Cleaning of Paper Artworks: Development of an Efficient Gel-Based Material Able to Remove Starch Paste

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S Supporting Information

ABSTRACT: The removal of old glue from paper artworks is of paramount importance for the preservation of its integrity during the restoration process. Wet cleaning is one of the traditional methods, although it may cause damages on artworks. In this work, an advantageous alternative method, based on the use of a rigid hydrogel, for a simple and localized removal of starch paste from paper supports is presented. The use of an appropriate hydrogel allows to overcome many of the problems faced by restorers minimizing damages, through a controlled release of water to the artwork, and a simple and not invasive application and removal. At the same time, the specific and targeted enzyme activity leads to a significant reduction in the application time of the cleaning procedure. In this context, experiments were carried out applying Gellan hydrogel carrying α-amylase enzyme on several paper samples soiled with starch paste. To assess the cleaning efficacy of the proposed hydrogel, we have used a multidisciplinary approach, by means of spectroscopic techniques, scanning electron microscopy, chromatographic analysis, and pH investigations.

SEM image of paper sample coated with starch paste

ENZYMATIC GELLAN GEL

SEM image of paper sample coated with starch paste and cleaned with the gel

KEYWORDS: amylase, paper artwork, gellan, hydrogel, cultural heritage, paper restoration
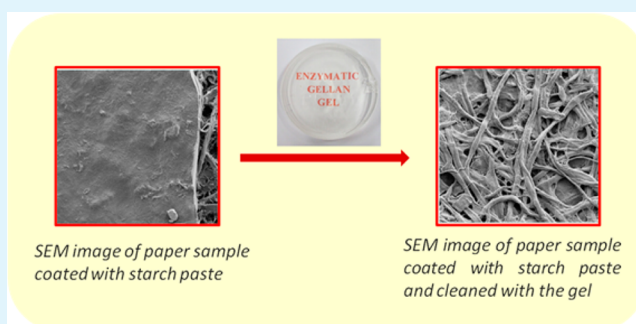
1. INTRODUCTION

Paper artifacts are difficult to restore because of their inherent fragility, degradation processes, and multicomponent composition. Moreover, past maintenance operations can complicate the scenario, as often ancient paper artworks are strengthened by gluing of external material, like wooden or cardboard lining paper, to the original piece. This step adds further problems; for example, the glue used for this assembling undergoes chemical transformations that cause a loss in compactness, yellowing, and an increase of acidity, thus accelerating the mechanical fragility of the artwork itself. Wet cleaning of the sheets represents a critical but fundamental step during paper artwork restoration;[1] the standard cleaning technique involves washing with water and can cause swelling and solubilization of some components.[2] The use of hydrogel represents an alternative to overcome the side effects already reported. Because of the high retention power and viscosity of gels, the penetration of the liquids into the paper artworks is significantly reduced, therefore minimizing damages.[3−7] However, to avoid dangerous microbial growth,[8,9] a complete removal of the gel is required and such a procedure often requires invasive mechanical action (i.e., removal with a brush) or solvents,

often unsafe for the artwork. In this contest, a smart possibility to avoid damages is represented by the use of rigid hydrogels[3,5,10,11] as they can be completely and easily removed in one step, as one body, without leaving residues after their application. The use of rigid gel based on the deacylated polysaccharide Gellan gum and calcium acetate (Gg) has been recently assessed to be as a new efficient wet cleaning agent for paper samples.[2,12,13] Gg is a linear anionic heteropolysaccharide produced by *Pseudomonas elodea*, with a repeating unit of (1, 3)-β-D-glucose, (1, 4)-β-D-glucuronic acid, (1, 4)-β-D-glucose, (1, 4)-α-L-rhamnose. In the presence of calcium ions, it generates hard and rigid hydrogel that is homogeneous, transparent, and stable to pH variations.[11,14−17] The pH stability assures that the gel can be applied to every paper sample, whatever its acidity or in the presence of alkaline reservoir. However, it should be pointed out that being the chemical-physical properties of paper contaminants and degradation products very different (i.e., hydrophilic, hydro-

phobic, or as for the glue, polymeric compounds) multisteps treatments are often required to complete the cleaning procedure. For starch paste removal, for example, traditional methods involve the use of water or mixed water/ethanol packs; innovative cleaning methodologies based on application of viscous media like cellulosic pack[18,19] containing enzymes able to hydrolyze the glycosidic bonds of the starch have been also proposed.

In this paper, we report the results obtained using the rigid Gg as carrier of α-amylase for a localized and selective removal of starch from paper; the aim of the work is thus to establish if Gg loaded with α-amylase enzymes (EGg) produces an efficient removal of starch paste from paper. To this end, experiments concerning a detailed characterization of hydrogel properties in terms of mechanical and structural properties, water loss, translational mobility and activity of the enzymes into the gel during time have been evaluated. Cleaning capability of EGg has been tested on filter paper samples soiled with starch paste, artificially aged or not. The results of our proofs have been analyzed by using several techniques like Fourier transform infrared spectroscopy (FTIR), UV–vis absorption, and fluorescence spectroscopy, optical microscopy, scanning electron microscopy (SEM), pH measurements, and high-performance liquid chromatography (HPLC).

2. EXPERIMENTAL SECTION

Materials. α-Amylase [EC-232–560–9; 30500U/ml], fluorescein isothiocyanate-dextrans (FITC-dextrans) of different molecular weights (average molecular weights: 10 000, 40 000, 70 000, and 15 0000 uma), wheat starch, potassium iodide, calcium acetate, calcium chloride and HEPES buffer were obtained from Sigma (Sigma-Aldrich, Mo, St. Louis, USA). Iodine were from Carlo Erba Reagenti (Carlo Erba Reagenti srl, Milano, Italy). Gellan gum is KELCOGEL CG-LA product by CP Kelco (Atlanta Georgia, USA).

Paper samples, called in the text P1 and P2, were from ALBET (ALBET LabScience, Hahnemühle FineArt, Germany). Their characteristics are density, 80g/m²; rate of filtration, 1196 s/100 mL (P1) and 35 s/100 mL (P2); retention rate, 3–5 μm (P1) and 35–40 μm (P2).

All reagents used were of analytical grade and used without further purification. In all cases, in the preparation of buffer solutions we used bidistilled water (Millipore, Billerica, MA, USA).

