Cold, warm, warmer: use of precision heat transfer in the optimization of hydrolytic enzyme and hydrogel cleaning systems

Tomas Markevičius, Terje Syversen, Emma Chan, Nina Olsson, Carola Skov Hilby and Rytė Šimaitė

Introduction

In the past decade, conservators have devised multiple methods for the confinement of water, enzymes, chelating agents and detergents for more efficient control over the cleaning process, and reduced impact of water on the artwork. Enzymes suspended in solid gels allow for highly selective cleaning due to their characteristic active site, which 'locks' only to specific substrate molecules. By putting the substrate into a short-lived transitional substrate/enzyme state ES (equation 1) and straining intra-molecular bonds, the enzymes permit reactions under mild conditions.[1] In optimal conditions where the enzymes and substrate are dispersed in water medium the reaction follows Michaelis-Menten dynamics (equation 2) and the reaction rate υ is limited by the amount of the substrate only:

$$E + S \rightleftharpoons ES \longmapsto E + S \rightleftharpoons ES \longmapsto \mathrm{E} + \mathrm{P} \quad (1)$$

$$\upsilon = \frac{V_{\max}[S]}{K_{\mathrm{M}} + [S]} \upsilon = \frac{V_{\max}[S]}{K_{\mathrm{M}} + [S]} \quad (2)$$

where E = enzyme, S = substrate, ES = enzyme/substrate complex, P = product, V_{max} = the maximum rate of the system at (maximum) saturating substrate concentrations, K_M = Michaelis constant, substrate concentration at which the reaction rate is 50% of V_{max}, [S] is the concentration of the substrate S.

However, in the real conditions of a cleaning treatment, which aims to remove undesirable organic materials (glue, starch, oil, mould etc.) the enzymes must react with a solid phase substrate, which includes pigments, hard particulates, dust, soot, metal ions and other materials present on the surface beneath. Such actual treatment conditions are very inefficient, since enzymes, being fairly large molecules (with dimensions over 200 nm), do not diffuse well when placed on complex interfaces. In these challenging conditions, enzyme dynamics are limited by diffusion, convection and enzyme kinetics.

Diffusion, enzyme kinetics and temperature

The performance of enzymes and hydrogels can be optimized by considering several factors: the concentration of the hydrogel, enzyme concentration, pH, conductivity, cofactors (Ca^{2+}, Mg^{2+}, Mn^{2+} ions), chelators, detergents, etc. (Wolbers 2000; Campani *et al.* 2007; Stavroudis and Doherty 2013). Further optimization can be achieved by accurately controlling the temperature, which has a direct impact on the diffusion, reaction rate, capillary action and enzyme kinetics, but has not been exploited in the past due to the absence of sufficiently precise heat transfer instrumentation. A rise in temperature increases the speed at which molecules move, resulting in more frequent and more energetic collisions. Molecular vibration increases with temperature, stretching and bending the intra-molecular bonds, hence enzyme activity increases rapidly, ascending to the optimum temperature (expressed as a curve of activity and temperature) where the enzyme performs at its best. Having surpassed the peak, the enzymatic activity drops rapidly as the vibrational forces denature the shape of the active site. For example, at room temperature (21 °C) the protease Alcalase 2.4L is only 15% active, yet achieves 100% activity at 60 °C.[2]

Yet another important factor in the hydrogel cleaning mechanism is the transport of the active content (water,

