

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

3 Francesca Gherardi,^{*†} Lyudmila Turyanska,^{†,‡} Enrico Ferrari,[†] Nicola Weston,[§] Michael W.
4 Fay,[§] and Belinda Colston^{*†}

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

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

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

8 *Email: lyudmila.turyanska@nottingham.ac.uk*

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

10 *Email: eferrari@lincoln.ac.uk*

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

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

13

14

15 Corresponding authors: bcolston@lincoln.ac.uk

16 fgherardi@lincoln.ac.uk

17

1 **Abstract**

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

15

16

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

18

1 **1. Introduction**

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

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

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

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

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

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

3

4 **2. Materials and Methods**

5 *Preparation of α -amylase dispersion:* Type II-A from *Bacillus sp.* (Sigma-Aldrich A6380) was
6 dissolved in a buffer solution (20 mM HEPES pH=7.3, 100 mM NaCl) at a concentration of 5
7 mg/mL. The enzyme has an estimated molecular weight of 50-55 kDa by SDS-PAGE and
8 $\geq 1,500$ units/mg protein (biuret) according to the technical data sheet.

9 *Preparation of α -amylase coated gold nanoparticles:* α -amylase coated gold nanoparticles
10 were prepared using passive adsorption followed by removal of protein excess by
11 centrifugation. The citrate-capped gold nanoparticles (1 mL, HD.GC20, BBI Solutions) with a
12 diameter of 20 nm and an optical density (OD) at the extinction peak of 5 were pelleted at
13 5000xg for 20 min and the storage buffer replaced with a 250 μ L of α -amylase solution with
14 excess concentration of ~ 25 μ g/mL diluted in 10 mM HEPES buffer, pH=7.3, 10 mM NaCl.
15 After 60 min incubation at room temperature, Au nanoparticles were pelleted at 5000xg for 20
16 min and the solution containing excess enzyme was removed, washed by resuspension in 500
17 μ L of 10 mM HEPES buffer, pH=7.3, 10 mM NaCl, then centrifuged and suspended again in
18 500 μ L of the same buffer solution. The buffer contains a lower concentration of NaCl (10
19 mM) compared to the bugger used for free amylase (100 mM). The enzymatic dispersions were
20 stored at $T = 4$ $^{\circ}$ C.

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

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

3 The optical density (OD) of the gold nanoparticles suspensions at 520 nm was measured by
4 NanoDrop (NanoDrop 2000, Thermo Fisher Scientific). The reference concentration at OD =
5 1 provided by the supplier is $7.0 \times 10^{11} \text{ mL}^{-1} \text{ OD}^{-1}$. The concentration of α -amylase adsorbed on
6 the nanoparticle suspension was estimated from the concentration of the nanoparticles
7 measured from the OD at the extinction peak and assuming complete coverage of the surface
8 (see Supporting Information S11).²⁷⁻²⁸

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

18
19 *Evaluation of the enzymatic activity and the removal of the enzymatic formulations:* Fourier
20 Transform Infrared (FTIR) spectroscopy studies were performed on PerkinElmer Spectrum
21 100 FTIR Spectrometer equipped with a DTGS detector and an Attenuated Total Reflection
22 (ATR) diamond crystal accessory. Dispersions of 1% and 2% (w/v) of potato starch (Sigma
23 Aldrich) boiled in water with stirring for 30 minutes were applied as thin film on silicon
24 polished windows (Crystran Ltd, United Kingdom). Following 30 min and 120 min of exposure
25 to enzymatic dispersions, the FTIR spectra were acquired (64 scans, resolution 4 cm^{-1}). The

1 background spectra were recorded with silicon windows and subtracted from the sample
2 spectra. All spectra were normalized on the intensity of the C-H stretching vibration at ~2930
3 cm^{-1} . The starch hydrolysis (*SH*) was calculated using the following equation: $SH =$
4 $(R_f - R_i)/R_i \times 100\%$, where R_i and R_f are the intensity ratios of the 1045/1022 cm^{-1} peaks
5 before and after treatment with the enzymatic dispersions. Each experiment was repeated three
6 times.

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

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

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

11 *Evaluation of the storage stability of the enzymatic formulations:* The enzymatic dispersions
12 were stored in the laboratory at $T = 23\text{ }^\circ\text{C}$ for 30 days and were exposed to ambient light. The
13 activity in starch hydrolysis was evaluated after 7, 15 and 30 days using FTIR spectroscopy.
14 The activities at different times were compared with the initial activity and the residual activity
15 (%) was calculated.