Hydrogel Preparation. We followed, with slight modifications, the hydrogel preparation general protocols reported elsewhere.[2,13] To prepare the hydrogel containing calcium ions an aqueous solution of deacylated Gellan gum (20 g/L) and calcium acetate (0.40 g/L) was mixed for almost a minute in a microwaver at 600W (Mars Microwave, CEM Corporation, Matthews, NC, USA) until it boils and becomes transparent; it was than left to cool on a Petri dish. To prepare gels loaded with the α-amylase (EGg), we added an aliquot of a concentrated enzyme solution (490 μM in PIPES buffer (PIPES 25 mM, CaCl₂ 8 mM, pH 6.9) to Gg at 60 °C while it is cooling in a Petri disk. The final enzyme concentration is 24.6 μM (1030 U/mL). To prepare the hydrogel containing sodium acetate (GgNa), we used an aqueous solution of deacylated Gellan gum (20 g/L) and sodium acetate (0.26 g/L), following the same protocol above-described for Gg.

Different dextran polymers were used to load Gg, differing for the molecular weights; in all cases the dextran were labeled with fluorescein isothiocyanate (FITC), as fluorescent dye. To load hydrogels with FITC-dextran, we added an aliquot of a 10 mg/mL of dye aqueous solution to the hydrogel one just before cooling it in a Petri disk. The final dye concentration into the hydrogels was 0.5 mg/mL.

Hydrogel Characterization: Texture Analyzer Experiments. A software-controlled dynamometer, TA-XT2*i* Texture Analyzer (Stable Micro Systems, UK), with a 5 kg load cell, a force

measurement accuracy of 0.0025%, and a distance resolution of 0.0025 mm, was used for the mechanical characterization of the gel samples.[20−23]

The samples, with a diameter of 50 mm and a height in the range 2.0–2.5 cm, were compressed twice with a recovery time in between. The experiments were carried out at room temperature with an ebonite cylinder probe (P/10, diameter of 10 mm), using the following parameters: trigger force, 0.098 N; acquisition rate, 50 points/s; pretest, test, and post-test speed, 1 mm/s; percentage of deformation, 20%; recovery time, 20 s. All experiments were repeated on at least six replicates of each sample and the results are reported as arithmetic mean ± SD.

From the compression test, several parameters can be determined, such as the Young modulus (E), which represents the elasticity of the sample. It can be evaluated in the linear range of deformation (i.e., when the percentage of deformation does not exceed 2–6%), as the slope of the stress/strain plot (see Figure S1 in the Supporting Information), where the stress (σ) and the strain (γ) are, respectively: $\sigma = F/A$, where F is the compression force (N) and A is the cross-sectional area of the probe (m²) and $\gamma = [(h_0 - h)/h_0] \times 100$, where h_0 is the initial height of the sample, and h the height at time t during the compression test.

When the data are reported in a force/time plot (see Figure S2 in the Supporting Information), other parameters can be obtained:

- the hardness, which represents the maximum force required to attain a given deformation, estimated for the first (F1) and for the second (F2) compression.
- the cohesiveness, which shows how well the sample withstands the second deformation in comparison to the first one. It is evaluated as the ratio A2/A1, where A1 and A2 represent the area under the curves.
- the resilience, which shows how good the sample is in recovering its original position during the first compression. It is defined as A4/A3, where A3 represents the area obtained during the compression up to the maximum value, whereas A4 represents the area described during the withdrawal step.

Hydrogel Characterization: Rheological Measurements. Rheological experiments were carried out by means of a controlled stress Haake RheoStress 300 Rotational Rheometer, equipped with a Haake DC10 thermostat. The geometry used for the tests was a cross-hatch plate device (Haake PP35 TI: diameter = 35 mm), in order to reduce the extent of the wall slippage phenomena.[24]

The hydrogel, with a thickness of about 1.0 mm, was laid with care on the lower plate of the rheometer. The upper plate was then lowered until it reached the hydrogel surface. The procedure followed for the sample characterizations was already described.[25] Gap setting optimizations have been undertaken according to the procedure described elsewhere.[26]

Frequency sweep experiments were performed on the hydrogel at 25 °C in the range 0.01–10 Hz, in the linear viscoelastic region, preliminary assessed by stress sweep experiments. The mechanical spectra were then recorded applying a constant deformation ($\gamma = 0.01$) in the linear regime. Stress sweep experiments were performed (25 °C and 1 Hz) in the range 10–10 000 Pa.

Mobility in Hydrogel. The mobility of incorporated macro-molecules was investigated by means of fluorescence recovery after photobleaching (FRAP). Experiments were performed on Gg loaded with different FITC labeled dextrans of molecular weight 10 000 (10M), 40 000 (40M) 70 000 (70M) and 150 000 (150M) uma.[27] Dextrans were used as models of macromolecules having different sizes. FRAP experiments were performed on a Olympus Fluoroview 1000 Confocal Laser Scanning System equipped with an inverted microscope, Olympus IX-81 (Center Valley, PA, USA). Experiments were carried out using a 60×/1.35 oil immersion objective. Bleaching experiments were performed using the 488 nm line of a 30 mW argon ion laser at a maximum of its intensity. Experimentally, 20 prebleach images (resolution: 128 × 128 pixels) obtained at 13% of full laser intensity were collected; then a uniform region of interest (ROI), 40 μm in diameter, was bleached (bleaching time: 2 s). After this

bleaching, 500 images were collected with lower than 13% intensity in order to follow the fluorescence recovery inside the bleached ROI. The time interval between consecutive images was 0.2 s. Diffusion coefficients of dextrans was obtained from the experimental curves by using the equation $D = (w^2/(4\tau_{1/2}))\gamma_D$, where w is the radius of the bleached spot, $\tau_{1/2}$ is the recovery time of the half of the original intensity and γ_D is a constant whose value is 0.88 for circular beam.[28]

Starch Paste Preparation and Removal. The starch paste was prepared mixing 6 g of wheat starch in 15 mL of deionized water. The suspension was stirred for several hours until it became a paste. It was used within few days and stored at 4 °C. In the tests on paper samples 1.6 g of starch paste was spread on circular samples of filter papers (4.8 cm of diameter) by using a soft brush and left to dry for almost 48 h.

To perform our experiments the paper samples with the starch paste was fully covered with the gel; over them was applied PET film (Mylar), uniformly pressed to ensure a close contact between the gel and the sample (usually, on a gel of 6 cm diameter and 1 cm height a weight of 500 g has been applied). The time of the gel application for cleaning was 1 h with the exception of the specific cases below. Gg was also used as control to assess any problems related to the presence of the hydrogel itself in the assay. After treatment time, the gel was removed in one step only by taking it without the use of a spatula or of a liquid component. To perform tests on artificially aged samples, we left the papers coated with the starch for 8 days at 80 °C and 65% relative humidity[1] and then cleaned them with EGg.

