Electronic Supporting Information (ESI)

Biocatalytic Mechano-Responsive Polyelectrolyte Multilayers Based on Covalently Cross-linked Enzymes

Cesar Rios, Johan Longo, Sarah Zahouani, Tony Garnier, Cédric Vogt, Andreas Reisch, Bernard Senger, Fouzia Boulmedais, Joseph Hemmerlé, Karim Benmlih, Benoît Frisch, Pierre Schaaf, Loïc Jierry and Philippe Lavalle*

1. Chemicals and chemical modifications of polymers and enzymes

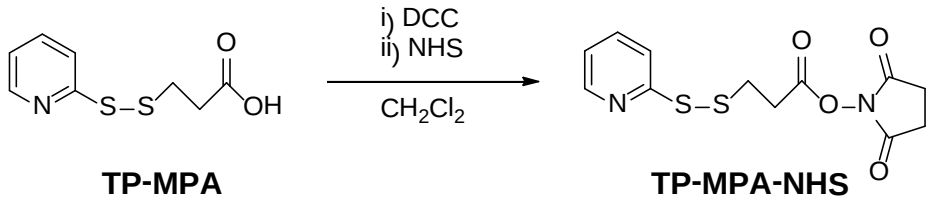
1.1. Chemicals

The polyelectrolytes poly(L-lysine) (PLL, $Mw = 2.60 \times 10^4$ Da) and hyaluronic acid (HA, $Mw = 1.32 \times 10^5$ Da) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) and Lifecore Biomedical (Chaska, USA) respectively. Coupling agents as *N*,*N*′-Dicyclohexylcarbodiimide 99% (DCC), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride ≥ 98% (EDC), *N*-Hydroxysuccinimide 98% (NHS), and *N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) ≥ 98% were all purchased from Sigma Aldrich. The enzyme β-galactosidase (β-Gal) from *Escherichia coli* Grade VI, lyophilized powder, 250-600 units/mg protein and the enzyme substrate fluorescein di(β-D-galactopyranoside) (FDG) were purchased from Sigma Aldrich. **2,4,6-Trinitrobenzenesulfonic acid** (TNBS), Tris(2-carboxyethyl)phosphine hydrochloride powder, ≥ 98% (TCEP), tris(hydroxymethyl)aminomethane (TRIS) and **4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid** (HEPES), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$) anhydrous solvents for synthesis dichloromethane (CH_2Cl_2), *N*,*N*-Dimethylformamide (DMF) were all purchased from Sigma Aldrich. Dialyzed cellulose ester membrane (MWCO 3500 Da) was purchased from Carl Roth (Lauterbourg, France) and bicinchoninic assay protein quantification kit was purchased from Uptima (Montluçon, France). Thiopyridone mercaptopropionic acid (TP-MPA) was synthesized by the procedure describe by Xie *et al.*[1] Maleimide succinimide ester was synthesized by the procedure describe by Thibaudeau *et al.*[2] Poly(dimethylsiloxane) (PDMS) sheets of 250 µm thickness (Specialty Manufacturing Inc., Saginaw, USA) and circular glass slides with 12-mm diameter and 150-µm thickness (Menzel-Gläser, Braunschweig, Germany) were chosen as substrates for the construction of polyelectrolyte multilayer (PEM) films.

1.2. Chemical modification of PLL with thiopyridone protecting groups (PLL-S-TP)

The modified poly(L-Lysine) with thiopyridone groups (PLL-S-TP) was obtained in a two-step reaction. The first step consists in the activation of the thiopyridone mercaptopropionic acid (TP-MPA) with the *N*-hydroxysuccinimide (NHS) followed by the second step where the amines of the PLL will make a nucleophilic attack on the activated acid cited before.

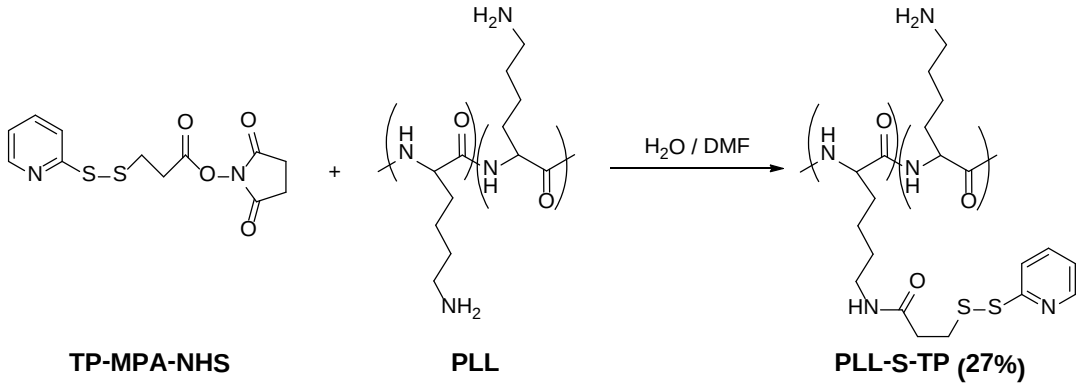
1.2.1. First step: activation of the TP-MPA acid with NHS



