled fluorescence tracing if applied in 0.5 % solutions by immersion. Aerosol application permitted local adhesive application, making it a suitable technique for stabilizing fragile papers. If applied to samples with low porosity, aerosol-misted gelatin was mainly deposited at the surface, whereas in porous filter paper, penetration dominated over surface deposition. Intermediate drying between repetitive applications apparently limited the penetration of aerosol-misted gelatin.

Keywords Fluorescence labeling · Gelatin · Methylcellulose · Stabilization · Pigment · Paper · Aerosols · Penetration · Size-exclusion chromatography

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Introduction

For a long time, artists have created drawings and paintings on paper that pose special problems in terms of preservation because they feature friable media. To achieve color applications that have a matte appearance, artists use paint or powdery drawing media

featuring pigments with a low ratio of binding medium or no binding medium at all. These kinds of artwork often show a partial loss of the pigment layer, indicating that the pigment layer lacks cohesion and is only weakly attached to the paper substrate. This is mainly due to the low content of binding medium, and can be amplified by degradation of the binding medium and fluctuations of the dimensions in the paper substrate, as caused by climatic changes or inappropriate handling of the artwork. To avoid irrevocable loss of media, and as a result, visual alterations of the artwork, preventing further loss is crucial. The stabilization of powdery paint layers is most commonly achieved by applying diluted (0.25–1 %), mostly aqueous, adhesive solutions. As the mechanical impact of an application tool such as a brush on the friable substrate surface can easily lead to further damage and dislocation of loose pigment particles, adhesive solutions used for the stabilization of powdery pigments are usually applied as aerosols—finely dispersed adhesive droplets, ranging from 1 to 5 μm, which allows a noncontact application method (Dierks-Staiger 1996; Michalski and Dignard 1997; Pataki 2006; Horie 2010).

Although application with aerosol has so far been mainly used for the stabilization of paint layers, it also offers several advantages for the stabilization of deteriorated and therefore structurally weakened paper. Paper becomes fragile due to intrinsic ageing processes; external influences like damp environments induce mold growth that weakens paper severely. The use of aerosol-misted adhesives offers an option for the local stabilization of even very small areas within a paper sheet; the conservator can control the amount of adhesive applied because the aerosol can be added in successive applications until the desired stability is achieved.

While stabilization treatments with aerosol-misted adhesives have been used for over 20 years in conservation, little information is available regarding the penetration and distribution of the adhesives within the substrate. However, this aspect is crucial, as the adhesive solution must be delivered precisely to the unstable regions to achieve sufficient stabilization. The quantity of adhesive applied must be low enough to avoid irreversible changes of the appearance of the artwork, such as color changes or the formation of glossy areas and tide lines, but high enough to ensure stabilization. Studying the penetration behavior allows observation of whether the adhesive is distributed to the desired position and permits adaptation and improvement of the application methods. As it is not possible to check the adhesive distribution with the unaided eye, the penetration behavior of adhesives has been studied with the goal of visualizing the distribution of the adhesive solutions within porous substrates.

In painting conservation, the penetration of binding media has been investigated through the preparation of cross- or thin-sections of a paint sample and subsequent histochemical staining (Plesters 1956; Wolbers and Landrey 1987; Schramm and Hering 1988; Wolbers 2000). The technique is limited due to the nonspecific staining of similar chemical groups of other materials present in the sample, making interpretation difficult (Wolbers and Landrey 1987). Although Fourier transform infrared spectroscopy (FTIR) has been frequently used to determine gelatin in paper using the amide I and amide II bands (Barrett 1989), it is not possible to visualize the distribution of gelatin within the paper. Rouchon et al. (2010) used FTIR microscopy on thin sections to characterize the distribution of gelatin in paper. However, the distribution was evaluated on samples that were stabilized with a 3 % w/w solution of gelatin, a concentration much higher than that used for the for stabilization of friable pigments and paper that requires concentrations of 0.25–1.0 % maximum.

A different technique used to characterize the distribution of adhesive solutions employed for stabilization treatments is the fluorescence labeling of the adhesive and its detection after application in sample thin sections with fluorescence microscopy. Labeling adhesives with a fluorescent dye offers significant advantages compared to the previously described techniques. The fluorescent dye is specific for chemical functionalities in the respective adhesive, and because it is chemically bound to the adhesive, there is no risk of separation during application. Originally used in the biomedical field, it has been employed in research in paper conservation (Ream 1995; Kessler et al. 1998) and painting conservation (Soppa et al. 2011). Soppa et al. labeled gelatin, methylcellulose, and a poly(isobutyl methacrylate) with fluorescent dyes to study the penetration behavior during stabilization treatments for flaking layers of oil paint on canvas. However, their adhesive concentrations of 3–5 % were much higher than those used for aerosol application to powdery paint on paper.

This study aims to verify whether fluorescence labeling is suitable to visualize the penetration behavior of diluted, aerosol-misted adhesives used for stabilization treatments. Gelatin and methylcellulose, which are the most frequently used adhesives for the stabilization of pigment layers, as well as for the stabilization of paper (Garlick 1986), were labeled with fluorescent dyes. A crucial point in applying fluorescent-labeled adhesives is the question of whether they are completely free from excess label (i.e. unbound monomer), which tends to get trapped in the polymer matrix and can therefore lead to an inexact interpretation of the penetration behavior. Hence, we have paid special attention to the purification of the labeled adhesives. The labeled adhesives were applied as aerosol on two sample sets with two different porous substrates, one featuring powdery pigment layers on paper, the second featuring papers of different water absorbencies. The distribution of the labeled adhesives was observed using thin sections cut perpendicularly to the plane of the sample papers. To determine whether fluorescence labeling allows differences to be observed between application techniques, an additional stabilization method—immersion in the adhesive solution—was used for one half of the sample set.

