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COMMUNICATION

A New Biomimetic Route to Engineer Enzymatically Active Mechano-Responsive Materials

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Using modified β -galactosidase covalently linked into cross-linked polyelectrolyte multilayers (PEM), catalytically active materials have been designed. Their enzymatic activity can be modulated, partially in a reversible way, simply by stretching. This strategy, based on enzyme conformational changes, constitutes a new tool for the development of biocatalytic mechano-responsive materials.

Chemo-mechano-responsive materials emerge as a new class of materials that respond chemically to a mechanical stress by triggering a chemical reaction.¹ For example, new types of polymeric materials were designed that include mechanophores which change color under mechanical stress, systems that release protons under compression² or trigger catalytic reaction under the action of a force.³ Whereas in these materials, the chemical reaction results from stress-sensitive chemical groups incorporated in polymer chains which undergo a chemical transformation under high tension, nature uses rather less energy demanding processes based on protein conformational changes. For example, stretching domain-proteins such as fibronectin exposes cryptic sites that allow cell adhesion or trigger osteogenic differentiation.⁴ Enzymes constitute a class of proteins that act through a precise topology of the residues involved in their active site. Moreover, structural dynamics of enzymes play a major role in the enzymatic activity.⁵ Thus, enzyme activity should also be modulated by applying on them a mechanical stress. First indications about the potential validity of this idea were brought in 1974 by Berezin *et al.* who deposited enzymes directly on fibers and who observed variations of their catalytic activity by stretching the fibers.^{6,7} Recently, the influence of a mechanical force on enzyme conformation and thus enzyme activity was clearly demonstrated by the groups of Gaub and Blank who monitored the activity of a single enzyme submitted to a periodic stretch.⁸ This was also confirmed by Tseng *et al.* who used DNA molecular springs that are coupled on two positions of an enzyme to create a protein-DNA chimera, the DNA applying a mechanical stress on the enzymes.⁹ In two other very recent works, it has demonstrated that the conformation of Green Fluorescent Proteins (GFP) covalently

grafted into or onto a material can be changed simply by compression¹⁰ or stretching¹¹ respectively, as measured through a change of the fluorescence intensity.

Based on this idea, we develop here a new strategy to achieve enzymatically active mechano-responsive material whose activity can be modulated simply by stretching. It relies on the covalent coupling of enzymes into a cross-linked polymeric network. When stretch, the network is anticipated to apply constraints on the enzymes leading to enzyme-conformational changes and thus alteration of the enzymatic activity. In the field of mechano-catalytic materials design, it must be noted that this original approach is entirely different than a first one that we introduced in 2009, where the catalytic activity of a material was based on the control by uniaxial stretching of the accessibility of freely diffusing enzymes in a liquid-like film capped by a barrier.¹²

Polyelectrolyte multilayer (PEM) films offer a unique opportunity to achieve this goal. Based on the alternate deposition of polyanions and polycations onto a solid substrate, PEM can be used whatever the chemical nature and shape of the substrate and for these reasons widespread applications are foreseen.^{13,14,15} In particular, it allows conferring biologic and enzymatic activity to a film by incorporation of proteins or enzymes.¹⁶ Several requirements have to be fulfilled to create mechano-responsive enzymatically active materials: *i*) they should be elastic and stretchable,^{12,17} *ii*) the enzymes should remain active in the film and *iii*) the enzymatic substrate should be able to diffuse in the film in order to react with the enzymes.

We used poly(L-lysine)/hyaluronic acid (PLL/HA) exponentially growing PEM films deposited on silicone substrates because these films reach micrometer thicknesses after less than 15 deposition steps and allow for the immobilization of large amount of enzymes, as required for our purpose. β -galactosidase (β -Gal), a hydrolase enzyme that catalyzes the breakdown of lactose, has been chosen due to its tetramer structure comprised of four active sites and four polypeptide chains held together through non-covalent interactions (Fig. 1a).^{18,19} Each active site of the enzyme includes residues belonging to two different subunits. Because these subunits are non-covalently linked, the catalytic center is anticipated to be sensitive to