Scheme S1. Synthesis of TP-MPA-NHS

The TP-MPA (186 mg, 0.864 mmol) was dissolved in 8 mL of CH_2Cl_2 followed by the addition of DCC (178 mg, 0.864 mmol, 1 eq) and NHS (99 mg, 0.864 mmol, 1eq). The reaction was stirred at room temperature for 24 h. The solution was filtered and the solvent was removed under pressure until dryness to obtain a yellow solid compound TP-MPA-NHS in a quantitative yield which will be used in the next step without further purification. The characterization of this compound is described by Xie *et al.*[1]

1.2.2. Second step: coupling reaction between TP-MPA-NHS and the PLL



Scheme S2. Synthesis of PLL-S-TP

The PLL (300 mg, 1.44 mmol) was dissolved in 15 mL of Milli-Q water followed by the addition of a solution of TP-MPA-NHS (269.9 mg, 0.864 mmol, 0.6 eq) in 3 mL of DMF. The reaction was stirred at room temperature for 24 h. The solution was filtered and dialyzed (cellulose ester membrane (MWCO 3500 Da)) against a 2 L solution of NaCl 0.30 M for 1

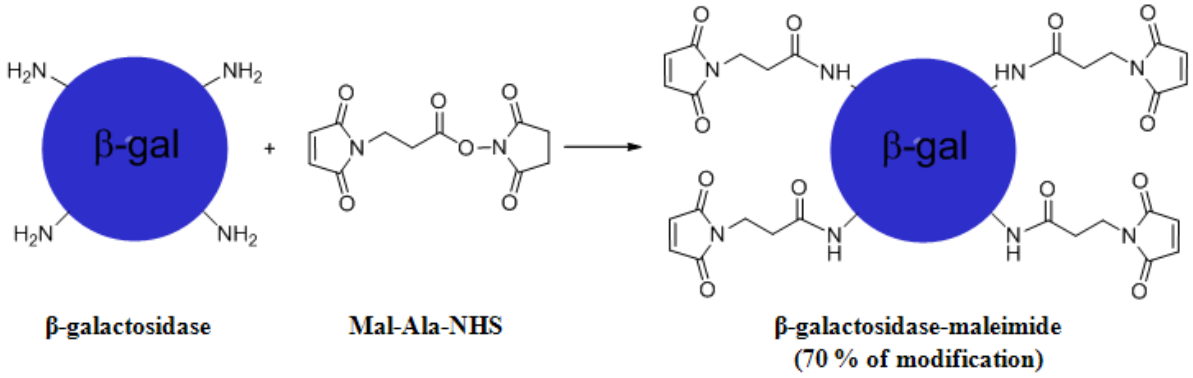
day at 4°C and then changed two times against 2 L of Milli-Q water for another 2 days at 4°C. Lyophilisation of the solution afforded PLL-S-TP as a white like foam with a 75 % yield.

^{1}H NMR (D_2O, 400 MHz, δ ppm): 8.30 (br s, 0.27H, Ar), 7.75 (br s, 0.54H, Ar), 7.20 (br s, 0.27H, Ar) 4.35 (br s, 1H, CαH), 3.05 (br s, 0.54H, CH_2 thiopyridyl), 2.99 (br s, 2H, CH_2 αNH_2), 2.70 (br s, 0.54H, CH_2 thiopyridyl) 1.85 (br m, 4H CH_2CH_2), 1.40 (br s, 2H, CH_2).

By comparison of the integration of one aromatic signal and the integration of the broad singlet at 4.25 (H on the carbon α of the lysine amino acid), the percentage of modification on the PLL by thiopyridone groups is estimated to be 27%

1.3. Chemical modification of β-galactosidase with maleimide groups (β-Gal-mal)

1.3.1. Chemical modification



Scheme S3. Synthesis of ß-Gal-mal.

