Immobilized Enzymes on Gold Nanoparticles: from Enhanced Stability to Cleaning of Textile Heritage

Francesca Gherardi,*[†] Lyudmila Turyanska,[†,‡] Enrico Ferrari,[‖] Nicola Weston,[§] Michael W. Fay,[§] and Belinda Colston*[†]

[†]*School of Chemistry, University of Lincoln, Lincoln LN6 7TS, UK;*

Email: fgherardi@lincoln.ac.uk, bcolston@lincoln.ac.uk

[‡]*School of Physics and Astronomy, University of Nottingham, NG72RD, UK;*

Email: lyudmila.turyanska@nottingham.ac.uk

[‖]*School of Life Sciences, University of Lincoln, Lincoln LN6 7TS, UK;*

Email: eferrari@lincoln.ac.uk

[§]*Nanoscale and Microscale Research Centre, University of Nottingham, NG7 2RD, UK*

Email: nicola.weston@nottingham.ac.uk, michael.fay@nottingham.ac.uk

Corresponding authors: bcolston@lincoln.ac.uk

fgherardi@lincoln.ac.uk

Abstract

Enzyme-based treatments are used in heritage conservation for the effective removal of glues and other damaging organic layers from the surfaces of historic and artisitc works. Despite their potential, however, the application of enzymatic treatments is currently limited due to their poor efficiency, and low operational and environmental stability. We demonstrate the use of α-amylase immobilized on gold nanoparticles to improve the efficacy of enzymatic treatments enchancing both the reactivity and the stability of the formulations. Gold nanoparticles coated with α-amylase exhibit significant advantages compared to free enzymes. We report up to 5-times greater resistance to environmental changes, up to 2-times higher efficacy towards removal of starch-based glues from textile, and deeper penetration through the fibres, without causing damage or inducing salt precipitation. These results offer exciting prospects for the development of ~~novel~~ enzymatic formulations, both for heritage conservation and the wider application of enzymes, such as in medicine, the detergent industry and green chemistry.

Keywords: gold nanoparticles; amylase; textiles; starch cleaning; cultural heritage

1. Introduction

Enzymes have been widely used as biocatalysts due to their chemo-, regio-, and stereo-specificity in applications ranging from medicine to the detergent industry and green chemistry.[1] Their application is often hindered, however, by lack of long-term stability, and difficulty in recovery and reuse.[2] Immobilization of enzymes on a carrier, (e.g. synthetic polymers, biopolymers, hydrogels, inorganic supports, nanoparticles), can be used to overcome these limitations.[2-4] Recent reports demonstrate that enzymes anchored to nanostructured materials with high surface area have improved enzyme loading and enhanced activity.[3-7] Among various nanomaterials, gold nanoparticles (AuNP) offer benefit of direct conjugation of enzymes without the need for further modification, making them an ideal model for enzyme immobilization studies.[5-6, 8]

Nanomaterials have brought significant technological advances in applications ranging from optoelectronics to medicine.[9] Recently, the use of nanomaterials was proposed for heritage conservation [10], and has the potential to effect a step change in the efficacy of interventive methods currently used for the conservation of historic artefacts.[11] One of the most delicate phases of interventive conservation is the cleaning process, which must exhibit selectivity in the treatment of unwanted layers, without compromising the original historic and artistic substrate. Current research focuses on the development of innovative cleaning materials, including microemulsions, solvents and rigid gels [12-13], and biological cleaning methods such as microorganisms and hydrolytic enzymes.[14-19] Enzymes exhibit exquisite molecular recognition [15, 19-20], and have been used to remove starch paste [18, 21-23], protein-based glues and adhesives [18, 24], lipid-based compounds [25], and aged acrylic coatings and inks [26] from historic textiles, paper and prints, wall-paintings and ceramic materials.

Starch and protein-based glues (e.g. collagen, casein) are widely used to consolidate brittle fibres. Hydrolysis/oxidation and cross-linking of these glues due to ageing, however, lead to

their layers becoming insoluble, and to the formation of cracks, tensions, discoloration and embrittlement of the fibres, and could be a source of future biodeterioration (fungi and bacteria).[19] The complete cleaning of aged starch is therefore essential for the conservation of textile artefacts, but presents significant challenges – the treatment agents need to exhibit both high selectivity and the ability to penetrate through the fibres without damaging the historic textile. In this context, enzymes are very promising. Their hydrolytic action provides selective treatments, whilst their application through aqueous formulations by brush or cotton swabs, absorbed on tissues and poultice, or embedded into viscous media and gels provides mobility and penetration. The use of enzymatic solutions, however, is limited due to their low environmental and operational stability (pH and temperature), difficulties in recovery and high cost.[19]

Here we report on the development of novel nano-formulations of enzymes for removal of starch-based treatments from textiles. Gold nanoparticles coated with α-amylase (αA) offer high efficiency in starch hydrolysis and enhanced environmental stability. We examine the long-term stability and shelf life of the formulations using Fourier-Transform Infrared (FTIR) spectroscopy on starch-treated silicon windows and wool. The enzymatic activity towards digestion and removal of starch by α-amylase immobilized on AuNP (αA-AuNP) is higher than observed in 'free' enzymes, especially once applied on textiles. In addition, αA-AuNP exhibits higher environmental stability compared to αA, which suffers from denaturation and consequent significant reduction of the enzymatic activity. By using Environmental Scanning Electron Microscopy (ESEM), we investigate the mechanism of interaction between enzymatic formulations and fibres, proving that the immobilization of amylase on nanoparticles fosters good penetration of enzymes into porous substrates, leading to a selective removal of starch glue, without compromising the fibres or leaving residues or salt precipitates. To the best of our knowledge, this is the first application of enzymes immobilized on nanoparticles in heritage

conservation, and this work will inform future development of functionalised nanomaterials for interventive conservation practices.

2. Materials and Methods

Preparation of α-amylase dispersion: Type II-A from *Bacillus sp*. (Sigma-Aldrich A6380) was dissolved in a buffer solution (20 mM HEPES pH=7.3, 100 mM NaCl) at a concentration of 5 mg/mL. The enzyme has an estimated molecular weight of 50-55 kDa by SDS-PAGE and ≥1,500 units/mg protein (biuret) according to the technical data sheet.

