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A NEW SUSTAINABLE AND INNOVATIVE WORK FOR PAPER ARTWORKS CLEANING PROCESS: GELLAN HYDROGEL COMBINED WITH HYDROLYTIC ENZYMES

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Abstract

Paper has been used as writing and drawing support for thousands of years. The conservation of paper artworks plays a fundamental role in the field of our cultural heritage. Moreover, restoration of paper artworks is difficult due to their inherent fragility, the presence of many components and their degradation state. Among the factors that may contribute to paper deterioration are the use of glue for the application of different materials (as a lining, mounting or as a repair intervention) on the paper artifact. During a natural ageing process, glue become yellow, acid and less compact, accelerating the degradation processes of the artwork itself. The removal of glues from paper artworks represents, therefore, an important procedure for their preservation. Here we present a sustainable alternative to the common removal systems (e.g. solvents or localized enzymatic packs on the support to be cleaned). For this goal we used a rigid Gellan hydrogel (totally removable in one step) containing hydrolytic enzyme, such as proteinase K. The enzyme works as a selective cleaning agent hydrolyzing animal glues into smaller fragments, soluble into the gel. Our system represents an effective alternative to the traditional techniques because it is easy to be prepared, eco-friendly and efficient.

Keywords: *Enzymatic Gellan gel; Glue; Paper artworks; Cultural heritage*

Introduction

Paper based artworks are very complex materials, difficult to be restored, due to its fragility, its degradation process and the presence of several components in it. The main paper component is cellulose, a polysaccharide made up of β-glucose units linked together by $\beta_{(1\rightarrow4)}$ glycosidic bonds [1]. Structural changes due to ageing can lead to a decrease in the stability of the material and in its strength, and a change in color [2, 3]. During restoring of paper artifacts, critical steps are the cleaning of the sheets, the pH change, the optimization of the degree of humidity, and the glue removal [4, 5]. **Gellan** gel made up of deacylated Gellan gum and calcium acetate has been used since 2003 as cleaning agent for paper artworks at the Istituto Centrale per il Restauro e la Conservazione del Patrimonio Archivistico e Librario (ICRCPAL,

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Rome, Italy) [6]. This gel is transparent, thermoreversible, rigid and stable to pH variations. The gel has a high level of the water uptake capability and high viscosity values; for these characteristics, when the gel was used for a cleaning treatment, the penetration of the liquids into the paper artworks was significantly reduced, minimizing damages [7-9].

The gel acts gradually releasing water [10], at the same time absorbing the water-soluble degradation products present on paper. Finally, due to its viscoelastic properties, its application and removal are fairly simple; indeed, it can be applied in one step as one body, thus avoiding residues. However, it should be pointed out that the chemical-physical properties of the paper contaminants and the degradation products are very different (i.e. hydrophilic, hydrophobic or, as for the glue, polymeric compounds), then a multi-step approach is often required to complete the cleaning procedure [10, 11]. In this contest, another important issue in the paper restoration field is represented by the presence of animal glue often present on paper artworks due to actual repair interventions of past restorations. Naturally, aged animal glue is often very difficult to remove by using Gellan Gel alone. The removal of old glue from artworks is very important for their preservation; during ageing, in fact, the structural transformations of the glue produce yellowing, a loss in compactness, and an acidity increase, thus accelerating the degradation of the artwork itself. In this study, we have evaluated the possibility of using Gellan Gel as a carrier of a tuned cleaning agent. In this case, a Gellan Gel containing hydrolytic enzymes (*proteases*) could be applied on paper samples to remove glues with a significant reduction in the cleaning application time due to the specific and targeted enzyme activity. In this system, indeed, the enzyme works as a selective cleaning agent while the rigid gel plays the role of support -water released system- and removal matrix for the enzymatic products. Hydrolytic enzymes, in a rigid hydrogel, allow a biocompatible cleaning and represent an interesting and alternative to traditional methods (i.e., use of water or mixed water/ethanol packs or enzymatic cellulosic packs) [12, 13].

In this work, preliminary results assessing the efficacy of this Gellan Gel containing hydrolytic enzymes has been presented. To this end, we have characterized glued paper samples before and after the cleaning process by means of several techniques like Fourier transform infrared spectroscopy (FTIR), UV-Vis absorption, scanning electron microscopy (SEM) and pH measurements.