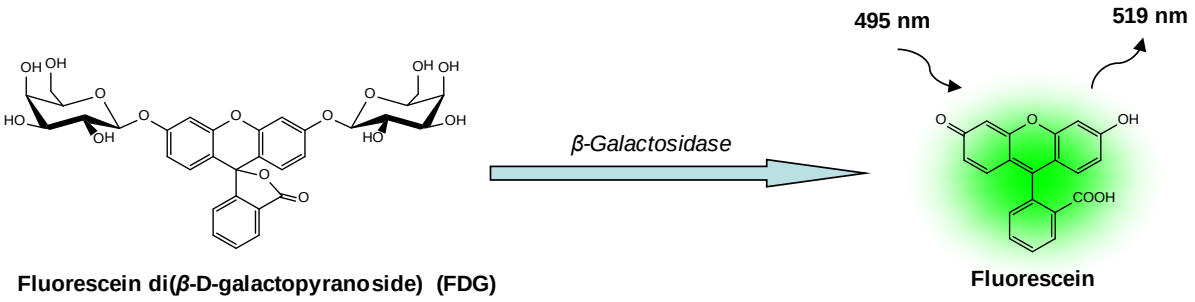
β-Gal from *Escherichia coli* (7 mg, 1.61 × 10^{-5} mmol) was dissolved in 1 mL of $NaHCO_3$ 0.1 M at pH 8.5 followed by 4 mL of HEPES 50 mM at pH 6. Then, 500 µL of a 1 mg/mL (0.5 mg, 1.87 × 10^{-3} mmol) 3-(Maleimido)propionic acid N-succinimidyl ester (Mal-Ala-NHS) solution in DMF is added to the β-Gal solution and stirred at room temperature for 3h. The solution was dialyzed (cellulose ester membrane (MWCO 10000 Da)) against a 2 L solution of 50 mM HEPES and two times against a 2 L solution 10 mM HEPES pH 6 at 4°C for 3 days. Aliquots of 250 µL were prepared and conserved at -20°C. The modified enzymes with maleimide groups (ß-Gal-mal) were not lyophilized after chemical modification to avoid the loss of enzymatic activity as observed in previous experiments where this lyophilization was performed.

1.3.2. Determination of the modified enzyme (ß-Gal-mal) concentration

We used the bicinchoninic acid (BCA) assay test to determine the concentration of the solutions of the modified enzyme.[3] A fresh set of protein standards, from 20 µg.mL^{-1} to 1 mg.mL^{-1}, were prepared using the bovine serum albumin (BSA) at 2 mg.mL^{-1}. β-Gal-mal and β-Gal (control) solutions at 1 mg.mL^{-1} were prepared and then diluted to 0.25 mg.mL^{-1} in $NaHCO_3$ 0.1 M solutions at pH 8.5. Then 2 mL of BC Assay reagent (mixture of 50 parts of bicinchoninic acid and 1 part of $CuSO_4$) were added to the test tubes, mixed and incubated at 60°C for 20 min. All the test tubes were mixed at room temperature and their optical absorbance read at 562 nm against the blank $NaHCO_3$ in a 96-well plate. Then the protein concentrations can be calculated with a reference curve obtained for a standard protein. Finally, we obtained a concentration of 0.4 mg.mL^{-1} for β-Gal-mal and 0.5 mg.mL^{-1} for β-Gal.

1.3.3. Verification of the catalytic activity of the modified enzyme

The enzymatic activity of the β-Gal-mal was verified against the unmodified enzyme β-Gal in presence of the substrate fluorescein di(β-D-galactopyranoside) (FDG). The principle of this experiment is the hydrolysis in 2 steps of the FDG in the presence of the enzyme to obtain fluorescein (Scheme S4).



Scheme S4. Hydrolysis reaction of the FDG by the β-Gal.

In a 96-well plate, we added 200 µL of FDG at 0.071 mg.mL^{-1} and 50 µL of the enzymes separately at 0.0048 mg.mL^{-1}. Then, the catalytic activity was recorded with the spectrofluorimeter equipped with a microplate reader ($\lambda_{ex/em}$: 495 nm / 519 nm; see 2.2 below). The modified enzymes appear 3.4 times less active than the non-modified β-Gal.