Preparation of α-amylase coated gold nanoparticles: α-amylase coated gold nanoparticles were prepared using passive adsorption followed by removal of protein excess by centrifugation. The citrate-capped gold nanoparticles (1 mL, HD.GC20, BBI Solutions) with a diameter of 20 nm and an optical density (OD) at the extinction peak of 5 were pelleted at 5000xg for 20 min and the storage buffer replaced with a 250 µL of α-amylase solution with excess concentration of ~25 µg/mL diluted in 10 mM HEPES buffer, pH=7.3, 10 mM NaCl. After 60 min incubation at room temperature, Au nanoparticles were pelleted at 5000xg for 20 min and the solution containing excess enzyme was removed, washed by resuspension in 500 µL of 10 mM HEPES buffer, pH=7.3, 10 mM NaCl, then centrifuged and suspended again in 500 µL of the same buffer solution. The buffer contains a lower concentration of NaCl (10 mM) compared to the bugger used for free amylase (100 mM). The enzymatic dispersions were stored at T = 4 °C.

Characterization of α-amylase coated gold nanoparticles: pristine gold nanoparticles and α-amylase coated gold nanoparticles were studied by Transmission Electron Microscopy (TEM). The nanoparticles were deposited on a graphene-oxide coated grid and TEM images were recorded on a JEOL 2100F FEGTEM microscope operating at 200 kV equipped with a Gatan

Orius SC1000 CCD camera. The average size of gold nanoparticles was analysed using ImageJ software.

The optical density (OD) of the gold nanoparticles suspensions at 520 nm was measured by NanoDrop (NanoDrop 2000, Thermo Fisher Scientific). The reference concentration at OD = 1 provided by the supplier is $7.0x10^{11}$ mL^{-1} OD^{-1}. The concentration of α-amylase adsorbed on the nanoparticle suspension was estimated from the concentration of the nanoparticles measured from the OD at the extinction peak and assuming complete coverage of the surface (see Supporting Information SI1).[27-28]

The hydrodynamic diameter distributions of pristine and α-amylase coated gold nanoparticles were measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern). The nanoparticles were diluted 10 times in 10 mM HEPES buffer, pH=7.3, 10 mM NaCl and the scattering was acquired from a micro-cuvette with 100 µL of the suspension using a backscatter angle of 173° and an automatically selected attenuator to optimize the photon count. Three measurements at a temperature of 20 °C were acquired and averaged, the size distributions were represented from the scattering intensity data using the Z-average (diameter) and the polydispersity index (PDI) from the cumulant analysis of the measured auto-correlation function.

Evaluation of the enzymatic activity and the removal of the enzymatic formulations: Fourier Transform Infrared (FTIR) spectroscopy studies were performed on PerkinElmer Spectrum 100 FTIR Spectrometer equipped with a DTGS detector and an Attenuated Total Reflection (ATR) diamond crystal accessory. Dispersions of 1% and 2% (w/v) of potato starch (Sigma Aldrich) boiled in water with stirring for 30 minutes were applied as thin film on silicon polished windows (Crystran Ltd, United Kingdom). Following 30 min and 120 min of exposure to enzymatic dispersions, the FTIR spectra were acquired (64 scans, resolution 4 cm^{-1}). The

background spectra were recorded with silicon windows and subtracted from the sample spectra. All spectra were normalized on the intensity of the C-H stretching vibration at ~2930 cm^{-1}. The starch hydrolysis (*SH*) was calculated using the following equation: $SH = (R_f - R_i)/R_i \times 100\%$, where R_i and R_f are the intensity ratios of the 1045/1022 cm^{-1} peaks before and after treatment with the enzymatic dispersions. Each experiment was repeated three times.

For further analysis, 1% and 2% (w/v) potato starch water dispersions were applied by brush (about 3 $\mu L/cm^2$) on wool specimens. Starch digestion by amylase-based dispersions was monitored by ATR-FTIR spectroscopy performed on the same area. All spectra were normalized on the intensity of the C=O stretching vibration of amide I band of wool at 1630 cm^{-1} and the starch hydrolysis was evaluated after 30 min exposure to the dispersions at room temperature (T = 23 °C). In addition, the percentage of removed starch was calculated from the intensity ratios of the 1022 cm^{-1} (characteristic of starch) and 1630 cm^{-1} (characteristic of wool) peaks before and after the application of the enzymatic formulations. Finally, the surface was treated with a cotton swab soaked with deionized water in order to remove the enzymatic formulations and the residues of starch digestions. ATR-FTIR measurements were repeated to assess the complete removal of amylase. Each result was calculated by averaging three individual experiments.

To assess the complete removal of the formulation based on gold nanoparticles coated with α-amylase, colour measurements were carried out by Konica Minolta CR-410 Chroma Meter instrument with a D65 illuminant. Measurements were elaborated according to the CIE L*a*b* standard colour system. Five measurements were performed on each area (about 4 cm^2) and the average results of L*a*b* were used to calculate the colour difference ΔE* between cleaned and uncleaned areas: $\Delta E^* = [(L^*_2-L^*_1)^2+(a^*_2-a^*_1)^2 + (b^*_2-b^*_1)^2]^{1/2}$).

ESEM-EDX on cotton fibres coated with potato starch: Environmental Scanning Electron Microscopy (ESEM) and Energy Dispersive X-Ray (EDX) analysis were performed on FEI Quanta 650 with an Oxford Instruments X-Max 150 EDX Detector. The 2% (w/v) potato starch water dispersions were applied by brush (about 3 μL/cm^2) on cotton specimens. Single fibres and woven textile were sampled from cotton textile and mounted on aluminium stubs. ESEM-EDX data were recorded before and after the application of dispersions, allowing 30 min of digestion time. Secondary Electron (SE) images were collected in order to monitor the removal of starch from the fibres while Backscattered Electron (BSE) images were used to study the surface distribution and the penetration depth of gold nanoparticles modified with amylase in cross-sections of cotton specimens.

Evaluation of the storage stability of the enzymatic formulations: The enzymatic dispersions were stored in the laboratory at T = 23 °C for 30 days and were exposed to ambient light. The activity in starch hydrolysis was evaluated after 7, 15 and 30 days using FTIR spectroscopy. The activities at different times were compared with the initial activity and the residual activity (%) was calculated.