Iodometric Assay. At the end of the cleaning time, the enzymatic activity was tested, by following, with slight modifications, the iodometric procedure.[29]

Briefly, the iodometric assay has been performed on circular EGg samples of 9 mm diameter picked up from the gel itself at fixed times (10, 20, 40, 50, 60, and 90 min) during cleaning treatment on the paper coated with starch paste. Every gel sample was mixed with 1 mL of distilled water and 100 μL HCl 1 M, and left at high temperature (150 °C) until it become a solution; then 100 μL of a solution containing KI and I_2, both 5 mM, was added. The solution was then analyzed by UV−vis absorption spectroscopy, using quartz cuvette of 1 mm optical length and monitoring the absorbance of the iodine-soluble starch complexes.

Paper Sample Characterization: FTIR and SEM Analyses. FTIR spectra were acquired on a Thermo-Nicolet (mod. Nexus 670) instrument (Thermo Scientific Inc., Madison WI), equipped with an attenuated total reflectance (ATR) ZnSe cell for measurement in the 4000−700 cm^{-1} region, at a resolution of 4 cm^{-1}. Spectra were collected by placing the paper samples directly on the ATR cell. A total of 256 scans were collected for each sample.

Scanning electron microscopy was performed using a field emission scanning electron microscope (FE-SEM), (SUPRA 35, Carl Zeiss SMT, Oberkochen, Germany). Punched samples were previously metalized to allow electronic conduction on the sample surface in order to have high quality images without deteriorating the samples or creating any kind of artifacts. The metallization, 1 min at 25 mA, was performed using a sputter coater (EMITECH K550X, Quorum Technologies Ltd., West Sussex, United Kingdom) with a gold target. The detector used was the Second Electron detector as the interest was mainly focused on the morphology of the paper fibers and on the presence of residues from the cleaning agents; the main operating parameters of the instrument were 10 keV as gun voltage and a working distance of about 8 mm.

Paper Sample Characterization: Chromatographic Analysis and pH Measurements. The HPLC system consisted of a modular CHROMQUEST spectra system from THERMOQUEST (San Joes, CA, USA), equipped with two LC-10AT Vp pumps, Schimadzu UV−vis spectrometer model (SPD-10AV) detector. A SCL-10A Vp controller operated the HPLC system working under control of software included in the CHROMQUEST module. The chromatographic separation was performed using a reverse phase C18 stainless steel column (5 μm 150 × 4.6 mm I.D - VYDAC™, W.R. Grace &Co, USA). The composition of the mobile phase was 25 mM phosphate buffer of aqueous solution at pH 2.4 and 1% (v/v) methanol with a flow rate of 0.7 mL/min and using a detection

wavelength equal to λ = 210 nm.[30] Analysis was performed before and after both cleaning treatments (by Gg and EGg). Each chromatographic analysis was repeated three times in the same day (reproducibility intraday RSD% = 2%) and on different days (reproducibility interday RSD% = 1%) for all the samples. Also the measurements were carried out three times on the same sample extract (RSD% = 2%) and on three different extractions of the same paper sample (RSD % = 4%) The chromatographic analysis was performed on extracts obtained by treating 1 cm^2 of each sample (paper or hydrogel) with 1 mL of distilled water, stirring overnight at room temperature. Measurements of pH were carried out on the aqueous extract, obtained as previously described, before and after hydrogel treatments[31] by using an Amel Instrument 334-B pHmeter with a combined glass electrode Ag/AgCl 6 mm (Amel Instrument, Italy).

Protein Assay. To evaluate the presence of enzyme residues on certified paper samples after gel treatment, we have performed a Bradford assay[32] on water extract of each paper sample, obtained as described in the previous section. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a color change.

The standard Bradford assay consists of mixing 1 part of the protein solution at different concentrations with 30 parts of the Bradford Reagent and measuring the absorbance at 570 nm at room temperature. The protein concentration is determined by comparison to a standard curve obtained using bovine serum albumin. The Bradford assay for paper extracts was carried out using a 96 well plate (polystyrene microtiter plates, MaxiSorp, purchased from NUNC - Roskilde, Denmark). A model 550-Microplate Reader (Bio-Rad Laboratories, CA, USA) was used to read the absorbance on ELISA plates at 570 nm.

3. RESULTS AND DISCUSSION

Hydrogel Mechanical and Rheological Characterization. The hydrogel under study must fulfill several requirements: (1) it must be sufficiently rigid to be applied and removed on a paper sheet as one body; (2) it must release water in a controlled manner, that is, paper artwork must receive from the gel enough water to be cleaned but in a less extent than a water bath, thus minimizing the swelling; (3) a weight must be applied on the hydrogel for almost 1 h without causing gel brittleness or fragmentation; (4) its mesh size must be sufficiently large to allow the diffusion of enzymes and of degradation products removed from paper. In this contest it is well-known in literature[11,14−17] that the properties of Gellan gum gels are deeply influenced by the presence of cations: an insufficient cation concentration leads to the formation of weak and extensive gels. At the same time, keeping the Gellan and cation concentrations constant, the ionic charge plays a fundamental role in the polysaccharide gelling properties. To this end, a detailed characterization of the hydrogel under study is necessary; furthermore, for an appropriate comparison, the compressibility and rheological properties of GgNa, a Gellan gel containing the same concentration of polysaccharide and an equivalent amount of sodium acetate have been evaluated.

The data reported in Table 1 show that the sample prepared with Gg has a high value of the Young modulus and of the hardness, indicating the strong interaction of the junction zones of the polymer with the bivalent salt. Moreover, compression tests (see Figure S1 in the Supporting Information), show that in the low deformation range (5−10%), Gg (or EGg) undergoes stress values in the 20−60 kPa range, significantly higher than the ones applied on the gel during the cleaning procedure. This finding suggests the fact that during the proposed paper treatment, the gel does not change its shape.