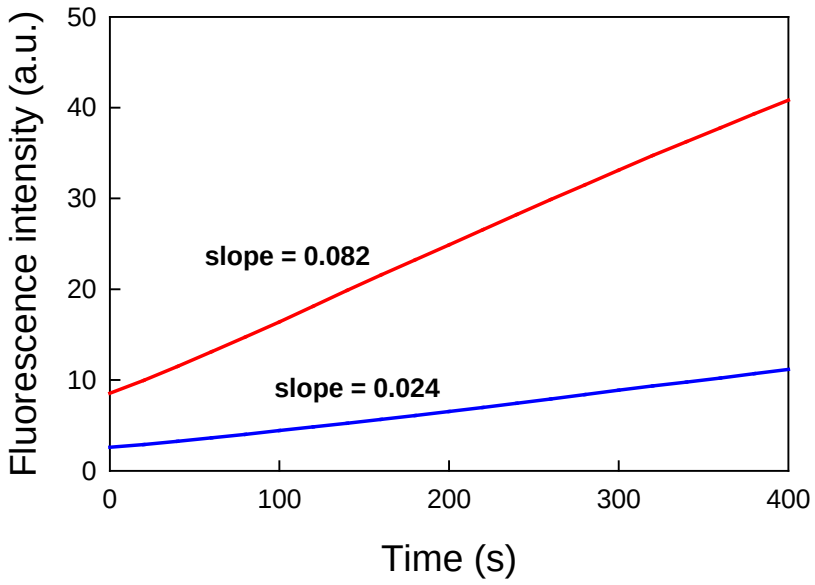
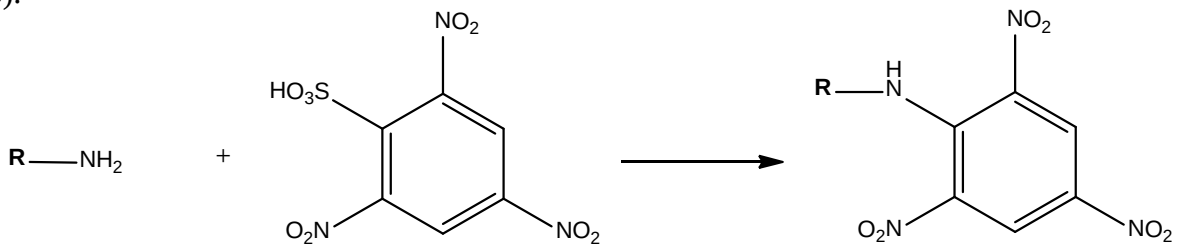


Figure S1. Comparison of the enzymatic activity of the modified (β-Gal-mal) (blue line) and unmodified (β-Gal) enzyme (red line).

1.3.4. Free-amines TNBS test

The grafting rate on the modified β-Gal with maleimide groups was obtained using the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) also known as picrylsulfonic acid. This test consists in the covalent coupling reaction between the picrylsulfonic acid and the primary amine groups that are present on the protein to form a highly chromogenic derivative (Scheme S5).[4]



Amine containing molecule | 2,4,6-trinitrobensenesulfonic acid (TNBS) | Orange colored derivative

Scheme S5. TNBS may be used to detect or quantify amine groups through the production of a chromogenic derivative.

Different concentrations of glycine (standard) were prepared ranging from 10^{-1} mM to 10^{-3} mM in 0.1 M of $NaHCO_3$ at pH 8.5 from glycine 1 mM. 1 mL of the standard solutions was mixed with 25 µL of a 30 mM picrylsulfonic acid solution. In the same way, 300 µL of

the β-Gal (control) and β-Gal-mal solutions at 0.1 mg.mL^{-1} in 0.1 M of $NaHCO_3$ pH 8.5 and 7.5 µL of a picric sulfonic acid solution were added into the Eppendorf tubes. They were incubated for 40 min at room temperature. After that 200 µL of the glycine (standard), β-Gal (control) and β-Gal-mal solutions were transferred into a 96-well plate and the optical absorption was measured at 420 nm with a spectrophotometer (see 2.2). In Fig. S1, the lysine concentration can be calculated with a reference curve obtained for a glycine solution (standard). The grafting ratio was calculated from the relation:

$$GR = \frac{modified\ \ groups}{total\ \ groups} = \frac{total\ \ groups\ \ -\ \ non-modified\ \ groups}{total\ \ groups}$$

where total and non-modified groups represent the UV absorbance of the β-Gal and β-Gal-mal, respectively. A grafting ratio of 70% was obtained after modification of the β-Gal with maleimide groups.

1.4. Chemical modification of enzymes with fluorophores

1.4.1. Chemical modification of β-Gal with fluorescein isothiocyanate (β-Gal^{FITC})

3.1 mg of β-Gal was dissolve in 3 mL of 100 mM $NaHCO_3$ buffer at pH 8.5 followed by the addition of 3.75 µg of fluorescein isothiocyanate (FITC) in methanol solution (100 µL). The reaction mixture was stirred for 3 h at room temperature and dialyzed with a cellulose ester membrane (MWCO 3500) against a 2 L solution of NaCl 0.3 M for 8 h and finally with 2 L of Milli-Q water for another 24 h. Lyophilization of the solution afforded 2.5 mg of β-Gal^{FITC} as a yellow powder.