Materials and methods

Chemicals

Chemicals were obtained from commercial sources and were used without further purification. Deionized water (HQ quality) was used for all aqueous solutions. *N,N*-Dimethylacetamide (DMAc) was obtained from Promochem Chemicals, Germany. Dimethylformamide (DMF) and methanol were purchased from Merck. Alexa Fluor™ 594 hydrazide and Texas Red™ C_2-dichlorotriazine were both purchased from Invitrogen. Texas Red™ (S3388) was obtained by Sigma-Aldrich. Methylcellulose (Methocel™ A4C, DP = 220; DS = 1.8, viscosity 400 cP (2 % in water at 20 °C) (Keary 2001) was provided by Dow Chemicals. Gelatin (photogelatin type restoration 1) was purchased from GMW Gabi Kleindorfer. It was derived from beef bone by alkaline hydrolysis (type B), completely desalted, with bloom number 267. To embed the samples for fluorescence microscopy Technovit™ 7100 (Heraeus Kulzer), a resin based on 2-hydroxyethylmethacrylate (HEMA), was used.

Preparation of the gelatin conjugate

The protein concentration was 10 mg ml^{-1} according to the protocol provided by the manufacturer, recommending a protein concentration of 5–20 mg ml^{-1} (Invitrogen 2007). The solution of gelatin in 0.1 M sodium bicarbonate buffer (pH 9.0) was prepared at room temperature: Gelatin was dispersed in the buffer solution and soaked for 3 h to hydrate it. The mixture was heated with moderate stirring for 3 h in a water bath at 45 °C until the gelatin was completely dissolved and the solution was clear. A stock solution of the fluorescent label in anhydrous DMF was prepared at 10 mg ml^{-1} and aliquots were added to the gelatin solution to obtain different dye-protein ratios. A 5.5 µl aliquot of the stock solution (equivalent to 0.055 mg label) allowed an excellent visualization of the labelled gelatine via fluorescence microscopy.

The fluorescent label was weighted in an oven-dried, amber-colored vial (1 ml); then, DMF was added with a micropipette. The vial was briefly vortexed and protected from light by wrapping in aluminum foil to avoid photodecomposition. During continuous, moderate stirring in subdued lightning conditions at 4 °C, aliquots of the stock solution were added to the gelatin solution using a micropipette. The solution was incubated in the dark for 3 days at 4 °C, since low temperatures increase the rate of reaction and the selectivity of the reaction (Brinkley 1992). Desalting of the gelatin conjugate was performed with ultrafiltration, using a stirred ultrafiltration cell (Model 8400, Millipore) with a polyethersulfone membrane disc filter (Omega™ ultrafiltration membrane disc filters, MWCO 10.000, 76 mm, Pall Life Science), and gas pressure was applied (nitrogen, 5 bar).

The conjugates[1] were purified from unreacted dye by aqueous size-exclusion chromatography (SEC) using the following setup: glass column EcoPlus™ (Kron-Lab) (column volume: 62.5 ml) packed with Sephadex™ G-25; pump: HPLC Pump Kontron 420, (Kontron Instruments); detection: Agilent FLD G1321A, λ_{ex} = 594 nm, λ_{em} = 623 nm; manual injection valve

[1] To lower the viscosity, the gelatin solution was kept at 25 °C during purification and analyses with SEC.

with a 5 ml sample loop (Rheodyne); fraction collector: FC 203B, Gilson. For quantitative preparations, 5 ml of the labeled gelatin was applied manually and eluted from the column with a flow rate of 4.3 ml min^{-1} at 16 bar pressure. A 0.025 M sodium phosphate buffer, pH 8.2, filtered through a 0.45 μm polyethersulfone filter (VacuCap^{TM} 60, Pall) was used as eluent.

Preparation of the methylcellulose conjugate

The methylcellulose was prepared in two different buffers: for the labeling with Alexa Fluor^{TM} 594 hydrazide, 100 mg of methylcellulose was dissolved in 10 ml 0.05 M sodium acetate buffer, (pH 5.6); for the labeling with Texas Red^{TM} C_2–dichlorotriazine, 100 mg of methylcellulose was dissolved in 10 ml 0.1 M sodium borate buffer (pH 9). The solutions were prepared as follows: 1/3 of the required buffer solution was heated to 90 °C and methylcellulose was dispersed in the buffer. The remaining buffer solution was cooled and then added. For labeling 5 mg of methylcellulose with Alexa Fluor^{TM} 594 hydrazide, 1.8 mg label was used. For labeling 5 mg methylcellulose with Texas Red^{TM} C_2-dichlorotriazine, 2 mg fluorescent label was added. Each of the fluorescent labels were weighted in an amber-colored vial (1 ml), then 50 μl of the buffer solution was added to the hydrazide label and 50 μl of DMF was added to the triazine label with a micropipette. The vials were briefly vortexed and wrapped in aluminum foil. During continuous stirring in subdued lightning conditions at room temperature, the respective fluorescent dye solution was added with a micropipette to solutions of methylcellulose. The solutions reacted for 4 days at room temperature, protected from light by wrapping in aluminum foil.

The methylcellulose conjugates were dialyzed for 3 days using an RC Spectra/Por^{TM} 6 dialysis tube with a molecular weight cutoff (MWCO) at 1,000 Dalton (Spectrum). The conjugates were purified from unreacted dye by aqueous SEC using prepacked, disposable columns: 2.5 ml of the conjugate was loaded on a Sephadex^{TM} G-25 PD-10 column (bed volume: 8.5 ml) (GE Healthcare) attached to a vacuum manifold (Phenomenex). The sample was eluted with 3.5 ml 0.05 M sodium acetate buffer at 3 bar pressure.

Size exclusion chromatography (SEC)

After purifying the gelatin and methylcellulose conjugates using the chromatographic methods described above, the degree of cleaning was determined by the following analytical setup.