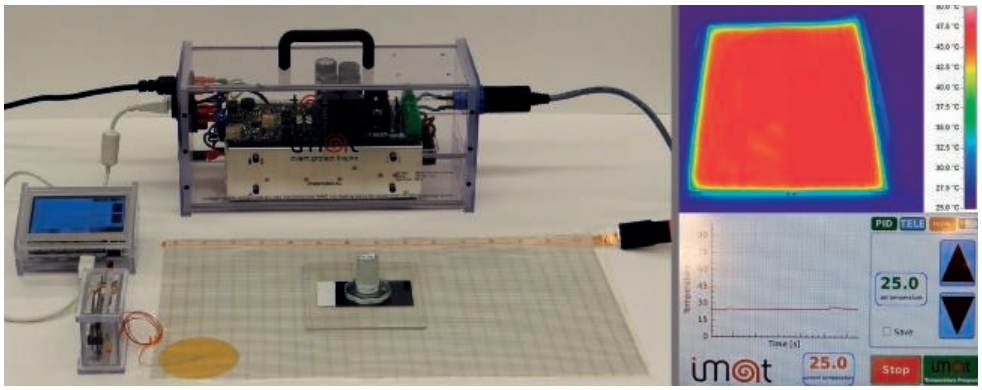

FIG. 1 IMAT precision heat transfer system (*left*), IR thermography showing even heat distribution over the surface (*top right*), and touch screen (*bottom right*) showing a steady heat transfer pattern.

enzymes, ions), which consists of movement of the fluid and within it. The former is determined by the fluid's physical properties, by the physical-chemical properties of the gel and of the substrate. Movement within the fluid depends on thermal motion and, at increased temperature, the diffusion is faster and more even. Hydrodynamic transport of globular enzyme molecules through any porous media could be described by the derivative Stokes-Einstein equation (equation 3), where diffusivity is directly proportional to temperature and inversely proportional to the frictional drag ff, which is a resistance force between the enzyme-carrying fluid and solid-state substrate (Amsden 1998):

$$\mathrm{Do} = \frac{\mathrm{kB\ T}}{\mathrm{f}}\mathrm{Do} = \frac{\mathrm{kB\ T}}{\mathrm{f}} \qquad (3)$$

where Do = diffusivity at infinite dilution; kB = Boltzmann's constant, T = temperature, f = frictional drag coefficient.

From this, it follows that increased adsorption is expected due to an accelerated diffusivity, and that the amount of adsorbed enzymes increases at elevated temperatures (Koutsoukos *et al.* 1983.

New accurate heat transfer method using conductive nanomaterials: IMAT heater

Most heating instruments employed by conservators lack accurate control and even heat distribution across the surface (Markevicius *et al.* 2013a). To control the temperature of hydrogels and enzymes, an innovative flexible transparent heat transfer mat (prototype) based on e-textile containing conductive nanomaterials and developed in the European IMAT project (2011–2014) was used.[3] The IMAT prototype mats employ the thermo-electrical properties of carbon nanotubes and other nanoscale materials to reach up to 85 °C at 36 V with an accuracy of 0.1 °C. The IMAT heat transfer system is composed of diverse lightweight, flexible, thin-profile transparent or breathable heating mats, and an associated main control unit with a touch-screen control console and a wireless temperature sensor (Fig. 1). The IMAT heater has woven-in parallel silver-coated yarns (electrodes) and when voltage is applied, the current is uniformly distributed over the layer of conductive nanomaterials, and heat is instantly generated evenly over the entire surface. If compared to conventional resistive heating elements (wound wire, etched foil) nanotubes and other conductive nanomaterials have an exceptionally low thermal mass, which allows for a rapid thermal response, an important factor to achieve even and steady heat transfer. The IMAT controller analyses the data coming from the wireless T-type thermocouple 40 times per second via a Bluetooth TC board, and regulates the temperature using the classical approach of PID-PWM regulation (proportional integrate derivative pulse width modulation), which permits ultra-steady heating with an accuracy of 0.1 °C. The thin and flexible IMAT profile allows it to be placed where the heat transfer is needed, and it can conform to diverse surface geometries (Markevicius *et al.* 2010, 2011; 2013a).

Coupling enzymatic hydrogels and heat transfer

To verify the feasibility of temperature-based optimization, three hydrogels were tested, with and without the hydrolytic enzymes, on mock-ups with rabbit skin glue and wheat starch substrates, followed by actual treatments.