1.4.2. Chemical modification of β-Gal-mal with FITC (β-Gal-mal^{FITC})

2 mg of β-Gal-mal was dissolved in 3 mL of 100 mM $NaHCO_3$ buffer at pH 8.5 followed by the addition of 3.75 µg of FITC in methanol solution (100 µL). The reaction mixture was stirred for 3 h at room temperature and dialyzed with a cellulose ester membrane (MWCO 3500) against a 2L solution of NaCl 0.3 M for 8 h and finally with 2L of Milli-Q water for another 24h. Lyophilization of the solution afforded 1.7mg of β-Gal-mal^{FITC} as a yellow powder.

2. Experimental methods

2.1. Stretching device

Figure S2 represents the homemade stretching device used for the experiments. It is made on stainless steel and it allows stretching manually the sample in an unaxial direction. The silicone sheet covered with the enzymatic active film is inserted in two jaws. These jaws can be moved continuously one with respect to the other. This stretching device is inserted in a black support made of poly(methylmethacrylate). This support was design to use the minimum amount of enzyme substrate (FDG). The strain, α, is defined by the relation $\alpha = 100 \times \frac{\ell - \ell_0}{\ell_0}$ (in %) where ℓ and ℓ_0 represent respectively the length in the stretched and non-stretched states. All the experiments were performed at room temperature with the PEM-coated silicone side facing down.

Figure S2. Devices used for the stretching of modified silicone sheets. The silicone sheets are placed between the two clamps and stretched manually.

2.2. Spectrofluorometer with microplate reader

A multidetector spectrofluorimeter (Xenius XC, SAFAS, Monaco) equipped with a microplate reader was used to monitor the fluorescein release in the supernatant which mirrors the catalytic activity of enzymes within the PEM films supported on silicone sheets in contact with its substrate, FDG ($\lambda_{ex/em}$: 495 nm / 519 nm). Thiopyridyl group deprotection of PLL-S-TP were also monitored by UV experiments at 343 nm.

2.3. Confocal laser scanning microscope (CLSM)

Confocal laser scanning microscope (CLSM) observations of PEM films were carried out with a Zeiss LSM 510 microscope using a × 40/1.3 oil immersion Objective and with 0.43

μm z-section intervals. FITC fluorescence was detected after excitation at λ = 488 nm with a cut-off dichroic mirror of 488 nm and an emission band-pass filter of 505–530 nm (green emission). An average of three images in the same location was acquired at 256 × 256 pixels. Virtual film section images were taken from the film in the presence of liquid (NaCl 0.15 / TRIS 10 mM), hence allowing the determination of the thickness of the film. All the experiments are performed in the presence of liquid (0.15 M NaCl/10 mM Tris, pH = 7.4) and the multilayer films were never dried.

3. Mechano-responsive film construction

3.1. Polyelectrolyte multilayer film construction

PEM films were built with an automated dipping robot (Riegler & Kirstein GmbH, Berlin, Germany) on silicone sheets (Specialty Manufacturing Inc., Saginaw, MI, USA) of 254 μm thickness or microscope slides. Silicone sheets of 18 × 18 mm^2 were previously cleaned with ethanol and then extensively rinsed with water. The polyelectrolytes used for the construction of the multilayers were dissolved in a 0.15 M NaCl solution prepared with ultrapure water (18.2 MΩ.cm Milli-Q plus system, Millipore) and used at a concentration of 1 $mg.mL^{-1}$. Silicon substrates were first dipped in a PLL-S-TP solution (polycation) for 4 min. Then, two rinsing steps were performed by dipping the sheets two times for 5 min in 0.15 M NaCl solution. The polyanion (HA) was then deposited in the same manner. The buildup process was pursued by the alternated deposition of PLL-S-TP and HA. After deposition of *n* bilayers, the film is denoted $(PLL\text{-}S\text{-}TP/HA)_n$. We used films constituted of 24 PLL-S-TP/HA "bilayers".