16

17 **3. Results and discussion**

18 **3.1 Characterization of α -amylase coated gold nanoparticles**

19 Gold nanoparticles coated with α -amylase were prepared following passive adsorption,
20 producing stable and active enzyme-nanoparticle conjugates without need for chemical
21 modification of the enzyme (Figure 1).²⁹⁻³⁰ The citrate-capped AuNP are used to form α A-
22 AuNP and the excess protein is removed by centrifugation. The final concentration of
23 nanoparticles is 3.9×10^{12} NP/mL, with an estimated 35 $\mu\text{g}/\text{mL}$ load of α -amylase (see
24 Supplementary Information, S11). Transmission Electron Microscopy (TEM) images revealed

1 that AuNP cores have average diameters of 18 ± 1 nm and 21 ± 3 nm for pristine and α -amylase
2 coated AuNP, respectively, both consistent with the 20 nm nominal diameter provided by the
3 supplier (Figure 1b). TEM images confirm that adsorption of α -amylase does not change the
4 size and morphology of the gold nanoparticles and leads to the formation of a stable colloidal
5 solution³¹⁻³² with only minimal aggregation (see the insets of Figure 1b), likely due to
6 electrostatic or van der Waals interactions between amylase on neighbouring nanoparticles.³³

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

14

15

16 **3.2 Evaluation of the enzymatic activity**

17 The activity in starch hydrolysis of free α -amylase (α A) and α -amylase immobilized on AuNP
18 (α A-AuNP) was evaluated by FTIR spectroscopy by following 30 min and 120 min digestion
19 of 1% w/v (P1) and 2% w/v (P2) potato starch applied as film on silicon windows (Figure 2
20 and Supporting Information SI2, Figure S1). We examined the bands in the spectral region of
21 $950-1100$ cm^{-1} characteristic for modifications of starch conformation³⁴⁻³⁸ and ascribed to C-
22 O and C-C stretching vibrational modes. The bands at 1022 cm^{-1} and 1045 cm^{-1} are associated
23 with amorphous and ordered/crystalline regions in starch, respectively.^{35-36, 38} With the
24 hydrolysis of starch by amylase, the chain length decreases and an increase of crystallinity is
25 expected resulting in a higher ratio of $1045/1022$ cm^{-1} peak intensities.^{37, 39}

1 In our studies, an increase in the ratio of 1045/1022 cm^{-1} peak absorbance is observed
2 following exposure to the enzymatic dispersions (Figure 2). The starch hydrolysis (SH) was
3 evaluated by measuring the percentage of increase of crystallinity, calculated from the
4 1045/1022 cm^{-1} peak ratio. The SH of α A after 30 minutes was higher compared to the
5 immobilized protein, suggesting faster activity (starch hydrolysis values after 30 minutes
6 exposure were about 13% and 6% for α A and α A-AuNP, respectively). No significant SH
7 increase was detected after 120 min for α A (Figure 2a). Instead, as shown in the Figure 2b, the
8 starch digestion by α A-Au was initially lower (at 30 min), however the activity was enhanced
9 over longer time compared to free enzymes. The sustained activity was especially evident on
10 silicon windows coated with P2 (increase of more than 20%), with SH values of \sim 6.5% for α A
11 and \sim 8% for α A-AuNP (Figure 2b), similar to those reported for silver nanoparticles and
12 calcium alginate gel beads.^{31, 40} The lower initial efficiency can be attributed to slower diffusion
13 of enzyme/NP dispersions compared to free enzymes. In addition, the presence of metal
14 nanoparticles has an impact on biomolecules, altering their catalytic activity either by
15 increasing or decreasing the affinity for enzyme-substrate formation.³³ Our results indicate that
16 gold nanoparticles could play a role as a nanocatalyst in the hydrolysis of starch by α -
17 amylase.^{33, 41} We also envisage that the enzymatic activity of immobilized amylase is not
18 affected but rather enhanced, possibly due to favourable orientation towards the substrate of
19 the catalytic sites of the enzyme.^{29, 42-43}

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

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

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

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

8 To evaluate the penetration depth of α A-AuNP, a cross-section of woven cotton with starch
9 was observed by ESEM with a backscatter electron detector (BSD) after the application of the
10 enzymes (Figure 5). The images revealed the presence of the enzymatic dispersion throughout
11 the full depth of the sample (Figure 5a). The cross-section of the woven cotton in the centre of
12 the sample also confirmed diffusion of α A-AuNP (Figure 5b) through \sim 200 μ m thick top layer
13 of the sample. Further improvement of the penetration depth could be achieved by treating the
14 woven cotton with a higher volume of a more diluted enzymatic dispersion.