3. Results and discussion

3.1 Characterization of α-amylase coated gold nanoparticles

Gold nanoparticles coated with α-amylase were prepared following passive adsorption, producing stable and active enzyme-nanoparticle conjugates without need for chemical modification of the enzyme (Figure 1).[29-30] The citrate-capped AuNP are used to form αA-AuNP and the excess protein is removed by centrifugation. The final concentration of nanoparticles is 3.9 x 10^{12} NP/mL, with an estimated 35 μg/mL load of α-amylase (see Supplementary Information, SI1). Transmission Electron Microscopy (TEM) images revealed

that AuNP cores have average diameters of 18 ± 1 nm and 21 ± 3 nm for pristine and α-amylase coated AuNP, respectively, both consistent with the 20 nm nominal diameter provided by the supplier (Figure 1b). TEM images confirm that adsorption of α-amylase does not change the size and morphology of the gold nanoparticles and leads to the formation of a stable colloidal solution [31-32] with only minimal aggregation (see the insets of Figure 1b), likely due to electrostatic or van der Waals interactions between amylase on neighbouring nanoparticles.[33]

The measurement of the hydrodynamic size of α-amylase AuNP and plain AuNP by Dynamic Light Scattering (DLS) confirmed the stability and minimal aggregation of the colloidal suspensions and revealed a marked shift towards larger diameters for α-amylase AuNP, which is consistent with extensive adsorption of the enzyme onto gold NP surface. The hydrodynamic diameter increased from 24.0 ± 0.6 nm for AuNP to 41.9 ± 2.1 nm for αA-AuNP. The polydispersity index (PDI) was 0.226 and 0.282, reflecting the slightly broader distribution of αA-AuNP size.

3.2 Evaluation of the enzymatic activity

The activity in starch hydrolysis of free α-amylase (αA) and α-amylase immobilized on AuNP (αA-AuNP) was evaluated by FTIR spectroscopy by following 30 min and 120 min digestion of 1% w/v (P1) and 2% w/v (P2) potato starch applied as film on silicon windows (Figure 2 and Supporting Information SI2, Figure S1). We examined the bands in the spectral region of 950-1100 cm^{-1} characteristic for modifications of starch conformation [34-38] and ascribed to C-O and C-C stretching vibrational modes. The bands at 1022 cm^{-1} and 1045 cm^{-1} are associated with amorphous and ordered/crystalline regions in starch, respectively.[35-36, 38] With the hydrolysis of starch by amylase, the chain length decreases and an increase of crystallinity is expected resulting in a higher ratio of 1045/1022 cm^{-1} peak intensities.[37, 39]

In our studies, an increase in the ratio of 1045/1022 cm^{-1} peak absorbance is observed following exposure to the enzymatic dispersions (Figure 2). The starch hydrolysis (SH) was evaluated by measuring the percentage of increase of crystallinity, calculated from the 1045/1022 cm^{-1} peak ratio. The SH of αA after 30 minutes was higher compared to the immobilized protein, suggesting faster activity (starch hydrolysis values after 30 minutes exposure were about 13% and 6% for αA and αA-AuNP, respectively). No significant SH increase was detected after 120 min for αA (Figure 2a). Instead, as shown in the Figure 2b, the starch digestion by αA-Au was initially lower (at 30 min), however the activity was enhanced over longer time compared to free enzymes. The sustained activity was especially evident on silicon windows coated with P2 (increase of more than 20%), with SH values of ~6.5% for αA and ~8% for αA-AuNP (Figure 2b), similar to those reported for silver nanoparticles and calcium alginate gel beads.[31, 40] The lower initial efficiency can be attributed to slower diffusion of enzyme/NP dispersions compared to free enzymes. In addition, the presence of metal nanoparticles has an impact on biomolecules, altering their catalytic activity either by increasing or decreasing the affinity for enzyme-substrate formation.[33] Our results indicate that gold nanoparticles could play a role as a nanocatalyst in the hydrolysis of starch by α-amylase.[33, 41] We also envisage that the enzymatic activity of immobilized amylase is not affected but rather enhanced, possibly due to favourable orientation towards the substrate of the catalytic sites of the enzyme.[29, 42-43]

Similar enhancement of the activity of αA-AuNP was observed on wool samples coated with potato starch (Figure 6b). Attenuated Total Reflection (ATR) FTIR spectra (Figure 3) revealed characteristic patterns of protein: amide I band at 1650 cm^{-1} (C=O stretching vibration), amide II at about 1540 cm^{-1} (N-H bending/C-N stretching vibrations) and amide III at about 1230 cm^{-1} (N-H bending/C-N stretching vibrations). A significant increase of the ratio of 1045/1022 cm^{-1} peak intensities was observed in spectra of enzyme-digested starches

(Figure 3). In this case, the starch was applied onto a 3D textile network, and treatment with αA-AuNP was more effective: after only 30 min an increase of the starch hydrolysis by 80% and 100% on P1 and P2, respectively was observed compared to free enzymes (Figure 3 and Supporting Information SI2, Figure S2). In particular, while free amylase leads to a lower digestion of P2 compared to P1, it is likely that the immobilization of the protein on nanoparticles could have facilitated a more favourable orientation of the active sites towards the substrate, thus improving the enzymatic selectivity and leading to a more efficient breakdown of starch (Figure 3b).[29] The higher effectiveness in starch hydrolysis of αA-AuNP is also mirrored by a significantly higher removal (~135% and 35% greater removal of P1 and P2, respectively) of starch from the textile (Supporting Information SI2, Table S1). In addition, ATR-FTIR spectra obtained after enzymatic digestion and cleaning of residues of starch and amylase proved the complete removal of P1 and P2 from wool treated with αA-AuNP, while P2 starch was still present on the surface on wool treated with αA (Supporting Information SI2, Figure S3).