Table 1. List of the Parameters Evaluated with a Compression Test by Using the Texture Analyzer Equipment: Young Modulus (E), Hardness (F1 and F2), Cohesiveness (A2/A1), and Resilience (A4/A3) for the Gg and GgNa samples

sample	E (N/m^2)	F1 (N)	F2 (N)	(A2/A1)·100	(A4/A3)·100
Gg	$(5.8 \pm 0.6) \times 10^3$	9 ± 1	4.7 ± 0.4	44 ± 6	45 ± 2
GgNa	$(6.5 \pm 0.9) \times 10^2$	1.4 ± 0.2	1.3 ± 0.6	97 ± 3	67 ± 3
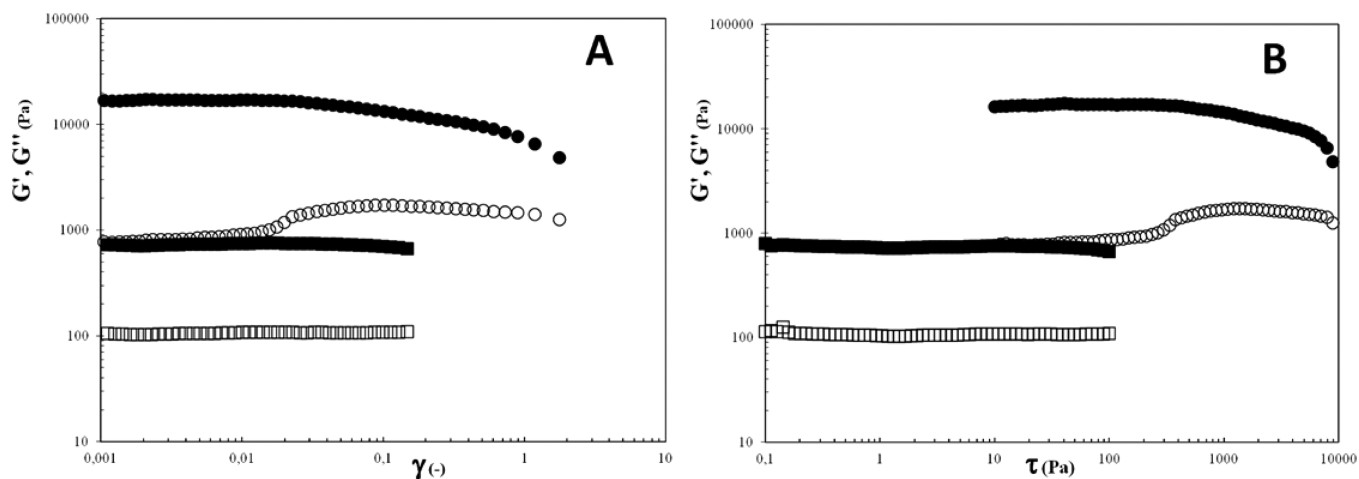
Figure 1. (A) Stress sweep and (B) viscoelasticity plots for Gg ($\bullet$, $\circ$) and GgNa ($\blacksquare$, $\square$) at cp = 2.0% (w/v) (1 Hz, T = 25 °C, G', full symbols; G'', empty symbols).

At the same time, the cohesiveness value is low, due to the rigidity of the sample, which is not capable to stand large deformations. For this reason, the F2 and A2 values of the samples prepared with the calcium ions are much lower for the second compression, than those recorded during the first compression (see Figure S2 in the Supporting Information and Table 1). At the same time, also the resilience value is low.

On the contrary, GgNa samples are characterized by high values of cohesiveness and resilience: the weak interaction between the junction zones and the monovalent ions leads to the formation of a more flexible gel, able to stand greater deformations, with Young modulus and hardness values characteristic of hydrogels much weaker, when compared to Gg.

Actually, the three-dimensional network built in the presence of sodium ions is significantly different from the gel obtained when the divalent calcium ions are involved. Thus, the Gg samples show high rigidities, which implies that when an appreciable deformation is applied, only a slight recovery of the original shape is observed. On the other side, the electrostatic interactions between the monovalent ions and the Gellan chains lead to a network relatively weak and flexible, which is capable of recovering the initial structure within a small period of time, thanks to the reversible nature of the physical interactions.

Figures 1 and 2 show the oscillatory tests of Gg and GgNa samples, recorded at 25 °C. As shown in Figure 1, Gg exhibits a polymeric network with a high rigidity and with a range of linear viscoelasticity that holds, approximately, up to $\tau_c \approx 476$ Pa (critical stress). Remembering that between the critical stress (τ_c) and the critical deformation (γ_c) the following relation holds:[24] $\gamma_c = \tau_c / ((G')^2 + (G'')^2)^{0.5}$, we obtain that $\gamma_c \approx 0.03$ for the Gg sample. Significantly different is the situation for the other sample, GgNa (Figures 1 and 2 and Table 2). In both cases much lower values for τ_c were calculated, indicating that the GgNa starts to deviate from the viscoelastic behavior
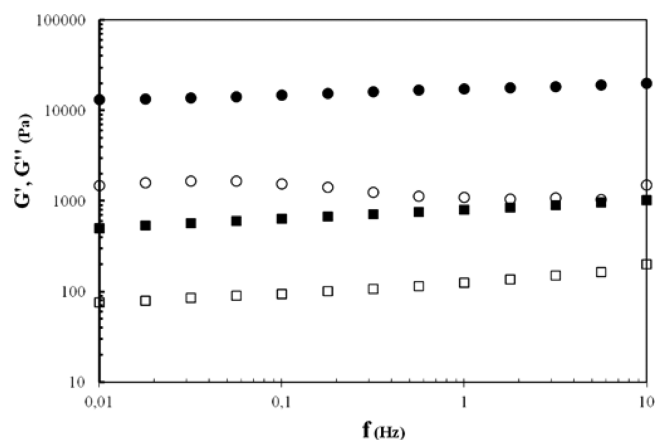
Figure 2. Frequency sweep for Gg ($\bullet$, $\circ$) and GgNa ($\blacksquare$, $\square$) at cp = 2.0% (w/v) (γ = 0.01, T = 25 °C, G', full symbols; G'', empty symbols).

Table 2. Storage and Loss Moduli (G', G'') Recorded at 1 Hz, Phase Differences between Stress and Strain (δ), Critical Stresses (τ_c), and Critical Strains (γ_c) for the Gg and GgNa Samples

sample	G' (1 Hz) (Pa)	G' (1 Hz) (Pa)	δ (1 Hz) (deg)	τ_c (Pa)	γ_c (%)
Gg	17450	1100	3.6	476.4	2.9
GgNa	807	125	8.8	70.4	9.9

when stresses much lower than those needed in the case of Gg sample are applied. Similarly, the percentage of elongation within the linear range is very low (2.9) for the most rigid network of Gg and significantly higher in GgNa (9.9), which shows characteristics typical of more flexible systems.