Organic SEC

For the methylcellulose conjugates, absence of unreacted dye was verified by SEC carried out on four PLgel mixed A LS (7.5 × 300 mm) columns. Gel permeation chromatography (GPC) measurements were carried out using the following components: a Dionex DG-2410 online degasser; a Kontron 420 pump; a pulse damper; an HP series 1100 autosampler; a Gynkotek STH 585 column oven; a Shimadzu RF 535 fluorescence detector; a Wyatt Dawn DSP multiple-angle laser light scattering (MALLS) detector, with an argon ion laser ($\lambda_0 = 488$ nm); and a Shodex RI-71 refractive index (RI) detector. The samples were lyophilized, dissolved in DMAc/LiCl (0.9 %, w/v), and filtered through 0.45 μm syringe filters (Phenomenex). They were injected automatically, separated on four GPC columns, and monitored by fluorescence, MALLS, and RI detection. The system used the following parameters: flow, 1 ml min^{-1}; fluorescence detection, $\lambda_{ex} = 585$ nm, $\lambda_{em} = 610$ nm; injection volume, 100 μl; and run time, 50 min. DMAc/LiCl (0.9 %, w/v), filtered through a 0.02 μm filter, was used as the eluent. Data evaluation was performed using Astra 4.72 software (Wyatt Corp.).

Aqueous SEC

The absence of unreacted dye in the gelatin conjugate was assayed by aqueous SEC. The system included the following components: a pulse damper; a Kontron 420 pump; an Agilent 1200 autosampler; an Agilent FLD G1321A fluorescence detector; a Wyatt Heleos MALLS detector ($\lambda_0 = 658$ nm); and a Shodex RI-101 RI detector. The detector delay was determined with bovine serum albumin (BSA, $\lambda_{ex} =$ 295 nm, $\lambda_{em} = 320$ nm). Samples of the purified gelatin conjugate were prepared by diluting the conjugate from 10 to 5 mg ml^{-1} with the mobile phase. The pure gelatin was prepared at 5 mg ml^{-1} in sodium dodecyl sulfate (SDS) eluent (soaked and heated for 3 h at 45 °C). The fluorescent dye was

prepared in the SDS eluent. The samples were filtered with a syringe filter (0.2 μm) and injected automatically. The system used the following parameters: flow 1.0 mL min^{-1}; two PSS Gral 3,000 Å, 10 μm, 8 × 300 mm; fluorescence detection, $\lambda_{ex} = 594$ nm, $\lambda_{em} = 623$ nm; injection volume, 100 μl; and run time, 60 min. An SDS buffer solution (0.01 M NaH_2PO_4: 0.1 M Na_2SO_4 10:1, 1 % SDS (w/w), pH 5.3) filtered through a 0.02 μm filter (Whatman, Anodisc 47), was used as the eluent. Data evaluation was performed using Astra 5.3.4 software (Wyatt Corp.).

Solid phase extraction (SPE)

SPE was performed with Strata^{TM}-X columns (33 μm, 85 Å, RP, Phenomenex) equipped with a polymeric sorbent (surface modified styrene divinylbenzene). The column was conditioned with 1 ml of methanol followed by 1 ml of water. Using a vacuum manifold (3 bar), 1 ml of the conjugate was loaded and then eluted with 0.5 ml water.

Model papers

To obtain samples imitating fragile papers, sample papers with different rates of water absorbency were used.

Paper A: modern rag paper

The modern handmade paper is composed of chlorine-free bleached hemp fibers (Gangolf Ulbricht, Berlin). The paper is surfaced sized with photographic gelatin and is nonabsorbent to slow absorbent. A water droplet is absorbed after 14 min.

Paper B: Whatman filter paper

Whatman filter paper no. 1 consists of almost pure α-cellulose and is unsized. The paper is absorbent—a water droplet is absorbed immediately.

Pigment layers on paper

To obtain samples featuring friable pigment layers on paper, a pastel (Kremer Pigmente, synthetic ultramarine blue, 881571) was applied to a modern handmade rag paper (same as paper A but without surface sizing). One layer of the pastel was applied by hand in vertical, slightly intersecting streaks with a width of 2 cm. Pastels mainly contain pigment particles and only a very low amount of binding medium, creating friable, powdery pigment layers when applied on paper. Pigment particles are transferred when the samples are touched; the pigment layer remains stable if the samples are held upside down and gently shaken. Ultramarine blue was chosen because it is known to have a weak initial cohesion, contributing to the friable character of the samples (Michalski and Dignard 1997).

Adhesive application

Immersion

The dry papers were immersed in solutions of 0.5 % gelatin at 40 °C and 0.5 % methylcellulose conjugates for 2 min. They were then removed from the bath and the excessive adhesive was allowed to drain for 20 s. The samples were transferred to synthetic mesh, air dried until the surface gloss was gone, and then dried between a polyester web, blotter, and weight for 3 days.

Aerosol application

Aqueous solutions of the conjugates (gelatin 0.5 and 1 %, methylcellulose 0.25 %) were applied with an aerosol generator (AGS 2000, ZfB Leipzig) using a Teflon nozzle (nozzle diameter: 4 mm). Adjustments for aerosol output and air flow were maintained during the whole application. To achieve a consistent application, the conjugates were applied with a custom-built cardboard frame (39 × 14 × 4 cm), which allowed the same distance of the nozzle to the sample surface (1.5 cm) to be maintained during every application. The misted adhesive solutions were applied starting from the upper left corner of the sample, moving in adjacent streaks over half of the sample. The right half stayed untreated as a reference and was covered with a polyester sheet during the application. On the samples imitating fragile papers, the adhesive solution was applied four times without intermediate drying (referred to as "4×"). The samples imitating powdery pigment layers on paper were treated four times without intermediate drying ("4×") and four times with intermediate drying (referred to as "4×d").

Fluorescence microscopy

For image analysis, samples were adjusted in embedding capsules (BEEM, Size 0–large), embedded with resin (Technovit^{TM} 7100) and cured at room temperature overnight. Thin sections (10–30 μm) were cut using a rotary microtome (HM 360, Microm) with a steel knife. The sections were viewed using an epifluorescence microscope (MZF LIII, Leica) equipped with a 1.0 × objective (Leica) and a mercury vapor lamp. Digital images were acquired with a DC 500 color digital camera (Leica). An optical filter set (DsRed, Leica) with a 545/30 excitation filter and a 620/60 suppression filter was used.