Mock-ups

Two types of samples with rabbit skin glue and wheat starch were prepared. For the α-amylase gels, strips of Munktell filter paper (batch 1678) and Canson drawing paper (123 g/m^2) measuring 5 × 10 cm were sized on both sides with 20% GMW wheat starch. In addition, samples of a 19th-century handmade paper from the Strandmøllen paper mill, Denmark, were used. The samples were coated with 7.5% Fagron Tritici amylum wheat starch and artificially aged according to ISO 5630-3 (14 days at 80 °C, 65% RH in an ESPEC LHU-113 climatic chamber).

Staining

An iodine test was selected to render the cleaning results visible, and was tested on the same paper sheet with and without the starch sizing. For protease testing, 5 × 10 cm strips of commercially primed off-white linen canvas coated with 20% rabbit skin glue (Kremer) with carbon black were used. The samples were examined visually and photographed under normal conditions.

Enzymatic hydrogels

Agarose and gellan hydrogels at 1.5 wt% containing α-amylase Amplify 12L,[4] BAN 480L[5] and protease Alcalase 2.4L[6] were prepared. Hydrogels were prepared with 0%, 0.25%, 0.5% and 1% (v/v of stock solution) of protease Alcalase 2.4L (buffered at pH 7, with conductivity adjusted to 2000 µS) and α-amylase Amplify 12L (buffered at pH 6, with conductivity adjusted to 2000 µS).

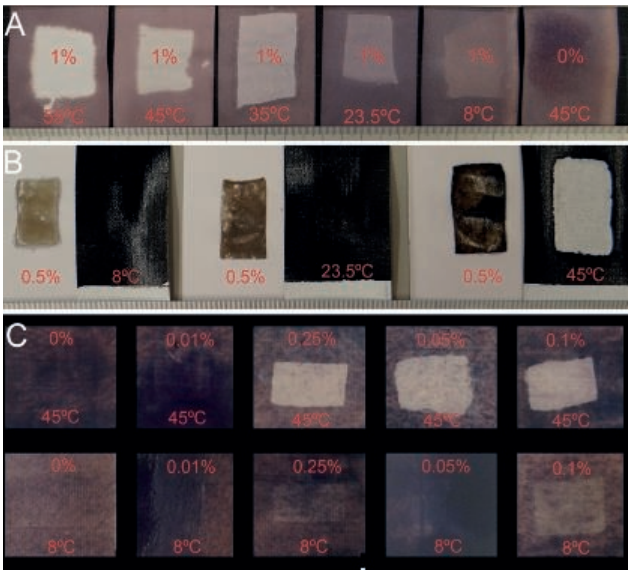

FIG. 2 (A) Ten-minute cleaning treatments at different temperatures using a 1.5 wt% agarose hydrogel containing 1% α-amylase Amplify 12L, or (B) 0.5% Alcalase 2.4L. (C) Agarose (1.5 wt%) gel containing 0–0.1% α-amylase BAN 480 at 8 °C and 45 °C after 5-minute treatments.

Testing

An experimental treatment was carried out by placing the enzymatic gel on a mock-up with starch/glue substrate. The samples were treated at: 4 °C, 8 °C, 12 °C, 23.5 °C, 35 °C, 45 °C and 55 °C for 2 , 6, 10 or 24 minutes. To render the result visible, paper samples with starch were rinsed with iodine solution, which stained the starch a purple-blue color, whereas the areas where the enzymes hydrolysed the starch resulted in a lighter stain. The samples coated with the rabbit skin glue containing carbon black pigment were treated following the above procedure. After exposure, the liquid phase residues of hydrolysed glue substrate were absorbed with filter paper and the result could be observed directly.