The interaction of enzymatic formulations with textile fibres and their cleaning effectiveness in the removal of starch from cotton was confirmed by ESEM. Representative ESEM images of the same area before and after the application of amylase-based products are presented in Figure 4. A clear difference in the mechanism of action of free amylase and amylase coated gold nanoparticles is evident: following treatment with αA-AuNP, the formulation was able to penetrate through the fibres and the glossy layer of starch accumulated between the fibres was completely removed, leaving empty spaces. In addition, the removal of starch-based glue did not damage the cotton fibres and restored their flexibility, detaching them from the main compact threads (Figure 4a). On the contrary, fibres treated with αA were still surrounded by starch as the formulation was not able to efficiently diffuse though the fibres but it was accumulated on the starch after the evaporation of the buffer (Figure 4b). In addition, intense

salts precipitation from the buffer medium was observed. Salts accumulate only in samples treated with free amylase, as high-salt content buffer is needed to solubilise enzymes, while αA-AuNP formulation is prepared in low salt content buffer. These results were confirmed on potato starch deposited on woven cotton and wool (Figure 6b). Almost complete breakdown of starch was detected on textile treated with αA-AuNP (Supporting Information SI2, Figure S4). To achieve a complete starch removal, the sample should be treated with the same dispersion twice.

To evaluate the penetration depth of αA-AuNP, a cross-section of woven cotton with starch was observed by ESEM with a backscatter electron detector (BSD) after the application of the enzymes (Figure 5). The images revealed the presence of the enzymatic dispersion throughout the full depth of the sample (Figure 5a). The cross-section of the woven cotton in the centre of the sample also confirmed diffusion of αA-AuNP (Figure 5b) through ~200 μm thick top layer of the sample. Further improvement of the penetration depth could be achieved by treating the woven cotton with a higher volume of a more diluted enzymatic dispersion.

Comparable removal of starch-based glues from textile and paper was previously achieved by incorporating enzymes in gels, in order to promote a slow release of the formulation in the substrate without causing swelling of fibres.[18, 23] Immobilization of enzymes on nanoparticles proved to be very effective in cleaning of textiles, thanks to their ability to diffuse in the textile and to interact with fibres, removing starch-based glues. However, new scientific investigations of the mechanism of action of enzymes and interaction with the supports are needed to select the best application procedures (time, concentrations, medium, etc.).

αA-AuNP formulations have light pink colour due to the plasmon resonance of the particles. Following the application of αA-AuNP, the textile becomes light pink colour, hence removal of these formulations following treatment is of great importance to preserve the original appearance of the fabric. Therefore, colorimetric measurements were carried out to assess the

removal of the formulations after cleaning with a cotton swab soaked with deionized water. The results indicate that αA-AuNP affect the surface color of wool with a reduction of the lightness (L*) and an increase of the red color intensity (a*) ($\Delta E_{\alpha A\text{-}AuNP}$ values of 4.18 and 6.21 for wool treated with P1 and P2) (Table 1). The final treatment with deionized water led to complete removal of the enzymatic treatment, restoring the initial color of wool, as evident from the color difference values between the cleaned surface and a reference untreated wool < 1, which is considered as ΔE value not perceived by human eye.

3.3 Evaluation of the stability of the enzymatic formulations

To simulate environmental conditions and evaluate the storage stability of the enzymatic formulations, αA and αA-AuNP were stored at 23 °C for 30 days. To test the residual activity of amylase in starch hydrolysis, the formulations were deposited on starch-treated silicon windows over a period of 30 days. For sample coated with 2% starch (P2), the SH value decreased to ~ 50%, ~30% and 15% after 7 days, 15 days and 30 days from the application of the formulations (Supporting Information SI2, Figure S5). Amylase immobilized on gold nanoparticles was more stable, with SH value of ~ 70% and 50% after 15 days and 30 days on P2 samples. Over the studied timeframe, residual activity of αA-AuNP was up to 5-times greater, compared to free enzyme.

Improved activity and shelf life of immobilized enzymes were further confirmed on wool samples coated with starch. After 7 days, lower residual activities of 82% was observed for free amylase on P2, compared to 94% for AuNP immobilized amylase (Figure 6). After 30 days, significant reduction of activity was observed for αA (17%), while αA-AuNP retained >80% of activity (Figure 6). These observations are consistent with the hypothesis that over time free enzymes undergo unfolding (denaturation) while the immobilization of enzymes improves the stability towards environmental change and prevents enzyme deactivation.[3, 32, 44-

[49] In addition, after 30 days, a significant reduction in starch cleaning from wool was evidenced for αA (values of starch removal of about 2% and 15% for P1 and P2, respectively), while αA-AuNP was still effective (more than 10-times and about 3-times greater removal on P1 and P2, respectively, with values of starch removal of about 25% and 45% for P1 and P2, respectively) (Supporting Information SI2, Table S1).

Our results highlight the advantages of enzymes immobilized on Au nanoparticles leading to increased storage stability of enzymatic formulations and their effectiveness in starch hydrolysis. The αA-AuNP formulations effectively removed starch from the fibres at room temperature after only 30 min from the application, without the need for water bath with optimized temperature to achieve the catalytic action of the enzyme.[21] In addition, amylase-coated AuNP allow the application of higher concentration of enzymes without their precipitation/agglomeration, hence enabling the cleaning process without swelling and damaging the fibres. The use of nanoparticles to graft enzymes fosters a good penetration into the fibres with no salt precipitation from the buffer detected. We believe that an increased enzymatic efficiency of the αA-AuNP formulation could justify the cost of this treatment for valuable heritage materials. We also note that further reduction of the cost of these formulations could be achieved by translating the approach developed in this work on other nanoparticles (e.g., SiO_2, TiO_2).

4. Conclusions

We have demonstrated that immobilization of α-amylase on gold nanoparticles provides a route for significant improvement of environmental stability and shelf life of the enzymes, high selectivity and enhanced treatment activity compared to free amylase. Efficient removal of starch glues from textiles without damage to the fibres and good penetration through the woven cotton were confirmed by microscopic investigations of treated textiles. These results

demonstrate for the first time the potential of novel immobilized enzymes for applications in heritage conservation. To fully evaluate the potential of these enzymatic formulations for treatment of historic fabrics, further work is needed to assess cleaning capability on a range of different fabrics and/or any potential damage to the dyes/discoloration of the fibres. The collaboration with end users will be paramount to tailor the properties of this formulation for the cleaning of textiles and artwork. Finally, the possibility to transfer our developments to other enzymes (collagenase, protease, and lipase) to remove animal glues and lipid-based coatings could significantly improve the cleaning interventions of historic and artistic textiles.

Supporting Information

Supporting methodology and characterization data (enzymatic activity and scanning electron microscopy).