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

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

1 removal of the formulations after cleaning with a cotton swab soaked with deionized water.
2 The results indicate that α A-AuNP affect the surface color of wool with a reduction of the
3 lightness (L^*) and an increase of the red color intensity (a^*) ($\Delta E_{\alpha A-AuNP}$ values of 4.18 and 6.21
4 for wool treated with P1 and P2) (Table 1). The final treatment with deionized water led to
5 complete removal of the enzymatic treatment, restoring the initial color of wool, as evident
6 from the color difference values between the cleaned surface and a reference untreated wool <
7 1, which is considered as ΔE value not perceived by human eye.

8

9 **3.3 Evaluation of the stability of the enzymatic formulations**

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

19 Improved activity and shelf life of immobilized enzymes were further confirmed on wool
20 samples coated with starch. After 7 days, lower residual activities of 82% was observed for
21 free amylase on P2, compared to 94% for AuNP immobilized amylase (Figure 6). After 30
22 days, significant reduction of activity was observed for α A (17%), while α A-AuNP retained
23 >80% of activity (Figure 6). These observations are consistent with the hypothesis that over
24 time free enzymes undergo unfolding (denaturation) while the immobilization of enzymes
25 improves the stability towards environmental change and prevents enzyme deactivation.^{3, 32, 44-}

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

6 Our results highlight the advantages of enzymes immobilized on Au nanoparticles leading
7 to increased storage stability of enzymatic formulations and their effectiveness in starch
8 hydrolysis. The α A-AuNP formulations effectively removed starch from the fibres at room
9 temperature after only 30 min from the application, without the need for water bath with
10 optimized temperature to achieve the catalytic action of the enzyme.²¹ In addition, amylase-
11 coated AuNP allow the application of higher concentration of enzymes without their
12 precipitation/agglomeration, hence enabling the cleaning process without swelling and
13 damaging the fibres. The use of nanoparticles to graft enzymes fosters a good penetration into
14 the fibres with no salt precipitation from the buffer detected. We believe that an increased
15 enzymatic efficiency of the α A-AuNP formulation could justify the cost of this treatment for
16 valuable heritage materials. We also note that further reduction of the cost of these formulations
17 could be achieved by translating the approach developed in this work on other nanoparticles
18 (e.g., SiO₂, TiO₂).

19

20 **4. Conclusions**

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

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

9

10 **Supporting Information**

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

13 **Conflicts of interest**

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

15 **Acknowledgements**

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

18

19

20

21

1 **References**

- 2 (1) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. Dispelling the Myths--Biocatalysis in
3 Industrial Synthesis. *Science* **2003**, *299* (5613), 1694-1697.
- 4 (2) Bornscheuer, U. T. Immobilizing Enzymes: How to Create More Suitable Biocatalysts.
5 *Angew. Chem. Int. Ed.* **2003**, *42* (29), 3336-3337.
- 6 (3) Homaei, A. A.; Sariri, R.; Vianello, F.; Stevanato, R. Enzyme immobilization: an update.
7 *Journal of chemical biology* **2013**, *6* (4), 185-205.
- 8 (4) Sheldon, R. A.; van Pelt, S. Enzyme immobilisation in biocatalysis: why, what and how.
9 *Chem. Soc. Rev.* **2013**, *42* (15), 6223-6235.
- 10 (5) Ansari, S. A.; Husain, Q. Potential applications of enzymes immobilized on/in nano
11 materials: A review. *Biotechnol. Adv.* **2012**, *30* (3), 512-523.
- 12 (6) Cipolatti, E. P.; Silva, M. J. A.; Klein, M.; Feddern, V.; Feltes, M. M. C.; Oliveira, J. V.;
13 Ninow, J. L.; de Oliveira, D. Current status and trends in enzymatic nanoimmobilization. *J.*
14 *Mol. Catal. B: Enzym.* **2014**, *99*, 56-67.
- 15 (7) Mei, S.; Shi, J.; Zhang, S.; Wang, Y.; Wu, Y.; Jiang, Z.; Wu, H. Nanoporous
16 Phyllosilicate Assemblies for Enzyme Immobilization. *ACS Applied Bio Materials* **2019**, *2*
17 (2), 777-786.
- 18 (8) Ma, W.; Saccardo, A.; Roccatano, D.; Aboagye-Mensah, D.; Alkaseem, M.; Jewkes, M.;
19 Di Nezza, F.; Baron, M.; Soloviev, M.; Ferrari, E. Modular assembly of proteins on
20 nanoparticles. *Nature Communications* **2018**, *9* (1), 1489.
- 21 (9) Daniel, M.-C.; Astruc, D. Gold Nanoparticles: Assembly, Supramolecular Chemistry,
22 Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and
23 Nanotechnology. *Chem. Rev.* **2004**, *104* (1), 293-346.
- 24 (10) Baglioni, P.; Carretti, E.; Chelazzi, D. Nanomaterials in art conservation. *Nature*
25 *Nanotechnology* **2015**, *10*, 287.
- 26 (11) Baglioni, P.; Chelazzi, D.; Giorgi, R. *Nanotechnologies in the Conservation of Cultural*
27 *Heritage: A compendium of materials and techniques*, Springer Netherlands: 2014.
- 28 (12) Mazzuca, C.; Micheli, L.; Carbone, M.; Basoli, F.; Cervelli, E.; Iannuccelli, S.; Sotgiu,
29 S.; Palleschi, A. Gellan hydrogel as a powerful tool in paper cleaning process: A detailed
30 study. *J. Colloid Interface Sci.* **2014**, *416*, 205-211.
- 31 (13) Bonelli, N.; Poggi, G.; Chelazzi, D.; Giorgi, R.; Baglioni, P. Poly(vinyl
32 alcohol)/poly(vinyl pyrrolidone) hydrogels for the cleaning of art. *J. Colloid Interface Sci.*
33 **2019**, *536*, 339-348.