3.2. Loading β-Gal enzymes into PLL-S-TP/HA films

3.2.1 Characterization of the loading by confocal microscopy

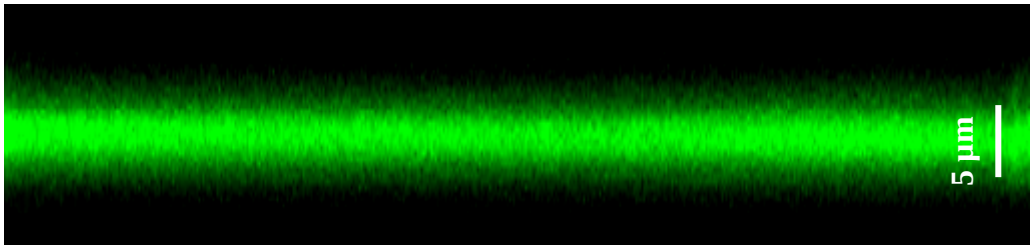


Figure S3. Confocal microscope section (x,z) images of $(PLL\text{-}S\text{-}TP/HA)_{24}$ films deposited on silicone sheets and brought in contact with a $\beta\text{-}Gal^{-FITC}$ solution at 500 µg.mL^{-1}. The image was taken after contact between the film and the $\beta\text{-}Gal^{FITC}$ solution before cross-linking following addition of TCEP. The thickness of the film is around 5 µm.

3.2.2. Determination of the amount of β-Gal-mal enzymes loaded in the cross-linked PLL-S-TP/HA film.

We adapted a method developed by Vodouhê *et al.*[5] to determine the amount of β-Gal-mal enzymes loaded in the cross-linked PLL-S-TP/HA film.

Once PLL-S-TP/HA film was built, the film was cross-linked through 20 mM EDC / 50 mM Sulfo-NHS solution as described above. Then, the film was brought in contact with an ethanolamine 1 M solution prepared in 0.15 M NaCl for 40 min. This treatment desactivates the activated carboxylic groups within the film. Later, a 400 µL of $\beta\text{-Gal-mal}^{FITC}$ solution at 0.5 mg.mL^{-1} was added for 1 hour, after that the enzyme solution was removed and replaced by 500 µL of 1 mM TCEP solution for 30 min. Finally, the PEM film was rinsed with the buffer solution 0.15 M NaCl / 10 mM TRIS at pH 7.4. Some (x,z) images were then taken by confocal microscopy at different locations in the film. From these images, the mean fluorescence was determined.

Then, we removed the film from the confocal microscope and replaced it with a glass slide onto which we deposited 100 µL of $\beta\text{-Gal-mal}^{FITC}$ at 0.5 mg.mL^{-1} solution. Successively, the concentration of the $\beta\text{-Gal-mal}^{FITC}$ was diluted with the addition of NaCl/TRIS solution. For each concentration, the fluorescence was determined from images

taken in the solution with the same experimental parameter setups as for the measurements in the film. This allowed to determine a calibration curve (figure S6) from which we calculated the concentration of β-Gal-mal^{FITC} inside the PEM film. Finally, the concentration of β-Gal-mal^{FITC} in the PLL-S-TP/HA film was about 850 µg.mL, when 400 µL of β-Gal-mal^{FITC} at 500 µg.mL^{-1} were deposited on it for 30 min and an extensive rinsing step was performed.

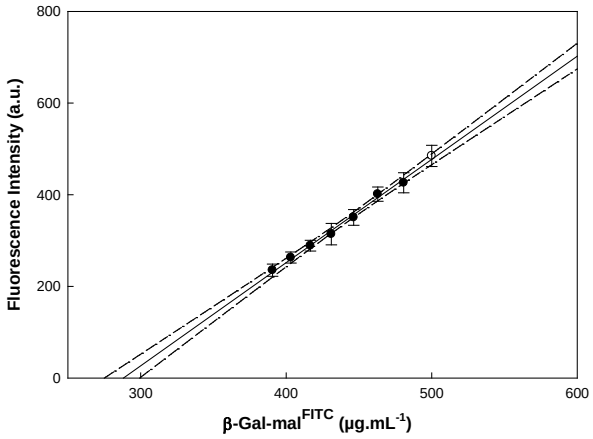


Figure S4. Calibration curve for the determination of the β-Gal-mal concentration in the PLL-S-TP/HA cross-linked film. The equation of the curve is $I = 2.25\ C - 648.92$, where I is the fluorescence intensity in a.u. and C the concentration of β-Gal-mal^{FITC} in µg.mL^{-1}. The solid line represents the linear regression and the dashed lines represent the 95%-confidence interval.

3.3. Deprotection of thiopyridyl groups on PLL-S-TP

The sample was put in the home made stretching device that was previously coated with parafilm to prevent leaking of the aqueous solution after its addition. A 100 µL β-Gal-mal (0.4 mg.mL^{-1}) in NaCl/TRIS solution was added on the sample containing the cross-linked PEM film (PLL-S-TP/HA) supported on PDMS for 1h. Later, the enzyme solution was replaced by 150 µL of TCEP 1 mM solution prepared in NaCl/TRIS. Sampling 150 µL of TCEP solution was done every 5 min and replaced by other 150 µL of TCEP solution. UV measurements at 343 nm wavelength were performed with the spectrofluorimeter equipped with microplates reader in a 96-well plate with 100 µL of the sampling solution for each period of time and total time of 30 min. Full deprotection of thyopiridone groups are completed after 30 min of treatment with TCEP solution (figure S5).