Conflicts of interest

The authors declare no conflicts of interest and no competing financial interest.

Acknowledgements

The work was supported by funding from the Engineering and Physical Sciences Research Council NanoPrime programme [grant number EP/R025282/1] and the University of Lincoln.

References

(1) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. Dispelling the Myths--Biocatalysis in Industrial Synthesis. *Science* **2003,** *299* (5613), 1694-1697.

(2) Bornscheuer, U. T. Immobilizing Enzymes: How to Create More Suitable Biocatalysts. *Angew. Chem. Int. Ed.* **2003,** *42* (29), 3336-3337.

(3) Homaei, A. A.; Sariri, R.; Vianello, F.; Stevanato, R. Enzyme immobilization: an update. *Journal of chemical biology* **2013,** *6* (4), 185-205.

(4) Sheldon, R. A.; van Pelt, S. Enzyme immobilisation in biocatalysis: why, what and how. *Chem. Soc. Rev.* **2013,** *42* (15), 6223-6235.

(5) Ansari, S. A.; Husain, Q. Potential applications of enzymes immobilized on/in nano materials: A review. *Biotechnol. Adv.* **2012,** *30* (3), 512-523.

(6) Cipolatti, E. P.; Silva, M. J. A.; Klein, M.; Feddern, V.; Feltes, M. M. C.; Oliveira, J. V.; Ninow, J. L.; de Oliveira, D. Current status and trends in enzymatic nanoimmobilization. *J. Mol. Catal. B: Enzym.* **2014,** *99*, 56-67.

(7) Mei, S.; Shi, J.; Zhang, S.; Wang, Y.; Wu, Y.; Jiang, Z.; Wu, H. Nanoporous Phyllosilicate Assemblies for Enzyme Immobilization. *ACS Applied Bio Materials* **2019,** *2* (2), 777-786.

(8) Ma, W.; Saccardo, A.; Roccatano, D.; Aboagye-Mensah, D.; Alkaseem, M.; Jewkes, M.; Di Nezza, F.; Baron, M.; Soloviev, M.; Ferrari, E. Modular assembly of proteins on nanoparticles. *Nature Communications* **2018,** *9* (1), 1489.

(9) Daniel, M.-C.; Astruc, D. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chem. Rev.* **2004,** *104* (1), 293-346.

(10) Baglioni, P.; Carretti, E.; Chelazzi, D. Nanomaterials in art conservation. *Nature Nanotechnology* **2015,** *10*, 287.

(11) Baglioni, P.; Chelazzi, D.; Giorgi, R. *Nanotechnologies in the Conservation of Cultural Heritage: A compendium of materials and techniques*, Springer Netherlands: 2014.

(12) Mazzuca, C.; Micheli, L.; Carbone, M.; Basoli, F.; Cervelli, E.; Iannuccelli, S.; Sotgiu, S.; Palleschi, A. Gellan hydrogel as a powerful tool in paper cleaning process: A detailed study. *J. Colloid Interface Sci.* **2014,** *416*, 205-211.

(13) Bonelli, N.; Poggi, G.; Chelazzi, D.; Giorgi, R.; Baglioni, P. Poly(vinyl alcohol)/poly(vinyl pyrrolidone) hydrogels for the cleaning of art. *J. Colloid Interface Sci.* **2019,** *536*, 339-348.

(14) Valentini, F.; Diamanti, A.; Palleschi, G. New bio-cleaning strategies on porous building materials affected by biodeterioration event. *Appl. Surf. Sci.* **2010,** *256* (22), 6550-6563.
(15) Cremonesi, P. Rigid Gels and Enzyme Cleaning. In *New Insights into the Cleaning of Paintings: Proceedings from the Cleaning 2010 International Conference*; Mecklenburg, M. F.; Charola, A. E.; Koestler, R. J., Eds.; Universidad Politecnica de Valencia and Museum Conservation Institute: Washington, DC, 2013; pp 179–183.
(16) Bosch-Roig, P.; Ranalli, G. The safety of biocleaning technologies for cultural heritage. *Frontiers in Microbiology* **2014,** *5* (155), doi: 10.3389/fmicb.2014.00155.
(17) Palla, F.; Barresi, G. *Biotechnology and Conservation of Cultural Heritage*, Springer International Publishing: 2017.
(18) Mazzuca, C.; Poggi, G.; Bonelli, N.; Micheli, L.; Baglioni, P.; Palleschi, A. Innovative chemical gels meet enzymes: A smart combination for cleaning paper artworks. *J. Colloid Interface Sci.* **2017,** *502*, 153-164.
(19) Mazzoli, R.; Giuffrida, M. G.; Pessione, E. Back to the past—forever young: cutting-edge biochemical and microbiological tools for cultural heritage conservation. *Appl. Microbiol. Biotechnol.* **2018,** *102* (16), 6815-6825.
(20) Decoux, S. Enzymes Used for Adhesive Removal in Paper Conservation: A literature review. *Journal of the Society of Archivists* **2002,** *23* (2), 187-195.
(21) Ahmed, H. E.; Kolisis, F. N. An investigation into the removal of starch paste adhesives from historical textiles by using the enzyme α-amylase. *Journal of Cultural Heritage* **2011,** *12* (2), 169-179.
(22) Mazzuca, C.; Micheli, L.; Cervelli, E.; Basoli, F.; Cencetti, C.; Coviello, T.; Iannuccelli, S.; Sotgiu, S.; Palleschi, A. Cleaning of Paper Artworks: Development of an Efficient Gel-Based Material Able to Remove Starch Paste. *ACS Applied Materials & Interfaces* **2014,** *6* (19), 16519-16528.
(23) Ferrari, M.; Mazzoli, R.; Morales, S.; Fedi, M.; Liccioli, L.; Piccirillo, A.; Cavaleri, T.; Oliva, C.; Gallo, P.; Borla, M.; Cardinali, M.; Pessione, E. Enzymatic laundry for old clothes: immobilized alpha-amylase from Bacillus sp. for the biocleaning of an ancient Coptic tunic. *Appl. Microbiol. Biotechnol.* **2017,** *101* (18), 7041-7052.
(24) Ranalli, G.; Alfano, G.; Belli, C.; Lustrato, G.; Colombini, M. P.; Bonaduce, I.; Zanardini, E.; Abbruscato, P.; Cappitelli, F.; Sorlini, C. Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes. *Journal of Applied Microbiology* **2005,** *98* (1), 73-83.