- 1 (14) Valentini, F.; Diamanti, A.; Palleschi, G. New bio-cleaning strategies on porous building
2 materials affected by biodeterioration event. *Appl. Surf. Sci.* **2010**, *256* (22), 6550-6563.
- 3 (15) Cremonesi, P. Rigid Gels and Enzyme Cleaning. In *New Insights into the Cleaning of*
4 *Paintings: Proceedings from the Cleaning 2010 International Conference*; Mecklenburg, M.
5 F.; Charola, A. E.; Koestler, R. J., Eds.; Universidad Politecnica de Valencia and Museum
6 Conservation Institute: Washington, DC, 2013; pp 179–183.
- 7 (16) Bosch-Roig, P.; Ranalli, G. The safety of biocleaning technologies for cultural heritage.
8 *Frontiers in Microbiology* **2014**, *5* (155), doi: 10.3389/fmicb.2014.00155.
- 9 (17) Palla, F.; Barresi, G. *Biotechnology and Conservation of Cultural Heritage*, Springer
10 International Publishing: 2017.
- 11 (18) Mazzuca, C.; Poggi, G.; Bonelli, N.; Micheli, L.; Baglioni, P.; Palleschi, A. Innovative
12 chemical gels meet enzymes: A smart combination for cleaning paper artworks. *J. Colloid*
13 *Interface Sci.* **2017**, *502*, 153-164.
- 14 (19) Mazzoli, R.; Giuffrida, M. G.; Pessione, E. Back to the past—forever young: cutting-
15 edge biochemical and microbiological tools for cultural heritage conservation. *Appl.*
16 *Microbiol. Biotechnol.* **2018**, *102* (16), 6815-6825.
- 17 (20) Decoux, S. Enzymes Used for Adhesive Removal in Paper Conservation: A literature
18 review. *Journal of the Society of Archivists* **2002**, *23* (2), 187-195.
- 19 (21) Ahmed, H. E.; Kolisis, F. N. An investigation into the removal of starch paste adhesives
20 from historical textiles by using the enzyme α -amylase. *Journal of Cultural Heritage* **2011**,
21 *12* (2), 169-179.
- 22 (22) Mazzuca, C.; Micheli, L.; Cervelli, E.; Basoli, F.; Cencetti, C.; Coviello, T.; Iannuccelli,
23 S.; Sotgiu, S.; Palleschi, A. Cleaning of Paper Artworks: Development of an Efficient Gel-
24 Based Material Able to Remove Starch Paste. *ACS Applied Materials & Interfaces* **2014**, *6*
25 (19), 16519-16528.
- 26 (23) Ferrari, M.; Mazzoli, R.; Morales, S.; Fedi, M.; Liccioli, L.; Piccirillo, A.; Cavaleri, T.;
27 Oliva, C.; Gallo, P.; Borla, M.; Cardinali, M.; Pessione, E. Enzymatic laundry for old clothes:
28 immobilized alpha-amylase from *Bacillus* sp. for the biocleaning of an ancient Coptic tunic.
29 *Appl. Microbiol. Biotechnol.* **2017**, *101* (18), 7041-7052.
- 30 (24) Ranalli, G.; Alfano, G.; Belli, C.; Lustrato, G.; Colombini, M. P.; Bonaduce, I.;
31 Zanardini, E.; Abbruscato, P.; Cappitelli, F.; Sorlini, C. Biotechnology applied to cultural
32 heritage: biorestitution of frescoes using viable bacterial cells and enzymes. *Journal of*
33 *Applied Microbiology* **2005**, *98* (1), 73-83.