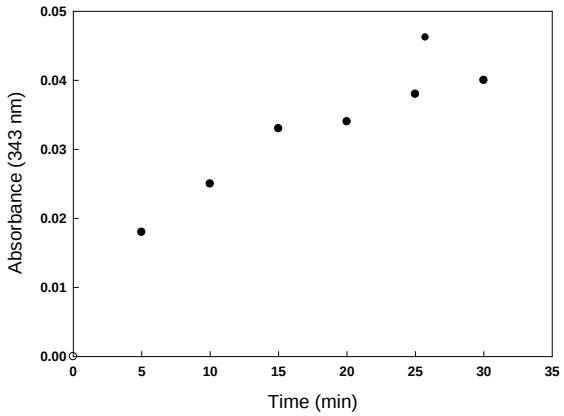


Figure S5. Deprotection of the thiopyridyl groups in contact with the TCEP followed by UV spectroscopy at 343 nm wavelength. Most of the reaction takes place over 30 min.

3.4. Cross-linking of the enzyme β-galactosidase-maleimide within the PEM film

The reticulated PEM film on silicone sheets was placed in a homemade stretching device. The homemade device was held together tightly with clips that were previously coated with parafilm to prevent leaking of the aqueous solution after its addition. 100 µL of β-Gal-mal at 0.4 $mg.mL^{-1}$ prepared in NaCl/TRIS buffer solution was then added for 1h. The modified enzyme was later pippeted and replaced by 200 µL of 1 mM TCEP prepared in 0.15 M NaCl/10 mM TRIS buffer solution for 30 min. Finally, the PEM film was rinsed with the buffer solution 0.15 M NaCl / 10 mM TRIS at pH 7.4.

3.5. Control experiments to verify the non-leaching out of non-covalently linked enzymes in cross-linked PLL-S-TP/HA films during stretching

To confirm the absence of leaching, we performed an experiment with β-Gal-mal enzymes loaded in the PLL-S-TP/HA film but without cross-linking these enzymes to the film (*i.e.* without TCEP, conditions similar to experiments in Fig. 3a). Then, two stretching/unstretching steps have been performed in pure buffer with return to the non-stretched state. Next, FDG, the enzyme substrate, was added to the supernatant. Enzymatic kinetics was monitored and an enzymatic activity was measured (Fig. S6a below). When the film was withdrawn from the cuvette, no more evolution in the fluorescence intensity was observed as depicted in Fig. S6b.