Results and discussion

Fluorescence labeling of gelatin and methylcellulose

Choice of fluorescent labels and optimizing conditions for labeling

In gelatin, various amino acids, mainly glycine, as well as proline and hydroxyproline, are joined by amide bonds to form a polymer with a M_w varying from 15,000 to 250,000 g mol^{-1} (Zhao, Furukawa, and Ohki 2009). The most common reactive groups of proteins for attaching the fluorescent label are aliphatic amines (Brinkley 1992). Therefore, Texas Red^{TM} (λ_{ex} 594 nm, λ_{em} 623 nm), an amine-reactive dye, was selected for labeling gelatin: It possesses sulfonyl groups, which can react with primary amines in gelatin to form stable sulfonamide bonds, cf. Fig. 1a (Hermanson 2008).

Compared to the range of fluorescent dyes available for proteins, the choice of fluorescent dyes for the fluorescence labeling of polysaccharides is rather small (Nordmark and Ziegler 2000). As a polysaccharide, Methocel^{TM} A4C possesses two possible reactive groups for the fluorescent label: reducing ends (aldehydes) and hydroxyl groups. Due to a degree of substitution (DS) of methyl groups of 1.8, free OH are available for labeling. To verify which reactive group allows the most successful conjugation of the fluorescent label, two different dyes were used:

1. Texas Red^{TM} C_2-dichlorotriazine (λ_{ex} 583 nm, λ_{em} 604 nm) can be directly attached to the hydroxyl groups of the polymer chain with a pH > 9, due to the reactive chlorine groups in the chlorotriazine, similar to textile dying reactions (Fig. 1b).
2. Alexa Fluor^{TM} 594 hydrazide (λ_{ex} 588 nm, λ_{em} 613 nm) is an aldehyde-reactive label that can be attached to the reducing ends of methylcellulose, forming a stable hydrazone linkage (Fig. 1c) (Hermanson 2008). Its fluorescence is insensitive to pH in the range of 4–10. It has good water solubility, permitting labeling without organic solvents.

As preliminary tests showed that an excitation above 400 nm for the attached label is necessary to circumvent the autofluorescence of the sample papers used, the selected labels have excitation maxima between 583 and 594 nm. Autofluorescence interferes with the fluorescence emission of the adhesive solutions and complicates a correct localization of the adhesive in the sample materials.

Choosing the concentration of the fluorescent dye used for preparing the conjugates involved a compromise between a sufficient fluorescence yield to guarantee detection of the diluted misted adhesives with fluorescence microscopy and minimizing structural changes to the adhesives, which could alter their penetration behavior. To determine the appropriate concentration for the reaction of the Texas Red^{TM} dye with gelatin, three conjugates with different fluorescent dye–reagent ratios (ranging from 0.055 to 1 mg label for 10 mg ml^{-1} gelatin) were prepared.

For the hydrazide label, the concentration of the fluorescent dye was calculated relative to the estimated amount of aldehydes at the reducing ends. A 20-fold excess was used. For the triazine label, the concentration of the fluorescent dye was calculated relative to the estimated amount of hydroxyl groups.

To get an optimal labeling result for the Texas Red^{TM} dye, an alkaline pH in the range of 8–9.5 is recommended during the reaction. That guarantees that most of the amines are not protonated, since amines only react with the fluorescence label in the free amine form. Therefore a 0.1 M sodium bicarbonate buffer pH 9 was used (Brinkley 1992).

Since the unbound label is very unstable in water—dilute solutions are completely hydrolyzed within 2–3 min in pH 8.3 aqueous solutions at room temperature—it was dissolved in anhydrous DMF, a water-

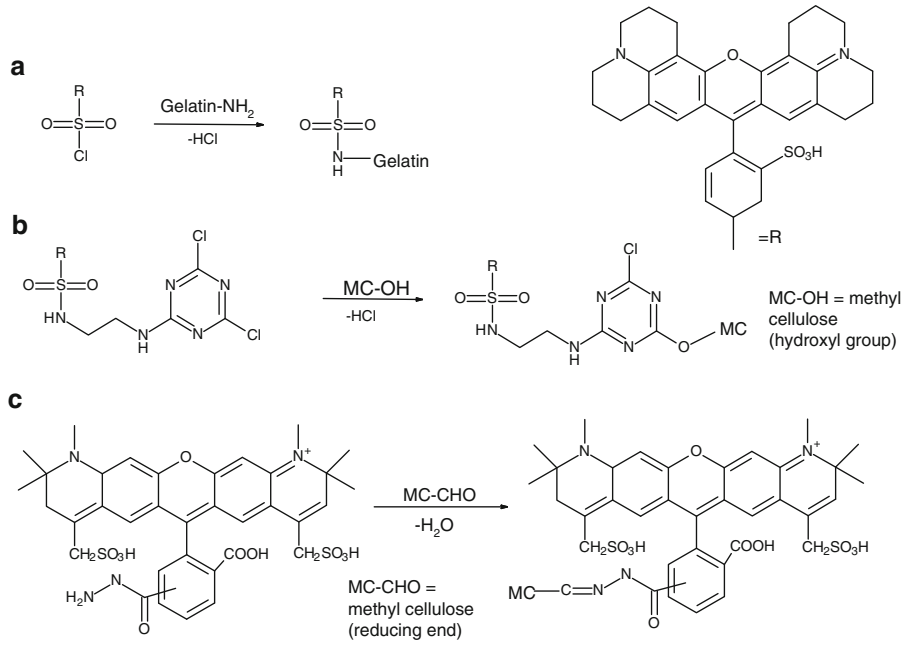

Fig. 1 Labeling of amino groups in gelatin with Texas Red™ (**a**) and labeling of hydroxyl groups in methylcellulose (DS 1.8) with Texas Red™ C_2-dichlorotriazine (**b**). Labeling of reducing end groups in methylcellulose with AlexaFluor™ 594 hydrazide (**c**)

miscible, organic solvent which prevents hydrolysis of the fluorescent label (Brinkley 1992; Haugland 2002).