Amplify 12L and BAN 480L (α-amylase) hydrogel formulations

Both hydrogels at 1.5 wt% without the enzymes swelled the substrate without any identifiable cleaning action at all temperatures tested. In samples containing enzymes no cleaning action was observed at 4 °C, although cleaning action was noticed at 8 °C with 0.5% and 1% α-amylase. At 23.5 °C considerably more starch material was hydrolysed, and the reaction rate increased further at 45–55 °C. It was also observed that the increase of temperature was linearly proportional to the degree of penetration of the cleaning solution into the paper substrate, which can be explained by the increased diffusion both within the gel and paper support. In the test sample, only the front of the paper was cleaned at 8 °C, while at 45–50 °C both sides of the paper were cleaned. BAN 480L at a low concentration (0.025%) at 8 °C showed little to no cleaning action, but iodine staining results revealed that considerably more starch substrate was hydrolysed at 45 °C, hence the reaction rate increased substantially. Further increase in enzyme concentration did not improve efficacy, since the specific activity of enzymes at lower concentration was already sufficient to catalyse the given amount of starch substrate in the sample, and the enzymatic activity did not increase any further following the Michaelis-Menten dynamics.

Alcalase 2.4L hydrogel formulation

Agarose and gellan gels without enzymes produced swelling of the glue substrate, but did not engage in visible cleaning action. Minor cleaning action was recorded at 6 °C and 10 °C. At 23.5 °C the amount of glue material absorbed by the gel increased, and some thinning of the glue substrate was noticed on the sample. At 45 °C, the glue was completely removed (Fig. 2B).

Diffusion and capillary force

The effects of temperature on the diffusivity and capillary action were tested on soiled, off-white, primed deaccessioned canvas from pre-1940, from the Munch Museum, Oslo, Norway. Agarose at 3 wt% and 6 wt% containing Richard Wolbers' iso-solution D[7] were applied in the liquid state at 70 °C, 50 °C, 40 °C and 35 °C for 1 minute. Based on visual observations, the results were twofold: at a higher application temperature, liquid hydrogel diffused further into the paint layer. However, the cleaning of surface soiling increased as the application temperature decreased and the capillary force increased, because of the decrease of agarose pore size.