According to the results obtained in the stress sweep experiments, the frequency sweep analysis was carried out in

the range of linear viscoelasticity at a constant deformation, $\gamma = 0.01$. Figure 2 shows that Gg behaves like a gel, being fulfilled the three conditions required for the definition of a gel from the rheological point of view:[33,34] the moduli are parallel to each other and independent from the frequency, and the storage modulus G' is considerably larger (preferably more than ten times larger) than the loss modulus G'' in the overall frequency window explored (three decades).[14,35,36] Moreover, the elasticity of the gel is very high as indicated by the value of the G' recorded, for example, at 1 Hz (17450 Pa). Also GgNa sample shows spectra typical of gels but now the storage and loss moduli values are more than ten times lower than the values recorded for Gg. Furthermore, also a slightly dependence of G' and G'' from the frequency can be detected. This is indicative of the presence of a three-dimensional network whose structure is actually "less strong", more loose than that observed for the Gg sample. As previously evidenced with the texture analysis, the rheological differences are strictly related to the change of connectivity obtained when monovalent (Na^+) or divalent (Ca^{2+}) ions interact with the junction zones of the Gellan chains. The solidlike character can be inferred also in terms of the δ value, where $\tan \delta = G''(\omega)/G'(\omega)$: thus, for a solidlike system δ is very low (for an ideal solid $\delta = 0°$), whereas for a liquidlike system δ assumes high values (for an ideal liquid $\delta = 90°$). Between the tested samples, Gg is the strongest gel, showing a smaller δ value (3.6) in comparison with that found (8.8) for the network prepared in the presence of Na ions.

All the data reported suggest that Gg represents a suitable gel for our purpose. Its high values of Young modulus and G' indicate that this gel is highly elastic and therefore more eligible for handling without risk of breakage and/or leaving of residues. GgNa, indeed, is more resistant to deformation, but the critical stress, from which starts to noticeably warp, is much smaller; therefore, there is a greater likelihood of loss of residues during the treatment. On the contrary, Gg can undergo stresses more compatible with those used in the cleaning procedure applied in the present work.

Finally, to definitely assess the validity of Gg for our purpose, we have measured the physical characteristic of the gel and the water uptake from P1 paper sample during the cleaning treatment. In 1 hour cleaning process, the weight and height decreases of the gel are 3.5 and 8.4%, respectively (see Figure S3A in the Supporting Information), confirming that the gel does not change significantly during the treatment. At the same time, the weight increment of paper samples, due to water uptake, is 150% (see Figure S3B in the Supporting Information), significantly lower with respect to a water bath (water uptake 314%); this means that the release of water from the gel to paper artworks is significantly reduced, therefore minimizing damages like swelling of cellulosic fibers.

Hydrogel Characterization: Fluorescence Recovery after Photobleaching. In this work, we have tested Gg as carrier for active α-amylase enzymes, able to degrade starch paste in order to facilitate its removal from paper samples.

The loading features of the hydrogel are therefore key properties for our purpose because, in the gel, α-amylase must remain active and have a good mobility to reach the gel surface and digest starch; moreover, the gel must be able to entrap the hydrolysis products.

Fluorescence recovery after photobleaching (FRAP) experiments have been used to evaluate the translational mobility of molecules into hydrogel, directly related with their availability on the hydrogel surface. FITC-labeled dextrans, with different molecular weight, have been exploited as model compound to evaluate the molecular diffusion in the proposed hydrogels.

As can be seen in Table 3, the mobility appears restricted in hydrogel phase, as the diffusion coefficient is reduced with

Table 3. Hydrodynamic Radii (R_H), Diffusion Coefficients in Free Water (D_0), the Obtained Mobile Fraction (k), and the Diffusion Coefficients in Gg of of FITC-Dextrans (D_E)

probe	$\tau_{1/2}{}^a$ (s)	R_H (nm)	D_0 ($\mu m^2/s$)	$D_E{}^a$ ($\mu m^2/s$)	k^a
10 M dextran	1	2.0^b	98^b	89	1.0
40 M dextran	2	4.8^c	70^c	45	1.0
70 M dextran	8	6.6^b	42^b	11	1.0
150 M dextran	15	8.3^d	27^d	6	0.7

^{a}Obtained from the experimental recovery profile. ^{b}See ref 19. ^{c}See ref 42. ^{d}See ref 40.

respect to the value obtained in solution especially for larger dextrans, probably due to a hindrance exerted on probes by polymer chains.[37,38] Moreover, the decrease in the content of the mobile fraction in the case of 150 M dextran inferable from the decrease of the k value in Table 3, suggests that the mesh size of the investigated hydrogel is roughly between the average dimension of the 70 and 150 M dextran (6.6 and 8.3 nm, respectively),[38,39] compatible with the dimension of our enzyme (which hydrodynamic radius R_H, is 3.2 nm).[40] Notwithstanding these differences, overall these measurements show that a high fraction of molecules is able to diffuse in Gg, making it useful as carrier for cleaning agents on paper samples.

SEM images (Figure 3) confirm these results: they show the presence of thick string networks forming pores of different sizes in the μm range, some of them are however well below 0.1 μm.

Hydrogel Application on Paper Samples: Starch Paste Removal by EGg. Gg represents per se a valid alternative to the traditional methods used in the restoring of paper. Paper samples indeed, belonging to different composition and degradation conditions, treated with Gg, are visibly cleared. Chromatographic and spectroscopic experiments assess the removal of dust and cellulosic degradation products from all the samples under examination.[2,5,13] However, its versatility makes possible another fascinating possibility, namely the ability to entrap enzymes, opportunely selected to improve the removal of specific contaminants. The efficacy of this cleaning method has been assessed by using several techniques on specimens made of filter paper and starch.

First, the activity of the enzyme into the gel has been assessed, using the procedure reported in the material and method section.

As shown in Figure 4, EGg not only is able to digest the starch paste, but also it retains a good activity with time, at room temperature; the amount of the solubilized starch, indeed, increases with the gel application time. Similar experiments, performed using Gg, have given not significant results (not shown), indicating that a high-molecular-weight starch is not able to diffuse into the gel.