- 1 (25) Girelli, A. M.; Salvagni, L.; Tarola, A. M. Use of lipase immobilized on cellulose support
2 for cleaning aged oil layers. *Journal of the Brazilian Chemical Society* **2012**, *23*, 585-592.
- 3 (26) Germinario, G.; van der Werf, I. D.; Palazzo, G.; Regidor Ros, J. L.; Montes-Estelles, R.
4 M.; Sabbatini, L. Bioremoval of marker pen inks by exploiting lipase hydrolysis. *Prog. Org.*
5 *Coat.* **2017**, *110*, 162-171.
- 6 (27) De Roe, C.; Courtoy, P. J.; Baudhuin, P. A model of protein-colloidal gold interactions.
7 *Journal of Histochemistry & Cytochemistry* **1987**, *35* (11), 1191-1198.
- 8 (28) Calzolari, L.; Franchini, F.; Gilliland, D.; Rossi, F. Protein–Nanoparticle Interaction:
9 Identification of the Ubiquitin–Gold Nanoparticle Interaction Site. *Nano Lett.* **2010**, *10* (8),
10 3101-3105.
- 11 (29) Rangnekar, A.; Sarma, T. K.; Singh, A. K.; Deka, J.; Ramesh, A.; Chattopadhyay, A.
12 Retention of Enzymatic Activity of α -Amylase in the Reductive Synthesis of Gold
13 Nanoparticles. *Langmuir* **2007**, *23* (10), 5700-5706.
- 14 (30) Ferrari, E. A plug and play approach for the decoration of nanoparticles with
15 recombinant proteins. *Nanomedicine* **2018**, *13* (20), 2547-2550.
- 16 (31) Khan, M. J.; Husain, Q.; Ansari, S. A. Polyaniline-assisted silver nanoparticles: a novel
17 support for the immobilization of α -amylase. *Appl. Microbiol. Biotechnol.* **2013**, *97* (4),
18 1513-1522.
- 19 (32) Defaei, M.; Taheri-Kafrani, A.; Miroliaei, M.; Yaghmaei, P. Improvement of stability
20 and reusability of α -amylase immobilized on naringin functionalized magnetic nanoparticles:
21 A robust nanobiocatalyst. *Int. J. Biol. Macromol.* **2018**, *113*, 354-360.
- 22 (33) Deka, J.; Paul, A.; Chattopadhyay, A. Modulating enzymatic activity in the presence of
23 gold nanoparticles. *RSC Advances* **2012**, *2* (11), 4736-4745.
- 24 (34) Goodfellow, B. J.; Wilson, R. H. A Fourier Transform IR study of the gelation of
25 amylose and amylopectin. *Biopolymers* **1990**, *30* (13 - 14), 1183-1189.
- 26 (35) van Soest, J. J. G.; Tournois, H.; de Wit, D.; Vliegthart, J. F. G. Short-range structure
27 in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-
28 transform IR spectroscopy. *Carbohydr. Res.* **1995**, *279*, 201-214.
- 29 (36) Bello-Pérez, L. A.; Ottenhof, M. A.; Agama-Acevedo, E.; Farhat, I. A. Effect of Storage
30 Time on the Retrogradation of Banana Starch Extrudate. *J. Agric. Food. Chem.* **2005**, *53* (4),
31 1081-1086.
- 32 (37) Lopez-Rubio, A.; Flanagan, B. M.; Shrestha, A. K.; Gidley, M. J.; Gilbert, E. P.
33 Molecular Rearrangement Of Starch During In Vitro Digestion: Toward A Better

1 Understanding Of Enzyme Resistant Starch Formation In Processed Starches.
2 *Biomacromolecules* **2008**, 9 (7), 1951-1958.