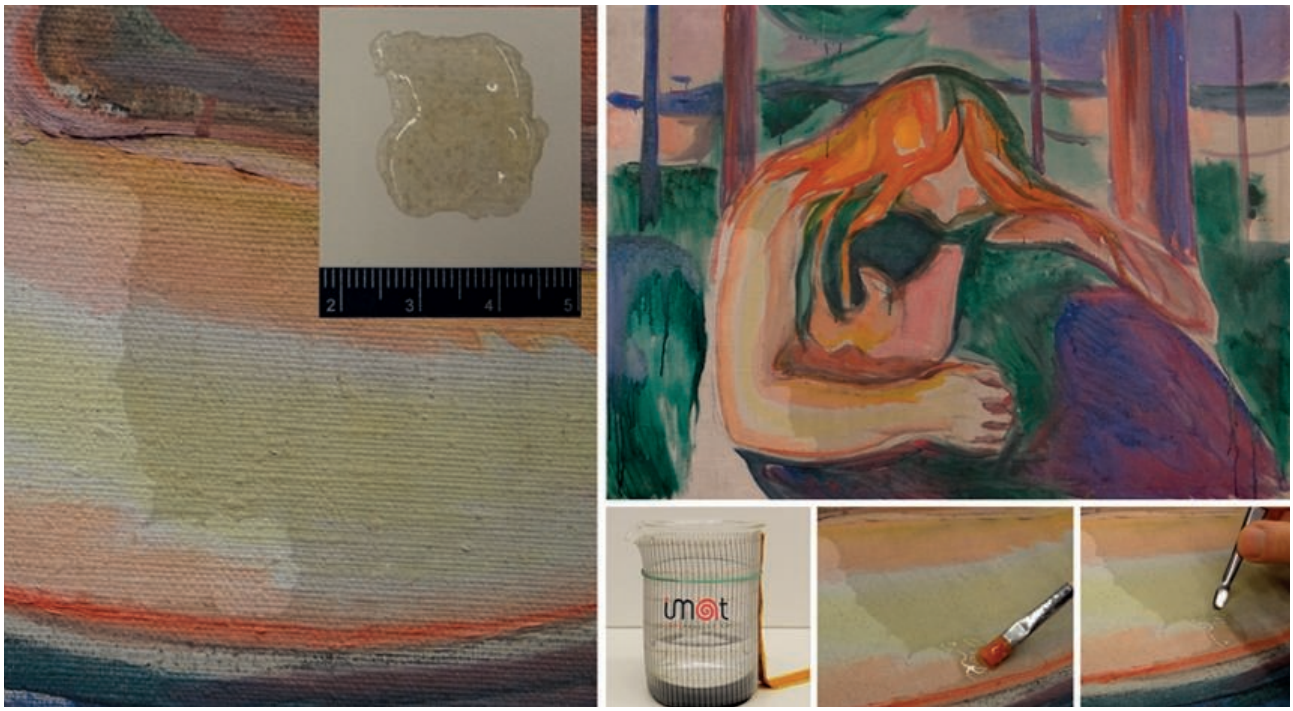

FIG. 3 Edvard Munch, *Vampire*, 1916–18, oil on canvas, 82 × 110 cm, Munch Museum, Oslo, catalogue raisonné no. M1173, and details with surface cleaning in progress. (*Lower centre*): beaker with IMAT sleeve to maintain constant temperature of the liquid-phase agarose gel.

FIG. 4 Gillian van der Gouwen, *Portrait of Cornelius Jansenius*, *c.*1670–*c.*1740, copper engraving, print on paper, 18.8 × 11.2 cm, inv. no. G-11040, Lithuanian Art Museum, Vilnius, Lithuania: showing removal of glue stains using the mild heat transfer with IMAT and a viscous enzymatic gel.

Surface cleaning of an early 20th-century painting by Edward Munch

Temperature-based capillary action of agarose hydrogel (5 wt%) containing Wolbers' iso-solution D with chelator (DTPA) was used to remove a layer of greyish surface dirt from a fragile unvarnished surface on Edvard Munch's *Vampire* (1916–18) (Fig. 3). Agarose gel, when applied as a solid-phase block, produced somewhat incomplete cleaning action due to the lack of contact with the porous surface. A different approach was tested and the agarose was applied in a liquid phase at 39 °C, which changed its phase to solid and after 1 minute, lifted as a film with the entrapped soiling, revealing the intact surface. Precisely controlled temperature was very efficient in controlling the diffusion and capillary action of the gel tailored for this specific case and allowed agarose to remain in a liquid phase during the treatment. Control of the exposure time and temperature was crucial, as minor temperature variations affected the cleaning action and resulted in uneven cleaning. To keep the agarose at a 39 °C (±0.1 °C), the IMAT heater was wrapped around the beaker (Fig. 3).

Removing aged glue stains from a Dutch 17th-century print by Gillian van der Gouwen

Since 2013, the IMAT has been used for diverse enzymatic treatments (e.g. removal of animal glue, starch and oil) of over 80 works on paper at the Lithuanian Art Museum, Vilnius, Lithuania. As an example, aged glue residues were removed from a 17th-century print. Alcalase 2.5L DX at 0.1% v/v was used in 5% Tylose MH300. The gel was applied to both sides of the print, which was covered with polypropylene film, and IMAT mats were positioned on both sides. While at room temperature (19 °C) the enzymes had little effect on the aged proteinaceous stains, treatment at 45 °C removed the stains completely (Fig. 4).