Experimental

Materials

Proteinase K from *Tritirachium album* [EC-3.4.21.64, ≥30U/mg], calcium acetate, calcium chloride, the Bradford assay kit, and PIPES buffer were obtained from Sigma (Sigma-Aldrich, Mo, St. Louis, USA). Gellan gum was KELCOGEL® CG-LA product by CP Kelco (Atlanta Georgia, USA). All reagents used were of analytical grade and used without further purification. In all cases, in the preparation of buffer solutions bidistilled water (Millipore, Billerica, MA, USA) was used. Animal glue, obtained from waste (such as bones and skins) of rabbit was a gentle gift of restorers.

Paper samples, called in the text P1 and P2, were from ALBET (ALBET® LabScience, Hahnemühle FineArt, Germany). Their characteristics are density: 80g/m^2; rate of filtration: 1196s/100mL (P1) and 35s/100mL (P2). Unless specified, experiments are performed on P1 paper samples.

Hydrogel preparation

The hydrogels were prepared following a general protocol already described in previous works, with slight modifications [7, 13, 14]. An aqueous solution containing deacylated Gellan Gum (20g/L) and calcium acetate (0.40g/L) was mixed for almost a minute in a microwave at 600W (Mars Microwave, CEM Corporation, Matthews, NC, USA) until the sample boiled and became transparent; then it was left to cool on a Petri dish. To prepare gels loaded with *proteinase K* (Eg), an aliquot of a concentrated enzyme solution (35μM in PIPES buffer, pH = 8 (PIPES 25mM plus $CaCl_2$ 8mM) was added to Gellan gum (Gg) at 60°C during the cooling step in a Petri disk. The final enzyme concentration was 1.2μM.

Paper samples preparation

The glue was prepared by mixing 2.0g of pellet in 14mL of deionized water. The suspension was left for 24 hours, at room temperature, and then stirred in a water bath at 50°-60°C until it became a paste. Aliquots of 2.4mL of animal glue were spread on circular samples of filter papers (diameter: 4.8cm) and left to dry for almost 48 hours. Samples have been used up to 2 years after their preparation.

Cleaning procedure

Paper samples were fully covered with the gel; on the top a PET film (Mylar®) was applied, and the whole system was uniformly pressed to ensure a close contact between the gel and the sample (usually, with gels of 6cm in diameter and 1.0cm height a weight of 500g has been applied). The time of the gel application for cleaning was one hour. Gg was also used as a reference sample to highlight possible problems related to the presence of the hydrogel itself in the tests. After the cleaning procedure, the gel was removed in one step as one body, only by taking it, without the use of spatula or liquid component [7, 15-19].

Paper sample characterization

Fourier transform infrared spectroscopy (FTIR) spectra of glued samples were acquired on a Thermo-Scientific (mod. Is50) instrument (Thermo Scientific Inc., Madison WI), equipped with an attenuated total reflectance (ATR) Diamond cell in the 4000-525cm^{-1} region, at a resolution of 4cm^{-1}. A total of 64 scans were collected for each sample. Spectra were collected by placing the paper samples directly on the ATR cell.

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 (1 minute at 25mA), using a sputter coater (EMITECH K550X, Quorum Technologies Ltd, West Sussex, UK) with a gold target. The detector used was the Second Electron detector; the main operating parameters of the instrument were 10keV as gun voltage and a working distance of about 8mm.

To evaluate whether the enzyme was able to keep its hydrolytic activity if entrapped into the gel, the Bradford assay was performed, following the procedure reported in literature [20, 21] on circular EGg samples of 9mm diameter picked up from the gel itself at fixed times (10, 20, 40, 50, 60 and 90 minutes) during cleaning process on the paper coated with a rabbit glue. Every gel sample was wetted with 1.0mL of distilled water, 100μL HCl 1M, and left at high temperature (150°C) until it become a solution; then 1 part of the protein solution at different concentrations was mixed with 30 parts of the Bradford Reagent and the absorbance was measured at 570nm at room temperature. The Bradford assay was carried out using a 96 well plate (polystyrene microtitre 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.

pH measurements were performed on water extract solutions of paper samples, prepared stirring overnight at room temperature 1 cm^2 of each sample in 1.0mL of bidistillated water. pH measurements were performed before and after the cleaning process with hydrogels [22] (Amel Instrument 334-B pHmeter with a combined glass electrode Ag/AgCl 6mm, Italy).

Results and Discussion

The cleaning efficiency of Gellan Gel containing the proteolytic enzyme *Proteinase K* (Enzymatic Gel) was tested on filter paper samples (used as reference paper) soaked with animal glue. In Fig. 1, the absorbance of the glue-dye complex is reported as a function of the enzymatic gel application time on paper soiled with rabbit glue. From these experimental data, it is evident how *proteinase K* keeps its hydrolyzing activity during the time (at least for 120 minutes). In particular, it can be noticed that the increase of the treatment time corresponds to an increase of the absorbance, indicating the percentage of glue protein removed. This trend confirms not only that the enzyme is active in the gel but also that its activity increases with increasing the cleaning time. In fact, the amount of solubilized glue into the gel increases with the application time.