On the basis of UV–vis absorption results, we have chosen 60 min as the optimal time for the application of EGg on paper samples because it represents a good compromise between an efficient cleaning procedure and an excessive treatment time.[2−5]

SEM and FTIR performed on differently treated paper strips are in accordance with UV–vis measurements. SEM images
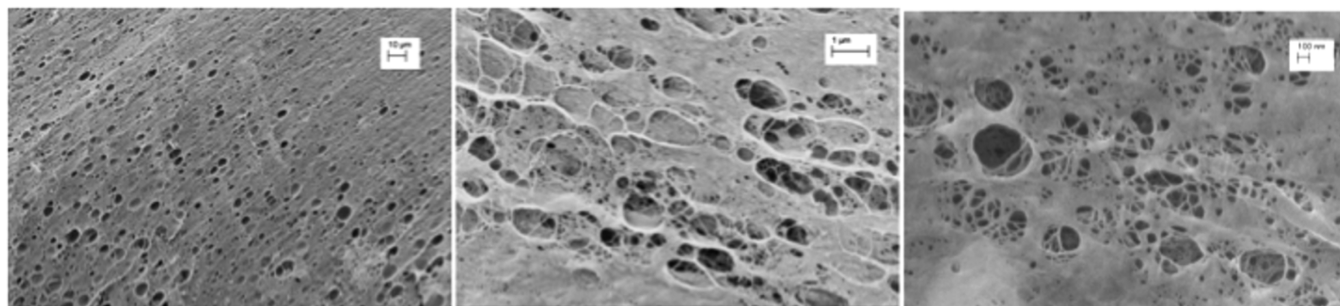
Figure 3. SEM images of freeze-dried Gg sample at different magnifications.
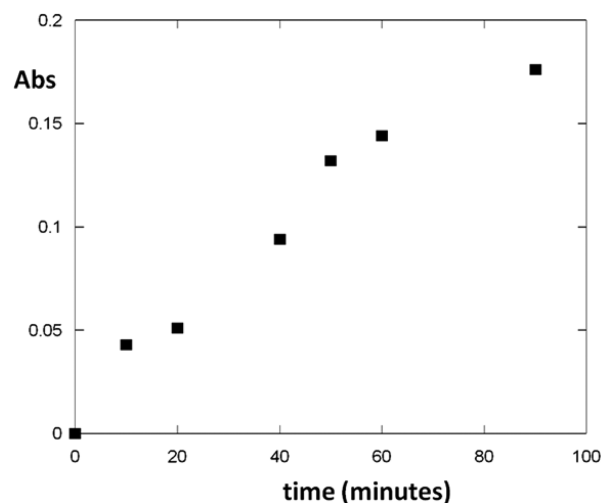

Figure 4. UV–vis absorption data (λ = 355 nm) of the starch-iodine complex into EGg as a function of the gel application time on starch-paste coated paper sample (RSD%=5%).

(Figure 5) show that starch coated paper, treated with the EGg, appears cleaned, and closely resembles the starting uncoated paper. At the same time, in the SEM image of starch-coated paper cleaned with Gg, the patina due to starch paste is clearly evident, although to a lesser extent with respect to the coated and not cleaned sample. The similarity between uncoated and starch coated paper, treated with the EGg, paper samples is confirmed also by optical microscope images (see Figure S4 in the Supporting Information) and visual inspection (see Figure S5 in the Supporting Information). In addition, optical microscope and SEM images demonstrate that gel treatment does not induce damage, i.e. swelling or fraying, in the paper.

As shown in Figure 6A, the FTIR spectrum of paper coated with starch paste and treated with EGg, is similar to that of the uncoated paper, showing that such cleaning procedure is effective in removing starch paste. On the contrary, the spectra of paper coated with starch paste untreated or treated with Gg, are comparable, indicating that Gg alone is not able to remove significantly the starch paste.

More in detail, the paper coated with starch paste shows an FTIR absorption spectrum different from that of the starting paper, because of the presence of the glue. Starch paste, indeed, shows a band at 998 cm^{-1}, whereas cellulose has an absorption maximum at 1024 cm^{-1}.[19] The absorbance ratio between these two peaks (Abs(1024)/Abs(998)), called R in the following, is therefore diagnostic to verify the efficiency of the cleaning
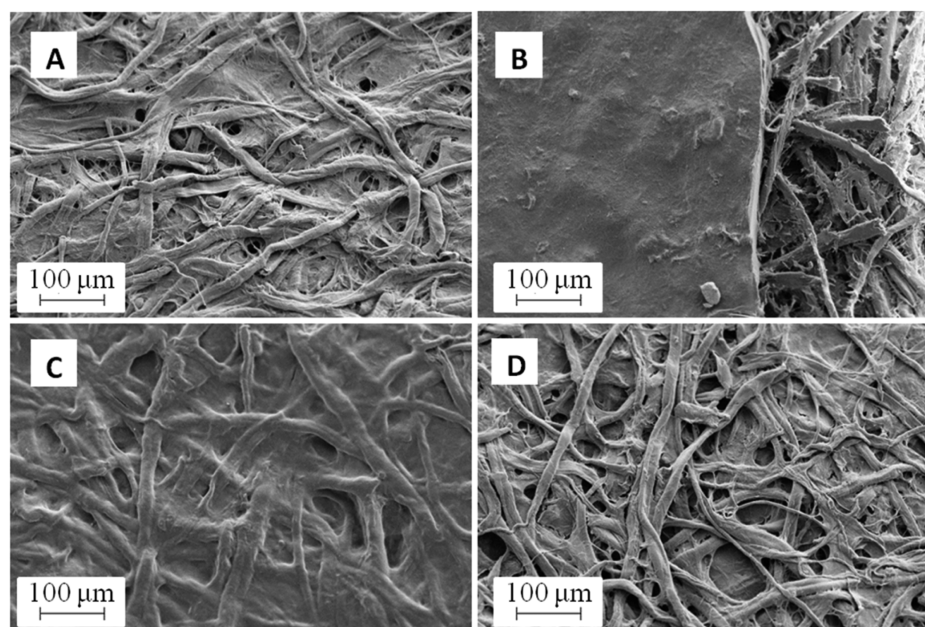