Conclusion

Conventionally, cleaning treatments are carried out in uncontrolled and variable temperature conditions. The transfer of energy to the system determines the liquid/solid phase of the materials, their dynamics, reaction rate, behaviour and the performance of the substance. Thus, heat transfer is a powerful tool in all conservation treatments, yet it cannot be fully exploited without accurate control. In the gel cleaning mechanism, precision heat transfer permits control of the diffusivity, capillary action, reaction rate and enzyme dynamics. In this preliminary study, the precision mild heat transfer system (IMAT) and flexible nanotube mats were shown to establish and regulate temperature conditions accurately; the results show the feasibility and potential of a temperature-controlled approach to optimize cleaning treatments using hydrogels and hydrolytic enzymes.

Initial testing shows that the IMAT provides precision temperature control and that modulating the temperature factor in cleaning treatments is feasible. Research focusing on accurate quantification of enzyme dynamics using fluorescent staining, including other enzymes and modelling methods of application is ongoing. The experimental cleaning tests demonstrate that when greater diffusivity of the liquid phase and enzymes is required, the temperature may be increased to enhance diffusion. Inversely, cooling the surface may reduce the diffusivity, which may be useful when the goal is to limit the cleaning action to the surface only. Capillary force may be controlled by both the concentration of the hydrogel and variation of the temperature. Enzymes are temperature-specific and they catalyse most efficiently in an optimal temperature range, which is frequently above room temperature, and the temperature factor is difficult to engage without precision heat transfer instrumentation. The described temperature-based approach, when coupled with other factors such as concentration of the hydrogel and enzyme, concentration and specific activity (enzyme units U/g, Anson units AU/g, etc.), pH, conductivity, cofactors, chelators, detergents, etc., offers valuable advantages in controlling the dynamics of gels and enzyme kinetics, improving the efficacy of gel cleaning mechanisms to be tailored to specific situations, and contributes to the increased safety of a cleaning treatment for the artwork and better selectivity and nuanced cleaning action.

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Materials and suppliers

- Alcalase 2.4L, DPTA, triethanolamine: www.sigmaaaldrich.com
- GMW wheat starch: GMW, gmw-shop.de
- Amplify 12L, BAN 480 L: Novozymes, www.novozymes.com
- ESPEC LHU-113 climatic chamber: ESPEC, www.espec.com
- Tylose MH300, rabbit skin glue, carbon black: Kremer Pigmente, www.kremer-pigmente.com/de

Notes

1. The classical Michaelis-Menten model includes three principal phases: enzyme [E] transports the substrate molecule [S] and binding to the substrate molecule with the active site (equation 1), forming a temporary enzyme-substrate complex [ES] (equation 2), breaking the intra-molecular bonds and releasing the free reusable enzyme [E] and the product of the reaction [P] (equation 3).
2. The optimum temperature curve for Alcalase 2.4L was provided by Novozymes. Assay prepared using Konelab 30 analyzer (Fisher Scientific).
3. IMAT: prototype/IMAT research project (2011–2014), European Commission's 7th Framework Program for Scientific Research/Coordinated by the University of Florence, Italy.
4. Amplify 12L α-amylase from *Bacillus lichenoformis* (EC: 232-565-6; CAS 900-90-2), hydrolyses (1.4)-alpha-D-glucosidic linkages in starch efficiently in the range 10–50 °Cat pH 6–9, with specific activity 12 SNU-A/g (standard novo units).
5. BAN 480L α-amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1; CAS 9000-90-2). Optimum pH of 6, temperature optimum 70 °C. Efficient at 10–90 °C.
6. Alcalase 2.4L is a proteolytic enzyme from *Bacillus licheniformis* (EC Number 232-752-2; CAS 9014-01-1). The main enzyme component, Subtilisin A, is an endoproteinase with a specific activity of 2.4 AU/ml (Anson units). Optimum pH is 7–8, optimum temperature is 60 °C.
7. Water, 0.5 % diethylenetriaminepentaacetic acid (DTPA), 0.5% citric acid, buffered to pH 8.0 with triethanolamine (TEA), conductivity adjusted to 2000 µS.

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