3 (38) Htoon, A.; Shrestha, A. K.; Flanagan, B. M.; Lopez-Rubio, A.; Bird, A. R.; Gilbert, E.
4 P.; Gidley, M. J. Effects of processing high amylose maize starches under controlled
5 conditions on structural organisation and amylase digestibility. *Carbohydr. Polym.* **2009**, 75
6 (2), 236-245.

7 (39) Palacios, H. R.; Schwarz, P. B.; D'Appolonia, B. L. Effects of α -Amylases from
8 Different Sources on the Firming of Concentrated Wheat Starch Gels: Relationship to Bread
9 Staling. *J. Agric. Food. Chem.* **2004**, 52 (19), 5987-5994.

10 (40) Yagar, H.; Ertan, F.; Balkan, B. Comparison of Some Properties of Free and
11 Immobilized α - Amylase by *Aspergillus sclerotiorum* in Calcium Alginate Gel Beads.
12 *Prep. Biochem. Biotechnol.* **2007**, 38 (1), 13-23.

13 (41) Ernest, V.; Shiny, P. J.; Mukherjee, A.; Chandrasekaran, N. Silver nanoparticles: a
14 potential nanocatalyst for the rapid degradation of starch hydrolysis by α -amylase.
15 *Carbohydr. Res.* **2012**, 352, 60-64.

16 (42) Dennison, J. M.; Zupancic, J. M.; Lin, W.; Dwyer, J. H.; Murphy, C. J. Protein
17 Adsorption to Charged Gold Nanospheres as a Function of Protein Deformability. *Langmuir*
18 **2017**, 33 (31), 7751-7761.

19 (43) Vahidi, S. H.; Bozorgmehr, M. R.; Morsali, A.; Beyramabadi, S. A. Study of alpha-
20 amylase and gold nanoparticles interaction at two different temperatures through molecular
21 dynamics. *J. Mol. Graphics Modell.* **2019**, 88, 273-281.

22 (44) Khan, M. J.; Husain, Q.; Azam, A. Immobilization of porcine pancreatic α -amylase on
23 magnetic Fe₂O₃ nanoparticles: Applications to the hydrolysis of starch. *Biotechnology and*
24 *Bioprocess Engineering* **2012**, 17 (2), 377-384.

25 (45) Homaei, A.; Saberi, D. Immobilization of α -amylase on gold nanorods: An ideal system
26 for starch processing. *Process Biochem.* **2015**, 50 (9), 1394-1399.

27 (46) Garcia-Hernandez, C.; Freese, A. K.; Rodriguez-Mendez, M. L.; Wanekaya, A. K. In
28 situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological
29 applications. *Biomaterials Science* **2019**, 7 (6), 2511-2519.

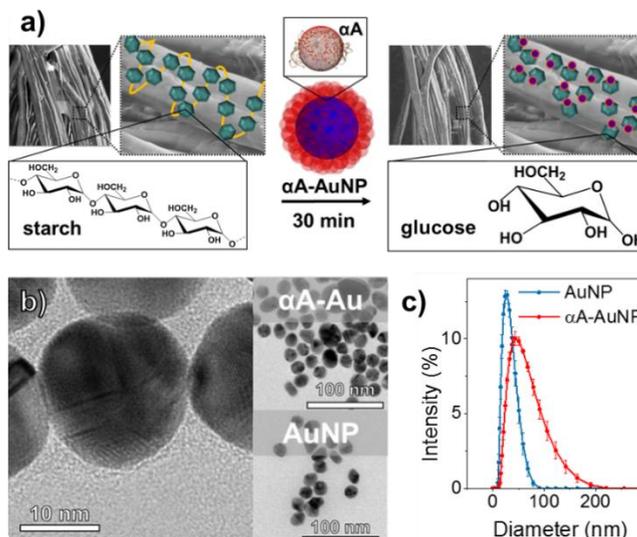
30 (47) Abbasi, M.; Amiri, R.; Bordbar, A.-K.; Ranjbakhsh, E.; Khosropour, A.-R. Improvement
31 of the stability and activity of immobilized glucose oxidase on modified iron oxide magnetic
32 nanoparticles. *Appl. Surf. Sci.* **2016**, 364, 752-757.

- 1 (48) Wang, H.; Huang, J.; Wang, C.; Li, D.; Ding, L.; Han, Y. Immobilization of glucose
2 oxidase using CoFe₂O₄/SiO₂ nanoparticles as carrier. *Appl. Surf. Sci.* **2011**, *257* (13), 5739-
3 5745.
- 4 (49) Zou, B.; Song, C.; Xu, X.; Xia, J.; Huo, S.; Cui, F. Enhancing stabilities of lipase by
5 enzyme aggregate coating immobilized onto ionic liquid modified mesoporous materials.
6 *Appl. Surf. Sci.* **2014**, *311*, 62-67.