The hydrazide label has good water solubility and is stable in aqueous solutions; therefore, it was dissolved in a small amount of 0.05 M sodium acetate buffer (pH 5.6) before adding it to the methylcellulose to ease distribution of the dye in the viscous solution. The triazine label was dissolved in dry DMF to avoid hydrolysis of the active group of the fluorescent label.

Purification of the conjugates by aqueous SEC

Since the fluorescent labels were added in excess and only a certain amount of the label is covalently bound to the protein, the labeled adhesive solutions contain the conjugate, as well as free fluorescent dye, which has not reacted with the protein/cellulose ether. The excess fluorescent dye must be removed as completely as possible from the conjugate, as it can be absorbed from the sample materials during the adhesive application and can cause nonspecific fluorescence emission.

As for rhodamine dyes like Texas Red™, it seems doubtful that dialysis is able to remove the free fluorescent dye completely (Nairn 1969). The conjugate was purified with SEC. A gel media, Sephadex™ G-25 Medium (particle size: 85–260 μm), was chosen because it allows a separation of proteins with a Mr > 5,000 from molecules with a Mr < 1,000. For purification, disposable columns prepacked with Sephadex™ G-25 media (PD-10 columns) were used, which prevent carryover effects from fluorescent dye nonspecifically fixed to the gel media. Using the prepacked columns for separation had two main disadvantages: First, with a bed height of 5 cm, only 2.5 ml of the conjugate could be purified per run. Second, an exact visual distinction between the purified conjugate and the free fluorescent dye was difficult. Therefore, an aqueous SEC system using Sephadex™ G-25 media in glass columns was set up; this included a fluorescence detector allowing an accurate monitoring of the separation and the purification of bigger sample amounts. As mobile phase, a 0.025 M sodium phosphate buffer pH 8.2 was used, since gelatin is soluble in alkaline phosphate buffers (Dauwe et al. 2001). Using the fluorescence detector, it was possible to distinguish between the conjugate and the unreacted fluorescent dye: the conjugate eluted 5 min after injection, whereas the free dye eluted 50 min after injection. Additional checking of the column with UV-light detected a weak fluorescence, which indicates that part of the unreacted fluorescent

label was nonspecifically bound to the gel medium. Therefore the column was flushed for 2 1/2 h after each run with the buffer solution to prevent cross contamination.

After purifying the methylcellulose conjugates with prepacked PD-10 columns, it became apparent that the separation was not completely successful: The conjugates still contained a small amount of free fluorescent dye. As a result, for the methylcellulose conjugates, the separation method was changed from size-exclusion to ionic interactions using SPE. A SPE column with a polymeric sorbent was chosen, which retains polar analytes with a molecular weight <10 kDa. Using SPE most of the fluorescent dye could be removed. A significant drawback of purification with SPE was the extensive separation time, which is due to the high viscosity of methylcellulose. Diluting the conjugate gave no significant improvement. As a compromise between a satisfactory removal of free fluorescent dyes and a feasible preparation time, the purification was carried out with SEC using prepacked PD-10 columns, to which a low vacuum (0.3 bar) was applied. This permitted a forced flow through the column and enhanced the penetration of the viscous methylcellulose solution in the column bed. Although PD-10 columns are mainly recommended for gravity flow, using a low vacuum did not seem to have a negative influence on the separation process.

Analysis of the purified conjugates with SEC

To verify whether the purification of the conjugates was completed successfully, samples of the purified conjugates, the free fluorescent dyes, the pure protein, and the pure cellulose ether, were examined by SEC coupled with fluorescence detection.

For the analysis of the purified gelatin conjugates, aqueous SEC was used. The choice of the mobile phase was based on a comparison of an alkaline phosphate buffer (0.07 M Na_2HPO_4 pH 9) (Dauwe, Reinhold, and Gores 2001) and a slightly acidic buffer solution with 1 % SDS (Ahlers et al. 2006). After running samples of the purified conjugate and of pure gelatin (100 µl, 5 mg ml^{-1} in the eluent) in the phosphate buffer, it became obvious that the sample protein could not be eluted from the column, indicating that the sample reacted with the column packing. A rise of pressure in the system was observed, which confirms the assumption that the gelatin might clog the stationary phase of the column (Zhao et al. 2009). As the use of SDS is often recommended as an addition to the mobile phase, the eluent was changed to an acidic buffer with addition of 1 % SDS. The anionic surfactant contributes in reducing ionic interactions between the mobile phase and the stationary phase. Additionally, SDS is able to form complexes with polypeptides, unfolding them to a "rod-shaped conformation" (Wu 1995; Dupont 2003). Type B gelatins have their isoelectric points (IEPs) in a pH range of 4.7–5.2 (Nordmark and Ziegler 2000). As the pH of the buffer is adjusted at the IEP, it can be assumed that at a pH of around 5, gelatin is uncharged, which might additionally reduce the adsorption effects of the gelatin in the column (Nordmark and Ziegler 2000). Compared to the alkaline buffer without surfactant, using an acidic buffer solution with SDS, samples of the purified conjugate—as well as of the pure gelatin and the fluorescent dye—could be eluted from the column. As a result, SEC was operated with an acidic buffer with SDS as the mobile phase; the same buffer was used for sample preparation.

As shown in Fig. 2, the gelatin conjugate could be almost completely purified from unreacted fluorescent dye by aqueous SEC using Sephadex™ G-25 media.

To analyze the methylcellulose conjugates, an organic SEC system was used (Röhrling et al. (2002).