Figure 5. SEM images of (A) P1 uncoated paper sample, (B) paper sample partially coated with starch paste, (C) paper coated with starch paste after cleaning with Gellan gel, and (D) paper coated with starch paste after cleaning with Enzymatic Gellan gel.
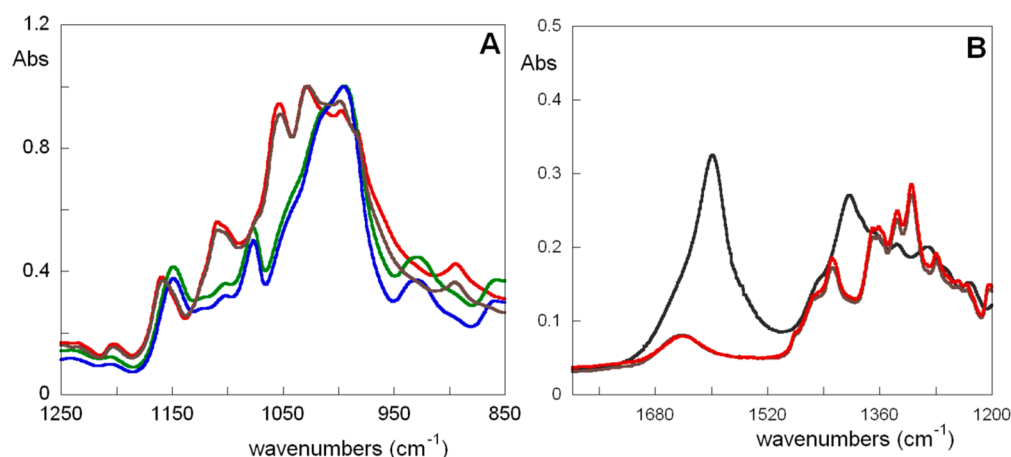
Figure 6. FTIR spectra of (A) P1 paper samples uncoated (red line), coated with starch paste (blue line), coated with starch paste after cleaning with Gg (green line), and coated with starch paste and cleaned with EGg (brown line). Spectra are normalized to the maximum for clarity. (B) Gellan gel (gray line), P1 paper sample, uncoated (red line), and coated with starch paste and cleaned with EGg (brown line).

procedure. This ratio is 1.10 ± 0.03 for untreated paper and decreases to 0.84 ± 0.05 when paper was coated with starch paste. Treatment with EGg causes an increment of this ratio to 1.05 ± 0.06, very close to that of the uncoated paper; this is not the case when the starch paste coated paper was cleaned with Gg (R: 0.82 ± 0.06).

To investigate about the presence of enzymes into paper after EGg treatment, we have performed the Bradford assay on paper samples before and after any treatments (coating with starch paste and cleaning procedures). The paper sample, used in this study, does not contain any proteins, therefore the color of the acidic solution of Coomassie Brilliant Blue G-250 does not change after the addition of water extracts of uncoated paper samples. As shown in Table 4, after coating with starch

Table 4. Absorption Values Obtained Performing Ten Times the Bradford Assay on Paper Samples

paper samples	uncoated paper	coated paper, not cleaned	coated paper cleaned with Gg	coated paper cleaned with EGg
P1	0.17 ± 0.01	0.31 ± 0.01	0.22 ± 0.02	0.23 ± 0.04
P2	0.19 ± 0.01	0.23 ± 0.02	0.21 ± 0.02	0.22 ± 0.03

paste, the absorbance increases, probably because the used glue contains proteins. Cleaning treatments, regardless the type of gel used (Gg or EGg) cause a decrease of the protein content. In addition, absorption values obtained after cleaning using Gg and EGg are also comparable, indicating that EGg does not release enzyme and not increase the contribute to the protein content on samples.

HPLC experiments (Figure 7) on water extracts of P1 paper samples, after treatment with hydrogels (with and without enzyme), were also performed. The results showed that the peaks due to glue (localized at 5 and 8 min) decrease more after application of EGg than Gg indicating that the cleaning treatment with EGg is more efficient than the application of the Gg alone.

Finally, the pH measurements confirm the efficacy of the cleaning methods adopted on fragments. The aging of paper samples are often coupled indeed, with increasing of acidic components, that, in turn, promotes further sample degradation.[1,41] For this reason, the removal of these components and the retrieval of safety pH values are crucial for the outcome of
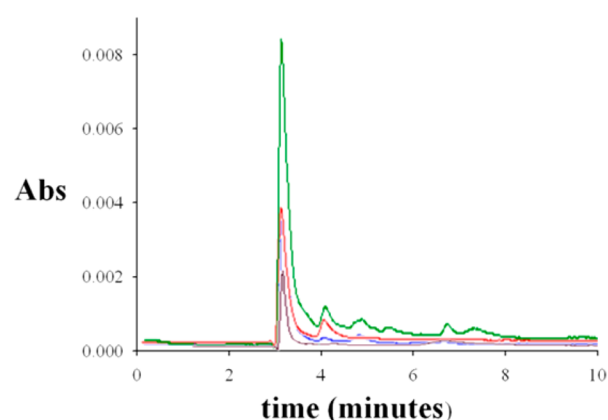

Figure 7. HPLC measurements on water extracts of P1 paper samples before and after treatment with enzymatic gel. Comparison of the obtained chromatograms for: (green) paper sample coated with starch glue; (blue) uncoated paper sample; (red) paper coated with starch paste after cleaning with Gg; (gray) paper with starch paste after application of EGg.

the maintenance and/or restoring operations. In all cases, the pH of the paper samples uncoated and soiled with starch paste after Gg and EGg treatments, increases slightly, passing from pH = 6.0 ± 0.2 to 6.5 ± 0.2 and 6.4 ± 0.2, respectively. The increase is due to the cleaning capability of the gel, able to remove patina and dust that cause a pH decrease.[1,2] The presence of starch paste does not change significantly the pH of the paper samples (data not shown).

Presence of Gel Residues and Bacterial Growth. Gg has been designed to suit the needs of restorers. To assess the efficacy of our proposed method, one other question must be taken into account, that is the presence of residues adsorbed on paper that can lead to bacterial growth on artworks over time after gel treatment. As regards the first investigation, the water extract of Gg (see Figure S6 in the Supporting Information) shows a chromatographic profile different to those of paper samples, characterized by two main peaks (retention times: 2.70 and 4.30 min), totally absent in the chromatograms obtained from water extracts of paper samples cleaned with the EGg (see Figure S6 in the Supporting Information). IR spectra (see Figures 6B and S7 in the Supporting Information) confirm these data, as the absorption peaks (localized at 1600 and 1404

cm^{-1}) due to Gg are not present. Furthermore, optical microscope (see Figure S4 in the Supporting Information) and SEM (Figure 5) images of P1 samples, untreated, starch coated, and cleaned with the EGg appear similar and no contaminants like gel residues are evident in the cleaned sample. The absence of residues makes it unlikely the possibility of bacterial growth; in this contest, the Bradford assays have been performed on paper samples, both immediately and several months after the treatment with the gels, giving comparable results; this finding suggests that there is not, on treated paper samples, an increase in protein content, index of bacterial growth. Such results are also confirmed by optical microscope images (performed on samples cleaned with EGg several months before). The FTIR spectra of paper samples coated with starch and cleaned with EGg about a year before (see Figure S7, left, in the Supporting Information) show no bands at 1650 and 1560 cm^{-1} (due to vibrational stretching and bending of amide groups in proteins) or bands at 1739 cm^{-1} (corresponding to the vibrational stretching of carboxylic groups from membrane lipids and fatty acids). This result confirms the absence of bacterial cultures on paper.[43]