7

8

1
2
3

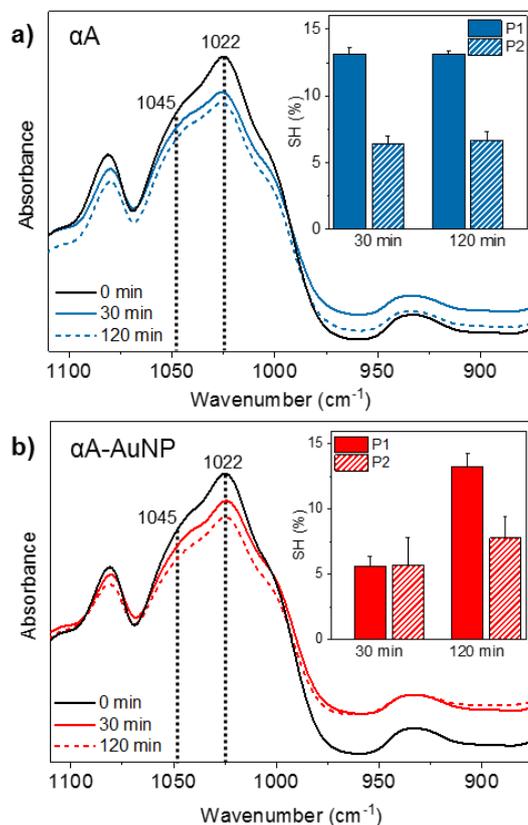


4
5
6
7
8
9
10
11

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

19

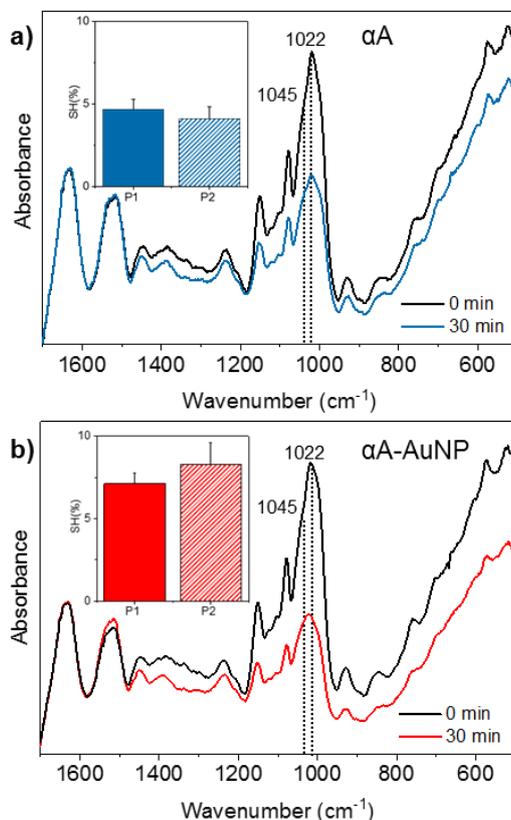
1
2
3



4
5
6
7
8
9
10
11
12
13
14
15

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.