From this analysis, it was obvious that a complete removal of free fluorescent dye from the triazine conjugate was not possible (Fig. 3): The conjugate

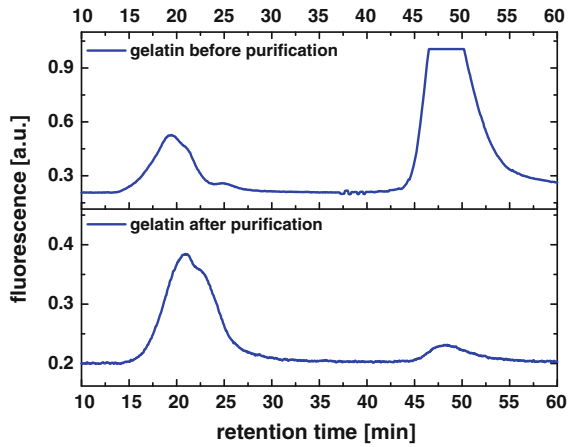

Fig. 2 Gelatin labeled with Texas Red™ before (*top*) and after (*below*) purification with aqueous size-exclusion chromatography (SEC) using Sephadex™ G-25. The amount of unreacted fluorescent label could be removed almost completely

still contained a small amount of unreacted fluorescent dye after purification with aqueous SEC using Sephadex™ G-25 media. Although the fluorescent label could not be completely removed from the conjugate with aqueous SEC, however, this method permitted acceptable purification combined with a short separation time; that is, 2.5 ml of the conjugate could be purified within 5 min.

For the hydrazide conjugate, the degree of labeling was so low that detection with fluorescence microscopy was not possible. This is suspected to be a result of the small number of bonding sites methylcellulose offers for the hydrazide label, since it is exclusively attached to the reducing end groups of the cellulose ether. Therefore, only the triazine conjugate was used for evaluation of the thin sections.

Evaluation of thin sections

To evaluate the distribution of the fluorescent-labeled adhesives in the sample papers, thin sections of the samples were prepared. The embedding media had to meet several criteria, specifically no autofluorescence in the excitation range of the fluorescent labels, curing at room temperature to avoid excessive heating of temperature-sensitive sample components like gelatin, and an adequate penetration of the resin in the sample to provides samples with a sufficient stability to allow cutting of thin sections. Technovit™ 7100, a HEMA, was chosen because it meets all of these requirements. Sectioning with an ultramicrotome showed, that the Technovit™ 7100 resin was compressed during sectioning, making it impossible to achieve a cut over the whole sample surface. A rotary microtome provided the desired results: It allowed a homogenous cut over the whole sample surface.

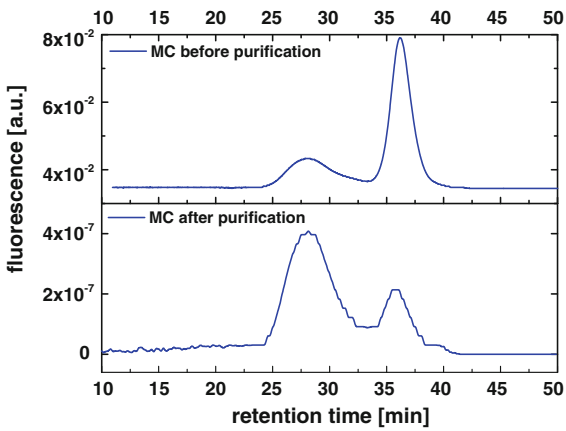

Fig. 3 Methylcellulose labeled with Texas Red™ C_2-dichlorotriazine before (*top*) and after (*below*) purification with aqueous size-exclusion chromatography (SEC) using prepacked PD-10 columns (Sephadex™ G-25) with vacuum (0.3 *bar*). After purification the conjugate still contained a small amount of unreacted fluorescent dye

Application as aerosol

The labeling conditions for gelatin proved to be sufficient to allow an adequate fluorescence emission for the detection of the adhesive solution with fluorescence microscopy. This allowed an excellent visualization of the distribution of the labeled gelatin when applied as aerosol. In contrast, the triazine conjugate, which had to be diluted from 1 to 0.25 % to permit misting with the aerosol generator, only led to a very weak fluorescence emission, which was too low for detection with fluorescence microscopy.

Figure 4 presents the distribution of the aerosol-misted gelatin conjugate (0.5 %) in two different sample papers: Whatman filter paper without surface sizing (paper B) (Fig. 4a) and a modern handmade rag paper with surface sizing (paper A) (Fig. 4b), which was included to clarify the effect of a preexisting surface sizing on the penetration of aerosol into paper. For both samples, the adhesive solution was applied four times as aerosol without intermediate drying (4×). Only the left half was treated with the gelatin conjugate (marked with arrow), while the right half remained untreated. From Fig. 4a, it is obvious that if applied to the unsized, water-absorbent filter paper, the aerosol-misted gelatin was distributed over the whole paper thickness (z direction). In contrast, if the aerosol was applied to the surface-sized paper (Fig. 4b), the adhesive rarely penetrated into the paper's interior and the adhesive was deposited exclusively at the paper surface, on the side where it was applied. Treated areas could be distinguished from untreated areas, although there was no sharp boundary visible.