Aging Effect. To further test the efficiency of cleaning of the proposed hydrogel, we have applied EGg to paper soiled with starch paste and then aged, as representative of old paper glued with this material. The FTIR spectra of artificially aged samples cleaned with EGg closely resemble those obtained with fresh samples, indicating that enzymatic gel is equally effective also on aged samples (Figure 8). The R value is 1.04 ± 0.03 for
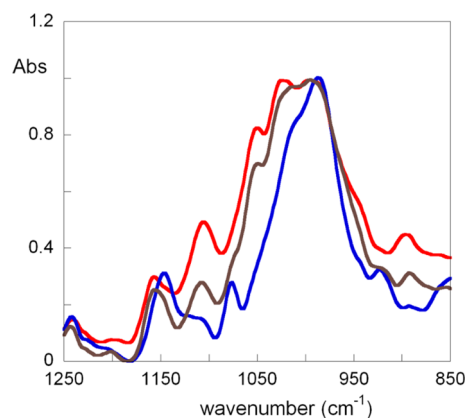

Figure 8. FTIR spectra of aged P1 paper samples uncoated (red line), coated with starch paste (blue line), and coated with starch paste and cleaned with EGg (brown line). Spectra are normalized to the maximum for clarity.

uncoated aged paper and decreases to 0.88 ± 0.05 when paper was coated with starch paste and aged. Treatment with EGg causes an increment of R to 1.00 ± 0.05, very close to that of the uncoated paper.

The cleaning procedure, also in this case, causes a slight pH increase, as its value is 5.7 ± 0.2 and 6.2 ± 0.2 for the aged paper sample uncoated and soiled with starch paste and treated with EGg.

Paper Sample Porosity Effect. Depending on the age and the raw materials used, the physical characteristics of the paper material can be very different; consequently, the gel treatment time to ensure cleaning can change according to these differences. Therefore, once assessed the activity of the EGg we applied our cleaning method on different paper strips (P2)

coming from the same company, characterized by the same composition and manufacturing procedure of P1 samples, but with different porosity, in order to test how cleaning time varies when the EGg was applied on papers with different characteristics (like porosity). FTIR spectrum of P2 paper strips coated with starch paste and then cleaned with EGg is different to those of coated but not cleaned or uncoated ones (Figure 9). At the same time, the R values for these three
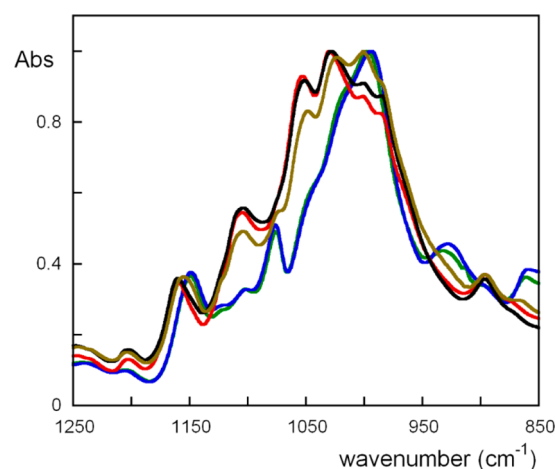

Figure 9. FTIR spectra of P2 paper samples uncoated (red line), coated with starch paste (blue line), coated with starch paste after cleaning with Gg (green line), and coated with starch paste and cleaned with EGg for 60 min (brown line) and for 90 min (black line). Spectra are normalized to the maximum for clarity.

samples are 0.93 ± 0.06, 0.74 ± 0.04, and 1.15 ± 0.06, respectively. These results indicate that after 60 min of treatment starch paste was only partially removed from paper samples. In order to remove the whole starch coating, the treatment time has been prolonged up to 90 min; the spectrum of P2 paper strips coated and cleaned with EGg for 90 min is very similar to that of the uncoated one (Figure 9) and the R values of this two samples are very close together (1.12 ± 0.04 and 1.15 ± 0.06 respectively).

These results confirm that the optimum treatment time depends on paper characteristics. As P2 is more porous than P1, the starch paste has been absorbed more deeply into the pores. For this reason, water released by EGg employs more time to penetrate inside pores and clean them. It should be pointed out that, also after 90 min treatment, the water uptake from the gel to P2 paper sample is 215%, significantly less with respect from a water bath (water uptake: 360%) thus reducing damages like swelling of cellulosic fibers. In this contest, SEM images (see Figure S8 in the Supporting Information) of starch coated paper treated with EGg is similar to that of the uncoated one, indicating the absence of swelling phenomena and the efficacy of the EGg treatment.

Also in this case, the spectra and the R values of the coated papers cleaned with Gg are similar to that of the not cleaned ones demonstrating the ineffectiveness of this treatment. It should be noted that, also in this case, a slight pH increase after Gg and EGg treatments is observed as pH varies from 6.0 ± 0.2 to 6.2 ± 0.2 and 6.5 ± 0.2, respectively. In addition, EGg does not induce enzymes adsorption on paper strips as protein contents on samples measured before and after cleaning remain substantially constant, and, also in this case the FTIR spectra of paper samples coated with starch and cleaned with EGg about a

year before (see Figure S7, right, in the Supporting Information) show no bands due to proteins or lipids, confirming the absence of bacterial cultures on paper.

Finally, preliminary experiments performed also on paper fragments coated with starch paste belonging to books of 18th and 20th centuries, indicates that the optimal time treatments are always in the range 60–100 min, assessing the general validity of the proposed cleaning procedure.

CONCLUSION

In this work, the efficacy of an innovative alternative to traditional methods for a simple and localized removal of starch paste from paper supports is assessed. The proposed systems are based on rigid hydrogels, made of the polysaccharide Gellan gum and calcium acetate spiked with hydrolytic enzymes. Their rigidity makes it easy to be totally removed as one body, without leaving fragments and avoiding damages to paper artwork; at the same time, the enzymes work as specific cleaning agents in order to remove starch paste with high yield in a reasonable time. These results indicate that our system represents a promising method to clean paper artworks from starch paste, as it is efficient, easy-to-prepare, and relatively cheap and safe. In this contest, our works constitute first but fundamental step for a deeper study focused on the development of hydrogels with peculiar characteristics for paper cleaning. Preliminary studies about the dependence of gel treatment as a function of paper characteristics have been performed.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures regarding the characterization of the gel under study, and the absence of gel residues and of bacterial growth on gel cleaned paper samples. The material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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