(25) Girelli, A. M.; Salvagni, L.; Tarola, A. M. Use of lipase immobilized on celluse support for cleaning aged oil layers. *Journal of the Brazilian Chemical Society* **2012,** *23*, 585-592.
(26) Germinario, G.; van der Werf, I. D.; Palazzo, G.; Regidor Ros, J. L.; Montes-Estelles, R. M.; Sabbatini, L. Bioremoval of marker pen inks by exploiting lipase hydrolysis. *Prog. Org. Coat.* **2017,** *110*, 162-171.
(27) De Roe, C.; Courtoy, P. J.; Baudhuin, P. A model of protein-colloidal gold interactions. *Journal of Histochemistry & Cytochemistry* **1987,** *35* (11), 1191-1198.
(28) Calzolai, L.; Franchini, F.; Gilliland, D.; Rossi, F. Protein−Nanoparticle Interaction: Identification of the Ubiquitin−Gold Nanoparticle Interaction Site. *Nano Lett.* **2010,** *10* (8), 3101-3105.
(29) Rangnekar, A.; Sarma, T. K.; Singh, A. K.; Deka, J.; Ramesh, A.; Chattopadhyay, A. Retention of Enzymatic Activity of α-Amylase in the Reductive Synthesis of Gold Nanoparticles. *Langmuir* **2007,** *23* (10), 5700-5706.
(30) Ferrari, E. A plug and play approach for the decoration of nanoparticles with recombinant proteins. *Nanomedicine* **2018,** *13* (20), 2547-2550.
(31) Khan, M. J.; Husain, Q.; Ansari, S. A. Polyaniline-assisted silver nanoparticles: a novel support for the immobilization of α-amylase. *Appl. Microbiol. Biotechnol.* **2013,** *97* (4), 1513-1522.
(32) Defaei, M.; Taheri-Kafrani, A.; Miroliaei, M.; Yaghmaei, P. Improvement of stability and reusability of α-amylase immobilized on naringin functionalized magnetic nanoparticles: A robust nanobiocatalyst. *Int. J. Biol. Macromol.* **2018,** *113*, 354-360.
(33) Deka, J.; Paul, A.; Chattopadhyay, A. Modulating enzymatic activity in the presence of gold nanoparticles. *RSC Advances* **2012,** *2* (11), 4736-4745.
(34) Goodfellow, B. J.; Wilson, R. H. A Fourier Transform IR study of the gelation of amylose and amylopectin. *Biopolymers* **1990,** *30* (13 - 14), 1183-1189.
(35) van Soest, J. J. G.; Tournois, H.; de Wit, D.; Vliegenthart, J. F. G. Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy. *Carbohydr. Res.* **1995,** *279*, 201-214.
(36) Bello-Pérez, L. A.; Ottenhof, M. A.; Agama-Acevedo, E.; Farhat, I. A. Effect of Storage Time on the Retrogradation of Banana Starch Extrudate. *J. Agric. Food. Chem.* **2005,** *53* (4), 1081-1086.
(37) Lopez-Rubio, A.; Flanagan, B. M.; Shrestha, A. K.; Gidley, M. J.; Gilbert, E. P. Molecular Rearrangement Of Starch During In Vitro Digestion: Toward A Better

Understanding Of Enzyme Resistant Starch Formation In Processed Starches. *Biomacromolecules* **2008,** *9* (7), 1951-1958.
(38) Htoon, A.; Shrestha, A. K.; Flanagan, B. M.; Lopez-Rubio, A.; Bird, A. R.; Gilbert, E. P.; Gidley, M. J. Effects of processing high amylose maize starches under controlled conditions on structural organisation and amylase digestibility. *Carbohydr. Polym.* **2009,** *75* (2), 236-245.
(39) Palacios, H. R.; Schwarz, P. B.; D'Appolonia, B. L. Effects of α-Amylases from Different Sources on the Firming of Concentrated Wheat Starch Gels: Relationship to Bread Staling. *J. Agric. Food. Chem.* **2004,** *52* (19), 5987-5994.
(40) Yagar, H.; Ertan, F.; Balkan, B. Comparison of Some Properties of Free and Immobilized α - Amylase by Aspergillus sclerotiorum in Calcium Alginate Gel Beads. *Prep. Biochem. Biotechnol.* **2007,** *38* (1), 13-23.
(41) Ernest, V.; Shiny, P. J.; Mukherjee, A.; Chandrasekaran, N. Silver nanoparticles: a potential nanocatalyst for the rapid degradation of starch hydrolysis by α-amylase. *Carbohydr. Res.* **2012,** *352*, 60-64.
(42) Dennison, J. M.; Zupancic, J. M.; Lin, W.; Dwyer, J. H.; Murphy, C. J. Protein Adsorption to Charged Gold Nanospheres as a Function of Protein Deformability. *Langmuir* **2017,** *33* (31), 7751-7761.
(43) Vahidi, S. H.; Bozorgmehr, M. R.; Morsali, A.; Beyramabadi, S. A. Study of alpha-amylase and gold nanoparticles interaction at two different temperatures through molecular dynamics. *J. Mol. Graphics Modell.* **2019,** *88*, 273-281.
(44) Khan, M. J.; Husain, Q.; Azam, A. Immobilization of porcine pancreatic α-amylase on magnetic Fe2O3 nanoparticles: Applications to the hydrolysis of starch. *Biotechnology and Bioprocess Engineering* **2012,** *17* (2), 377-384.
(45) Homaei, A.; Saberi, D. Immobilization of α-amylase on gold nanorods: An ideal system for starch processing. *Process Biochem.* **2015,** *50* (9), 1394-1399.
(46) Garcia-Hernandez, C.; Freese, A. K.; Rodriguez-Mendez, M. L.; Wanekaya, A. K. In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications. *Biomaterials Science* **2019,** *7* (6), 2511-2519.
(47) Abbasi, M.; Amiri, R.; Bordbar, A.-K.; Ranjbakhsh, E.; Khosropour, A.-R. Improvement of the stability and activity of immobilized glucose oxidase on modified iron oxide magnetic nanoparticles. *Appl. Surf. Sci.* **2016,** *364*, 752-757.