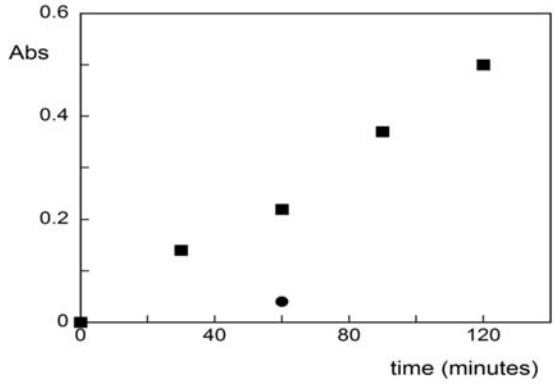

Fig. 1. UV-Vis absorption data (λ=570nm) of the Coomassie blue-protein complex into Enzymatic Gellan Gel (squares) or Gellan Gel (circle) as a function of the gel application time on coated paper sample with animal glue (RSD% = 5%).

SEM and FTIR experiments performed on glued paper samples, uncleaned or cleaned for 60 minutes with Enzymatic Gel (Eg) or Gellan Gel (Gg) alone indicate that gel containing enzyme is able to remove glue, while Gellan Gel alone not. Indeed, SEM images (Fig. 2) show that the glue-coated paper, treated with Eg (panel C) appears cleaned, similar to the original uncoated paper (panel B). On the contrary, the glue (coated and uncleaned paper is shown in panel A) is still present in samples cleaned with Gg (panel D). It's important to consider that SEM images indicate that gel treatment does not induce damages on paper fibers like fraying or swelling and no residues are present after gel treatment.

In figure 3 the FTIR-ATR spectra, recorded for all samples, uncleaned and cleaned with Eg or Gg for 60 minutes, are reported.

The FTIR analysis allows evaluating the efficacy of the cleaning treatment in removal the protein glue. Indeed, the main component of the glue is the gelatin whose spectrum is

characterized by amide I and amide II bands (at 1500-1700cm^{-1}) [23]; while, in the interval between 1200 and 950cm^{-1} the typical envelope of the absorption bands due to the cellulosic units is present [7]. If the gel is not able in removing the glue, the intensity of bands due to glue is similar to that obtained for the glued and uncleaned sample; it is the case of the spectrum of coated paper cleaned with Gellan Gel. At the same time, the intensities of Amide I and Amide II bands decreases appreciably when the glue is removed; in this case, the IR bands due to cellulose increases strongly because paper is not more covered by glue.

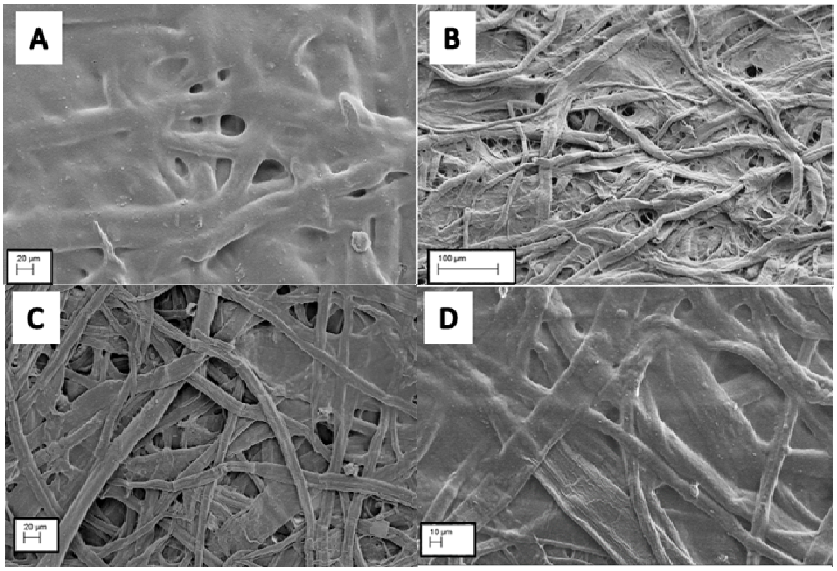

Fig. 2. SEM images of (A) paper sample coated with animal glue, (B) paper sample uncoated and uncleaned, (C) paper coated with animal glue after cleaning with Enzymatic Gel for 60 minutes and (D) paper coated with animal glue after cleaning with Gellan Gel for 60 minutes.