Obviously, if the adhesive was applied as an aerosol, the penetration behavior showed great variations depending on the water absorbency of the paper. The results may be explained by considering the preexisting surface sizing of the handmade rag paper and how it influences the penetration of the aerosol-misted gelatin conjugate. The sample paper was

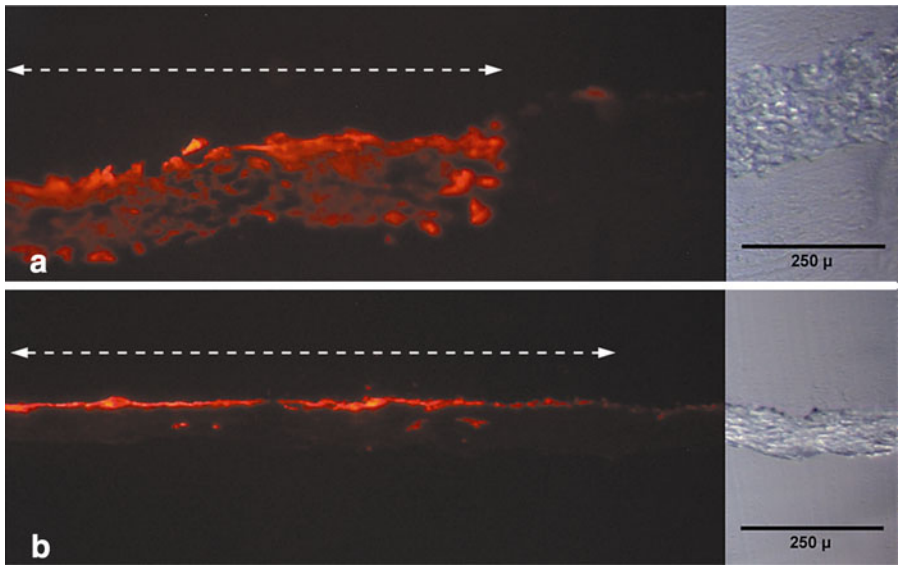

Fig. 4 Distribution of the gelatin conjugate in filter paper after an aerosol application (**a**) and in handmade rag paper, surface sized with gelatin (**b**). The 0.5 % gelatin conjugate was applied 4× in the areas marked by *arrow*; the right area includes a bright field image

surface sized with gelatin, which was sprayed on the surface of the dried paper sheet during manufacturing. Surface sizing with gelatin leads to an incoherent film on the surface, reducing the ability of liquid water to penetrate the capillary system inside the paper (Banik et al. 2011). This phenomenon is visible in Fig. 4b. Penetration was prevented by the preexisting surface sizing, which acted as a "barrier" and led to a superficial film on the paper surface. The paper surface was flooded with the gelatin solution because the delivery of the aerosol-misted adhesive in the air stream of the ultrasonic generator was faster than capillary penetration of the adhesive into the paper (Michalski and Dignard 1997). Furthermore, compared to the filter paper, the rag paper was denser and therefore less porous, which contributed to the reduced penetration of the aerosol. Additionally, the hemp pulp used for rag paper production is more complex, containing hemicelluloses and residual lignin, which could further limit the water absorption of the paper.

Using the prepared gelatin conjugate, it was also possible to visualize its distribution in friable pigment layers on paper after aerosol application. Variations within the aerosol application could be clearly distinguished.

The sample presented in Fig. 5a was treated four times with the aerosol-misted gelatin conjugate; between each application, the samples were allowed to air dry completely (4×d). The sample seen in Fig. 5b also received four aerosol applications of the gelatin conjugate, i.e., the same amount of gelatin overall, but without intermediate drying (4×).

Observing the distribution of the gelatin in Fig. 5a, it can be seen that if applied with intermediate drying, the aerosol-misted gelatin only penetrated into the pigment layer, but did not reach the paper support. In contrast, if the gelatin was applied without drying, it was deposited in the pigment layer as well as in the paper. Apparently, repeated aerosol applications that are allowed to dry inhibit the adhesive penetration. It is suspected that this is due to a reduction of the pore size of the pigment layer caused by an accumulation of the adhesive in the pigment layer during the stepwise application. This was already described by Michalski and Dignard (1997), who referred to this phenomenon as "choking," meaning that the adhesive is adsorbed in the pigment layer through chromatography interactions leading to a reduction of the pore diameters. Due to the reduced pore size of the pigment layer, the macromolecules of the adhesive are blocked during their way in the pigment layer (Michalski and Dignard 1997).

Immersion

The gelatin conjugate was also successfully used to visualize the distribution of the adhesive, if immersion was used as application technique.

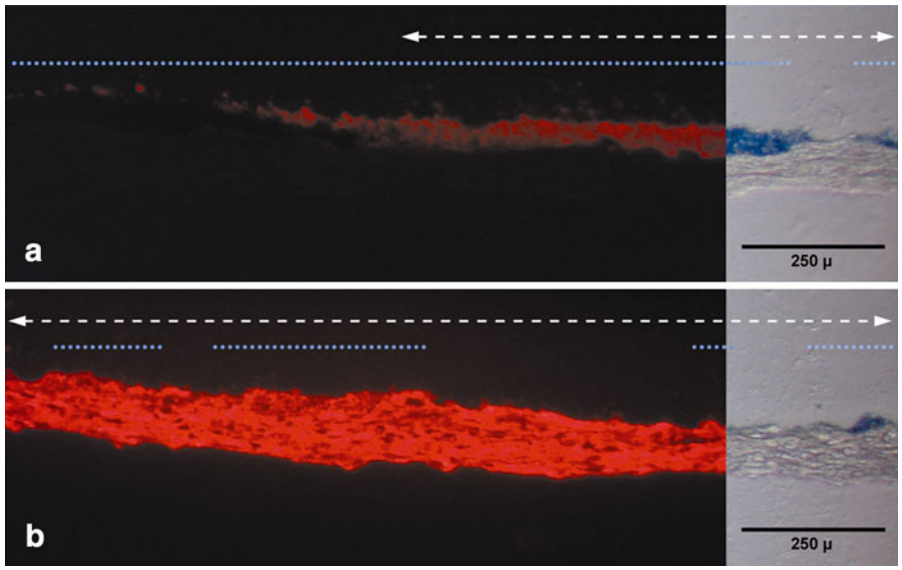

Fig. 5 Distribution of the gelatin conjugate in samples featuring a powdery pigment layer (*ultramarine blue*) on handmade rag paper. The 1 % gelatin conjugate was applied as aerosol in the areas marked by *arrow* using two different application methods: 4× with intermediate drying (**a**) and 4× without intermediate drying (**b**). The *blue* pigment covers the paper in areas marked by the *blue dotted line*; the *right area* includes a bright field image