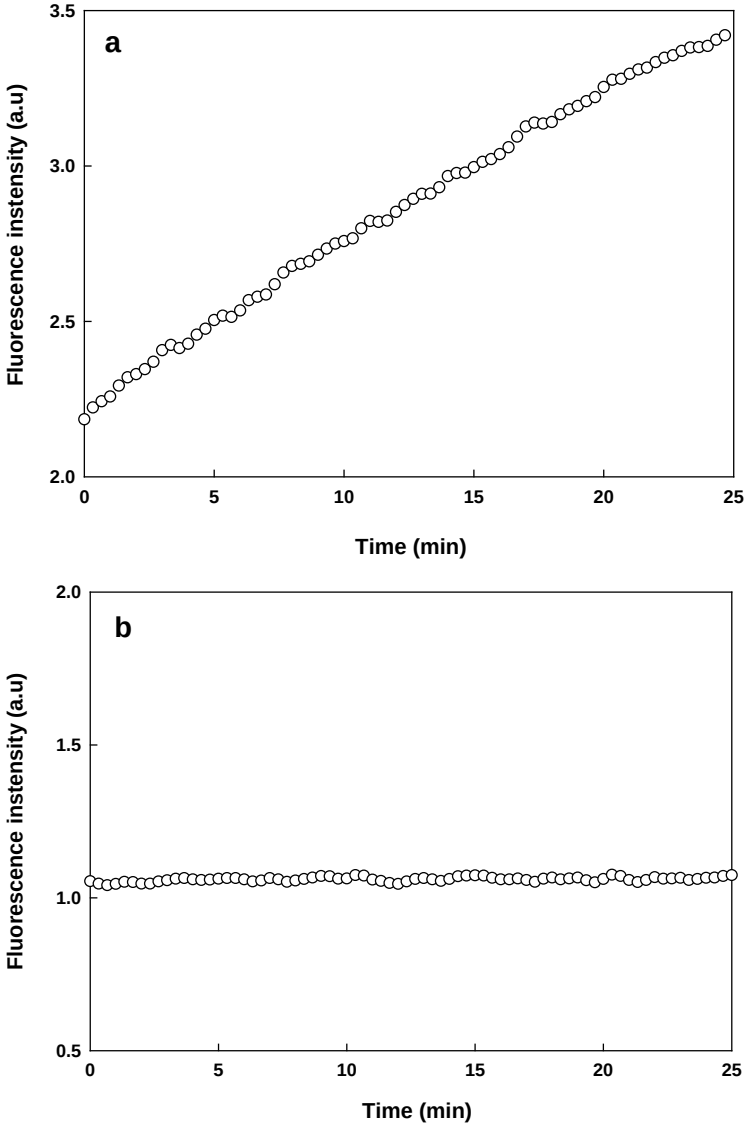


Figure S6. Evolution of the enzymatic activity monitored via production of fluorescence and measured in solution over time. a) Activity of a cross-linked $(PLL\text{-}S\text{-}TP/HA)_{24}$ film loaded with β-Gal-mal. The β-Gal-mal molecules were not linked to the film because the deprotecting agent TCEP was not introduced. The enzymatic activity was measured using FDG as the substrate. b) Following the experiment depicted in a), the supernatant was maintained in place but the enzymatic film was removed from the measurement's cuvette and the enzymatic activity was monitored again.

4. Localization of lysine residues on β-galactosidase

Chemical modification of β-Gal with maleimide groups as described in scheme S3 was performed on part of the lysine residues. A mapping of these lysine residues and in particular those appearing on the external face of the enzyme can be obtained from Protein Databank.

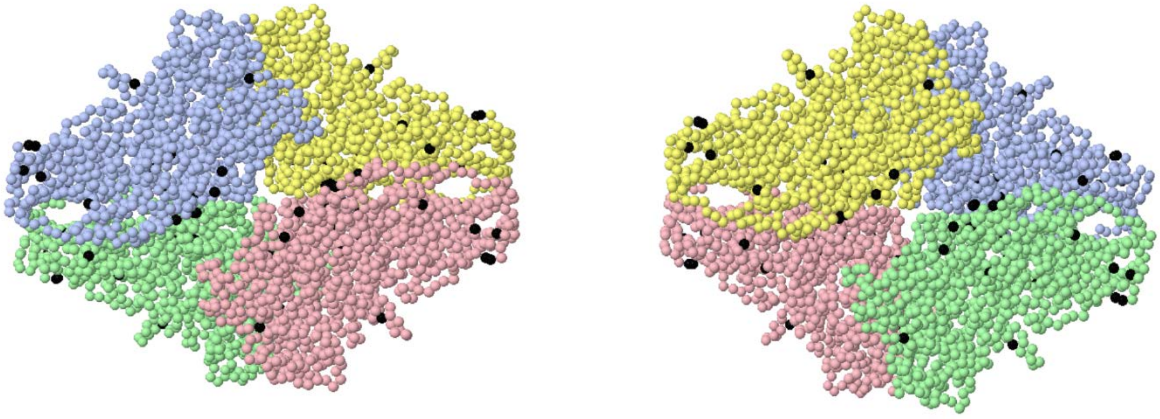


Figure S7. 3D representation of β-Gal residues with a labeling in dark of lysine residues on both faces (left and right representations) of the enzyme (PBD code: 1BGL). From these representations, it appears that a great number of lysine residues are distributed all over the external area, suggesting that the grafting of the enzyme to the film can occur. β-Gal contains 80 lysine residues but only few lysine residues exposed to the outer surface are visible on this figure.

References

(1) Xie, H. Z.; Braha, O.; Gu, L. Q.; Cheley, S.; Bayley, H. *Chem. Biol.* **2005**, *12*, 109-120.

(2) Thibaudeau, K.; Léger, R.; Huang, X. C.; Robitaille, M.; Quraishi, O.; Soucy, C.; Bousquet-Gagnon, N.; van Wyk, P.; Paradis, V.; Castaigne, J. P.; Bridon, D. *Bioconjugate Chem.* **2005**, *16*, 1000-1008.

(3) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76-85.

(4) Habeeb, A. *Anal. Biochem.* **1966**, *14*, 328-36.

(5) Vodouhê, C.; Le Guen, E.; Méndez Garza, J.; Francius, G.; Déjugnat, C.; Ogier, J.; Schaaf, P.; Voegel, J.-C.; Lavalle, P. *Biomaterials* **2006**, *27*, 4149-4156.