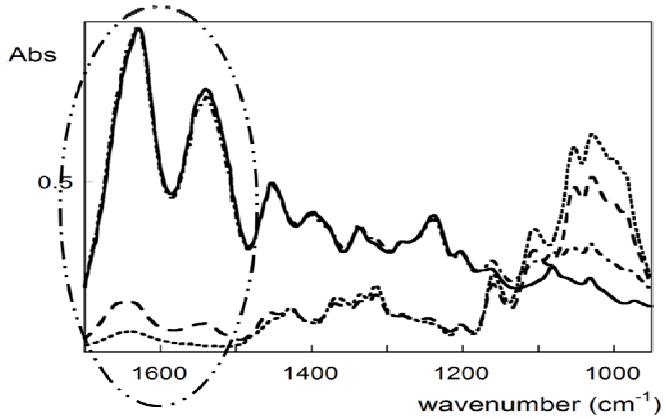

Fig. 3. FTIR spectra of paper samples uncoated (dotted line) coated with animal glue and uncleaned (continuous line), coated with animal glue and cleaned with Enzymatic Gel for 60minutes (straight line), coated with animal glue and cleaned with Gellan Gel for 60 minutes (straight and dotted line). Bands referring to protein are evidenced by a surrounding oval.

The absorbance ratio between the area of the bands referring to the glue and the area of the bands referring to cellulose is therefore diagnostic in order to verify the efficiency of the cleaning procedure. This ratio has been calculated for each sample; it has been found that Gg alone is not able in removing the glue; the ratio obtained (2.2 ± 0.1), indeed, is comparable to those obtained for the glued and uncleaned sample (2.5 ± 0.1); at the same time, the ratio value decreases appreciably when the glue was removed, as found in the case of glued paper cleaned with Eg (0.60 ± 0.07) (n = 3).These results have been confirmed by the Bradford assay on paper samples, before and after any cleaning (coating with animal glue and cleaning procedures). Cleaning process with Eg, indeed, induces a strong decrease of the protein content as indicated by the decrease in the absorbance of the dye-protein complex with respect to the glued paper sample (Table 1, *left column*). 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 in presence of uncoated paper samples. After coating with glue, the absorbance increases, because the used glue contains proteins.

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 Eg, we applied our cleaning method on different paper strips (P2) coming from the same company, characterized by the same composition and manufacturing procedure of previously paper (P1) samples, but with different porosity, in order to test how cleaning time varies when the Eg was applied on papers with different characteristics (like porosity).

The comparison of the results of the Bradford assay (Table 1) obtained for these two different porosity, showed that there were no significant differences on the application of the enzymatic gel for these different paper, suggesting that the cleaning process was little dependent on the degree of porosity of the sheet of paper.

Table 1. Comparison of ABS values (normalized to the glued paper with respect to the unglued one) obtained for P1 and P2 samples in function of the porosity

	Normalized ABS values ± σ (n = 3)	
	Sample P1	Sample P2
Uncleaned glued paper*	1.00 ± 0.03	1.00 ± 0.03
Cleaned with Eg (30' cleaning time)	0.38 ± 0.04	0.35 ± 0.03
Cleaned with Eg (60' cleaning time)	0.31 ± 0.03	0.32 ± 0.03
Cleaned with Eg (90' cleaning time)	0.15 ± 0.03	0.20 ± 0.02

**the amount of glued present onto the glued uncleaned paper samples is around 2 mg*

Finally, the pH measurements confirm the efficacy of the adopted cleaning method. In all cases, the pH of the glued and uncleaned paper samples and of the glued and cleaned (for 60 minutes), with Gellan Gel or Enzymatic Gel paper samples increases, going from pH equal to (5.8 ± 0.2) to (6.1 ± 0.1) and (6.4 ± 0.2), respectively. The pH increase is due to the ability of the gel in removing patina and dust that cause the pH decrease and to the glue removal, as the presence of animal glue has decreased the pH of the paper samples (pH of uncoated paper is equal to 6.0 ± 0.1).

Conclusion

In this work, the use of Gellan Gel containing the enzyme *Proteinase K* for the removal of animal glue from paper supports is presented. Gellan Gel alone is a powerful tool in paper cleaning process; however, in the presence of animal glue, the Gellan Gel application is not sufficient. The proposed systems combine the advantages of this rigid gel, with the use of appropriate cleaning agents (the enzyme *proteinase K* for the glue that can be easily loaded inside the gel). The results presented in this article indicate that the proposed systems represent an efficient method for cleaning paper artworks from glues they are effective, easy-to-prepare, and safe. In this contest, the reported study represents preliminary step focused in the development of hydrogel-based systems with peculiar characteristics for paper cleaning.

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