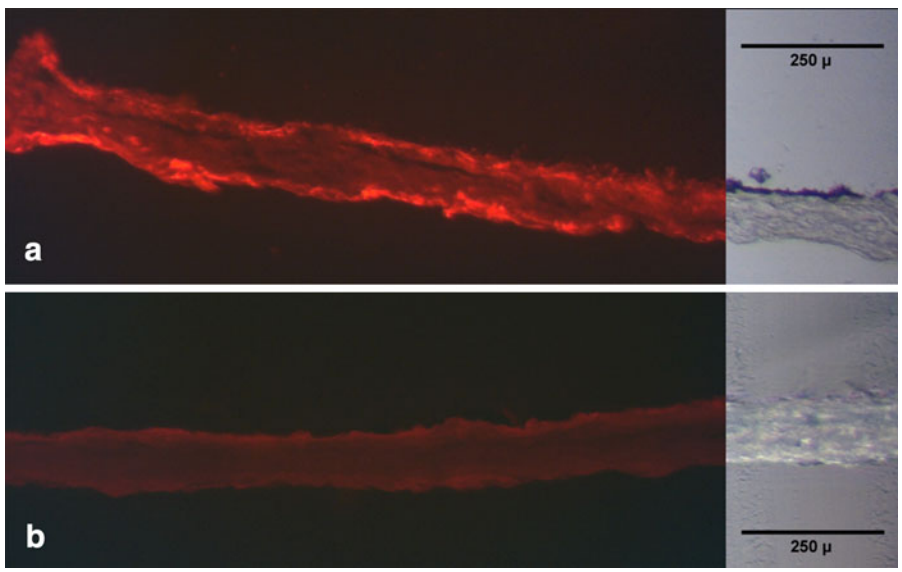

Fig. 6 Distribution of the gelatin conjugate (**a**) and the methylcellulose conjugate (**b**) after immersion in 0.5 % solutions in handmade rag paper, surface sized with gelatin

Figure 6a presents the distribution of the gelatin conjugate in a modern handmade rag paper with a preexisting surface sizing (paper A), after the sample was immersed in the labeled gelatin solution. The fluorescence emitted from the paper surface is more intense than that emitted from paper interior. This indicates that the adhesive solution was deposited on both sides of the sample paper's surface as a layer and at the same time had penetrated the paper interior to a smaller extent.

The triazine conjugate could be successfully used to visualize the distribution of methylcellulose, if the

samples were immersed in a 0.5 % solution (Fig. 6b), although the fluorescence intensity emitted was lower compared to the gelatin conjugate (Fig. 6a). With immersion, the distribution of methylcellulose and gelatin in a rag paper with preexisting surface sizing is very similar. Obviously, in the aqueous environment of the immersion bath, the gelatin surface sizing was swollen to an extent sufficient to allow significant penetration of the dilute gelatin solution from the surrounding bath into the paper interior. The slightly enhanced deposition of the labeled adhesive at the paper surface, which can be observed for gelatin as well as for methylcellulose, might be an effect of reverse migration occurring during drying of the sample papers between absorbent blotters. Although, the results indicate the formation of an adhesive film on the surface, no visual alterations, like surface gloss were noticed when the samples were observed in raking light.

Conclusion

Labeling gelatin and methylcellulose with fluorescent dyes and the subsequent detection with fluorescence microscopy proved to be a suitable method for investigating the distribution of aerosol-misted adhesive solutions used for stabilization treatments in porous substrates. The preparation of a gelatin conjugate with Texas Red™ allowed for an excellent visualization of the distribution of the dilute adhesive solutions (0.5–1 %), necessary for aerosol misting, via fluorescence microscopy. The gelatin conjugate could be purified from excess fluorescent dye, which is a mandatory step for the correct interpretation of the adhesive distribution. As for the methylcellulose conjugate, labeled with Texas Red™ C_2-dichlorotriazine, fluorescence detection was only possible when the methylcellulose was applied in a 0.5 % solution concentration via immersion. When the conjugate was diluted to a 0.25 % solution concentration for misting with the aerosol generator, it could no longer be detected via fluorescence microscopy. This was due to a significantly lower yield in the labeling reaction. To enable visualization of the aerosol-misted methylcellulose, labeling conditions for the triazine label have to be further optimized.

Evaluating the distribution of the fluorescent labeled adhesives brought two new insights relevant for improving stabilization treatments with diluted adhesive solutions. First the results indicate that the deposition of dilute gelatin solutions applied as aerosol is always governed by the same principles: For samples featuring a low porosity, like the sample paper with a preexisting surface sizing, the gelatin is mainly deposited on the surface, while for the highly porous unsized filter paper, penetration dominates over surface deposit. Second, it could be shown that there is a significant influence of the application method on the distribution of diluted adhesives in porous substrates. Using aerosol-misted adhesives the application can be small, which allows a local application to damaged areas, making it a suitable method for stabilizing fragile papers. Of particular interest is the comparison of samples that received aerosol-misted gelatin with or without intermediate drying between applications: The gelatin solution penetrated the pigment layer in both methods, but a deposition of gelatin inside the paper only occurred without intermediate drying. This indicates that the application with intermediate drying is suitable if the pigment layer but not the paper needs to be stabilized, e.g., for artworks featuring a moisture-sensitive paper support that is prone to water stains. It allows a gradual, controlled adhesive delivery: After each application, the artwork can be checked for visual changes and for the degree of stabilization achieved. In comparison, if the aerosol-misted adhesive is applied without intermediate drying, the number of wet-drying cycles the pigment layer undergoes during treatment is reduced, which can minimize the risk of color changes (Michalski and Dignard 1997). Fluorescence labeling also permitted monitoring of changes of the adhesive distribution if immersion was used instead of aerosol application. In contrast to aerosol application, immersion leads to a full penetration of the paper with adhesive, even for papers featuring low liquid water absorbency. This could be observed for gelatin as well as for methylcellulose.

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