(48) Wang, H.; Huang, J.; Wang, C.; Li, D.; Ding, L.; Han, Y. Immobilization of glucose oxidase using CoFe2O4/SiO2 nanoparticles as carrier. *Appl. Surf. Sci.* **2011,** *257* (13), 5739-5745.

(49) Zou, B.; Song, C.; Xu, X.; Xia, J.; Huo, S.; Cui, F. Enhancing stabilities of lipase by enzyme aggregate coating immobilized onto ionic liquid modified mesoporous materials. *Appl. Surf. Sci.* **2014,** *311*, 62-67.

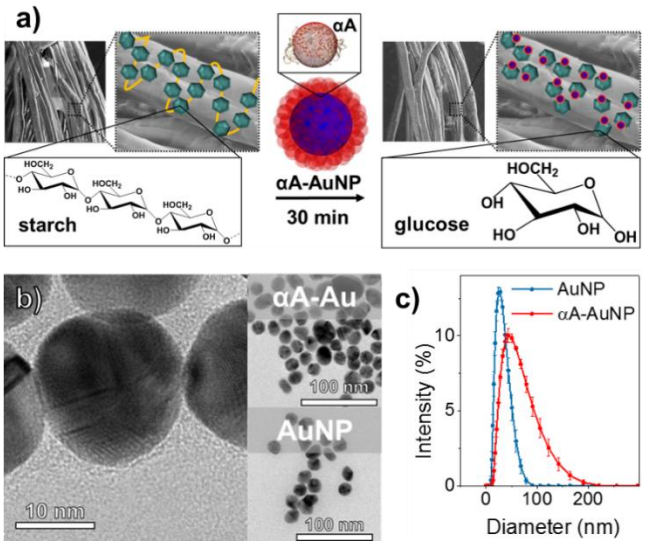

Figure 1 (a) Schematic representation of starch digestion from textile fibres by α-amylase (αA) immobilized on Au NP (αA-AuNP); α-amylase is represented by a red sphere approximating the protein structure; the structure used here for illustrative purpose is a representative α-amylase from *Bacillus licheniformis* (PDB ID: 1BLI). (b) High resolution TEM of αA-AuNP and (insects) TEM images and Au and αA-AuNP. (c) Hydrodynamic size distributions of Au NP and αA-AuNP assessed using DLS; data points are the average of three measurement and the error bars represent the standard deviation.

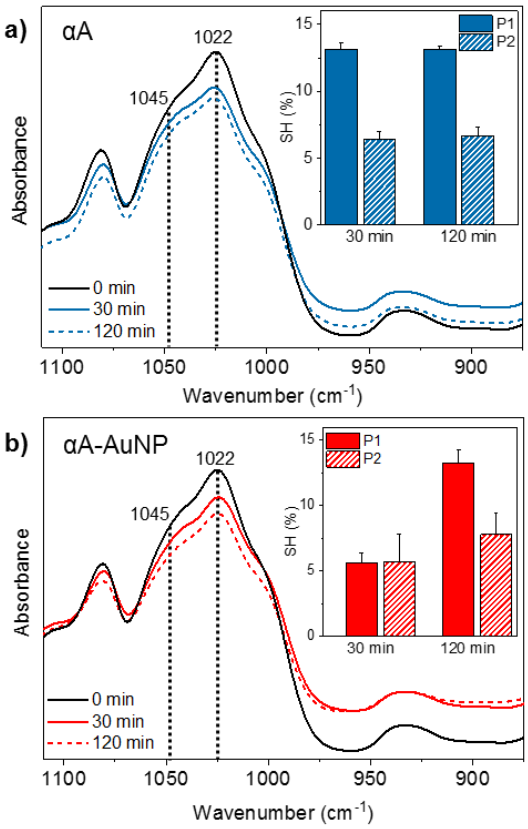

Figure 2 FTIR spectra of silicon windows coated with potato starch 2% (P2) and after 30 min and 120 min from the application of (a) free α-amylase (αA) and (b) α-amylase coated Au nanoparticles (αA-AuNP). Corresponding insets show the histograms of the activity of starch hydrolysis (SH%) of αA and αA-AuNP after 30 min and 120 min from the application on 1% (P1) and 2% (P2) potato starch. Error bars represent standard deviation of three independent repeats.

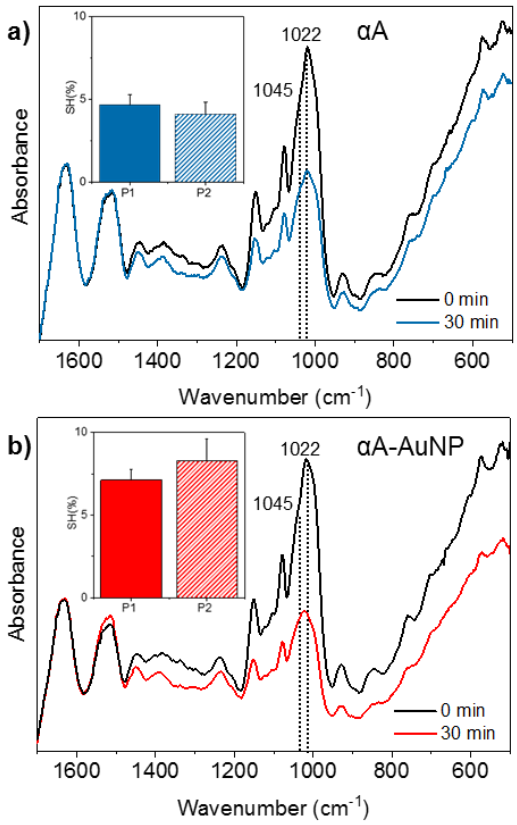

Figure 3 ATR-FTIR spectra of wool coated with potato starch 2% (P2) and after 30 min from the application of (a) free α-amylase (αA) and (b) α-amylase coated Au nanoparticles (αA-AuNP). Corresponding insets show the histograms of the activity of starch hydrolysis (SH%) of αA and αA-AuNP after 30 min from the application on 1% (P1) and 2% (P2) potato starch. Error bars represent standard deviation of three independent repeats.