1
2
3

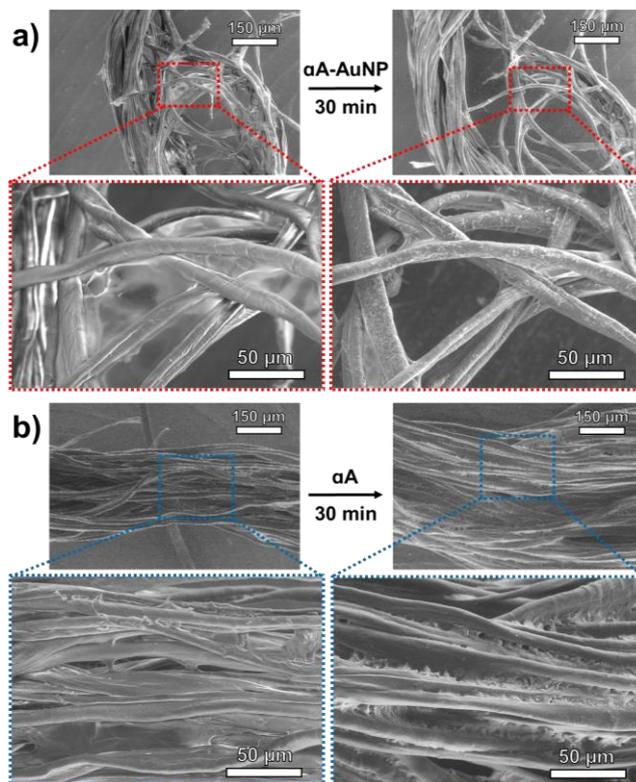


4
5
6
7
8

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

14
15

1
2
3



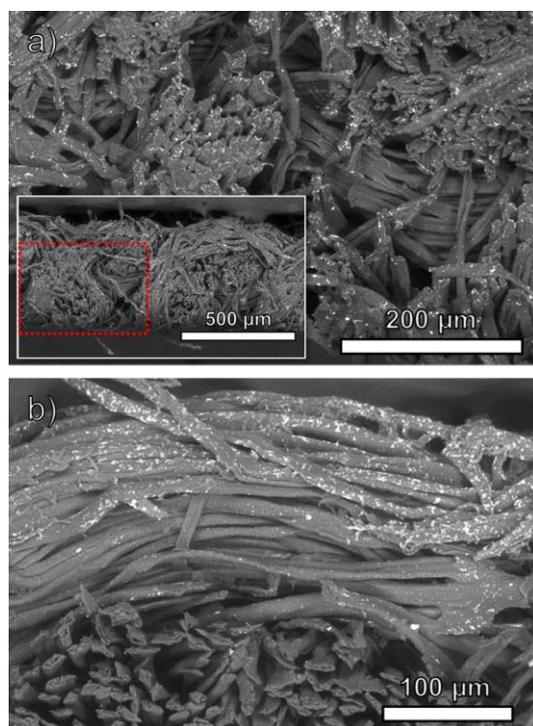
4
5
6
7
8
9
10
11
12
13
14
15
16

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 (α A-AuNP) and (b) α -amylase (α A).

1

2

3



4

5

6

7

8

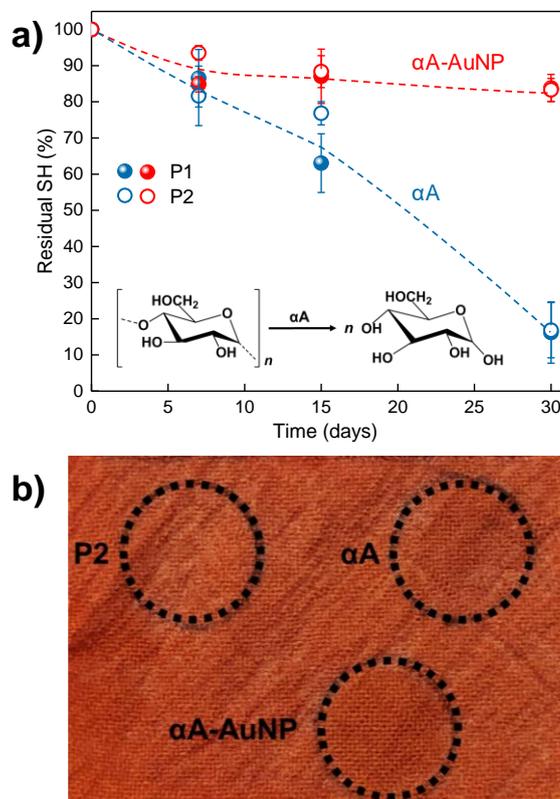
9

10

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

13

1
2
3



4
5

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

15

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29

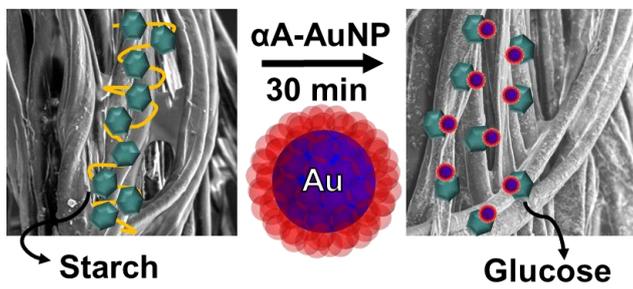
Table 1 Color difference data obtained from wool sample after the application of α A-AuNP ($\Delta E_{\alpha A-AuNP}$), after the removal of α A-AuNP with a cotton swab soaked with deionized water ($\Delta E_{\text{post-wash}}$) and comparison with a reference untreated wool sample (ΔE_{Ref}). Values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

	$\Delta E_{\alpha A-AuNP}$	$\Delta E_{\text{post-wash}}$	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

1

2



3

4

5