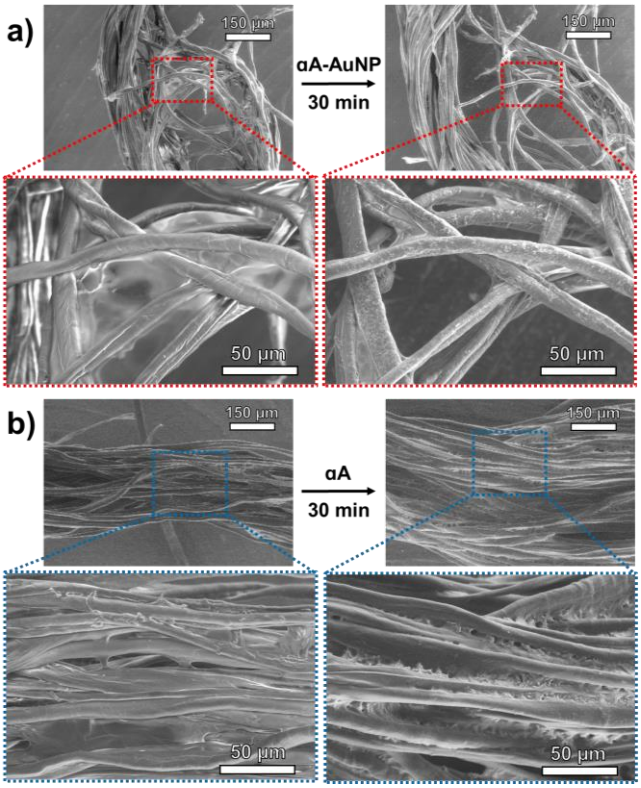

Figure 4 Secondary electron ESEM images of single cotton fibres coated with potato starch 2% before and after the application of (a) α-amylase coated Au NP (αA-AuNP) and (b) α-amylase (αA).

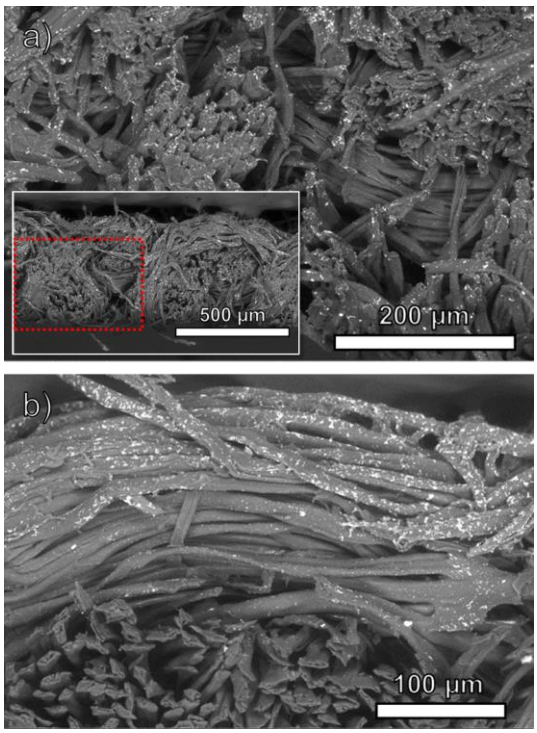

Figure 5 Backscattered electron ESEM images of external (a) and internal (b) sections of cotton woven coated with potato starch 2% after the application of α-amylase coated Au NPs.

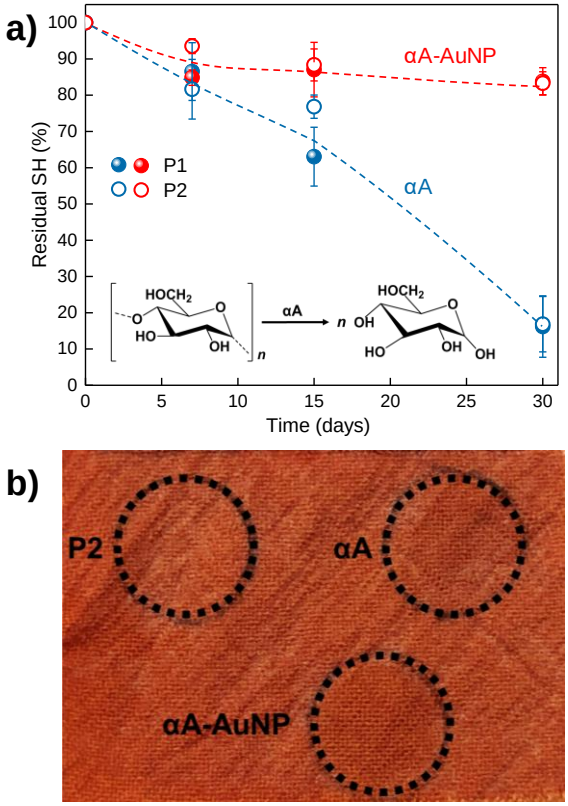

Figure 6 (a) Residual activity in starch hydrolysis (SH) (%) calculated from FTIR spectra of wool coated with potato starch 1% (P1, filled circles) and 2% (P2, empty circles) after the application of α-amylase (αA, blue circles) and Au nanoparticles coated with α-amylase (αA-AuNP, red circles) stored at 23 °C for 7, 15 and 30 days. The dashed lines are guide to the eye. Error bars represent standard deviation of three independent repeats. (b) Image of potato-starch coated wool sample, in which dotted circles are areas treated with alpha-amylase (αA) and Au nanoparticles coated with alpha-amylase (αA-AuNP). P2 dotted circle is a representative area treated with potato starch and characterized by white surface glazing due to starch accumulation.

Table 1 Color difference data obtained from wool sample after the application of αA-AuNP ($\Delta E_{\alpha A\text{-}AuNP}$), after the removal of αA-AuNP with a cotton swab soaked with deionized water ($\Delta E_{post\text{-}wash}$) and comparison with a reference untreated wool sample (ΔE_{Ref}). Values are shown as an average of five measurements on the same area and their standard deviation.

	$\Delta E_{\alpha A\text{-}AuNP}$	$\Delta E_{post\text{-}wash}$	ΔE_{Ref}
P1	4.18±0.52	3.39±0.76	0.94±0.34
P2	6.21±0.44	6.08±0.05	0.45±0.10

Table of Contents