external forces applying onto the enzyme. Fluorescein di(β -D-galactopyranoside) (FDG) is used as a substrate of β -Gal. This fluorogenic substrate is transformed into fluorescein and β -D-galactopyranoside under the action of β -Gal (see §1, Scheme S4 in SI). The reaction can thus be followed by monitoring the fluorescence of the solution in contact with the film. The enzyme had to be covalently linked to the multilayer network to respond to a mechanical stress. As a consequence, we used PLL chains chemically modified by thiopyridyl (S-TP), named PLL-S-TP (grafting ratio of 27%) and β -Gal enzyme modified by maleimide groups (mal, 70% of grafting ratio), named β -Gal-mal. The synthesis of these compound is given in §1 of the SI.

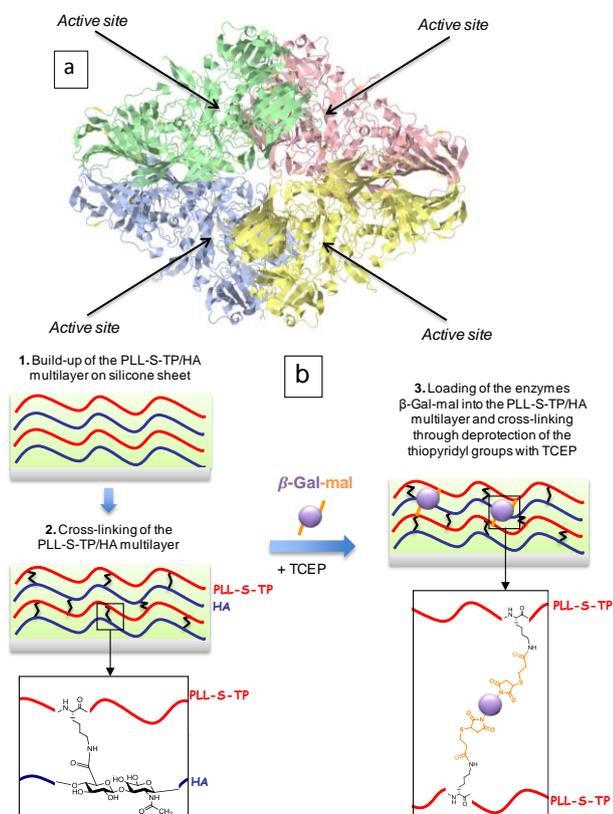


Figure 1. a) View of the β -Gal tetramers by coloring each subunit.^{18,19} Black arrows show the localization of the four active sites of the enzyme. Image taken from PDB (code: 1BGL). b) Description of the different stages necessary to cross-link β -Gal in the stretchable multilayer.

In a first set of experiments multilayers were grown on silicone substrates by alternating the deposition of PLL-S-TP and HA. After the deposition of 24 bilayers a thickness of 5 μm was reached (§3 in SI). These films were then cross-linked through carbodiimide chemistry by bringing them in contact with an ethyl(dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC-NHS) solution.²⁰ They were then brought in contact with a 500 $\mu\text{g}/\text{mL}$ β -Gal-mal solution without deprotection of PLL-S-TP, avoiding the covalent coupling of the enzymes to the multilayer. We first verified that the enzyme has diffused into the reticulated film. Using β -Gal-mal^{FITC}, confocal microscopy images show that the whole cross-linked PLL-S-TP/HA film section is labeled in green (Figure S5 in SI). When this film was brought in contact with the substrate, *i.e.* a FDG solution, fluorescence in solution increased linearly with time. This indicated

that despite the cross-linking of polyelectrolyte chains, the enzyme embedded into the film remained enzymatically active. When this film was stretched at 50% and then at 100%, no significant change in the enzymatic activity was observed (Fig. 2a). This result suggests that enzymatic activity of the film is not affected by stretching if enzymes are uncoupled to the polyelectrolyte chains. We also checked that the absence of variation of fluorescence intensity with stretching cannot be attributed to a leaching of enzymes out of the film during the stretching cycles (see §3.5 in SI).

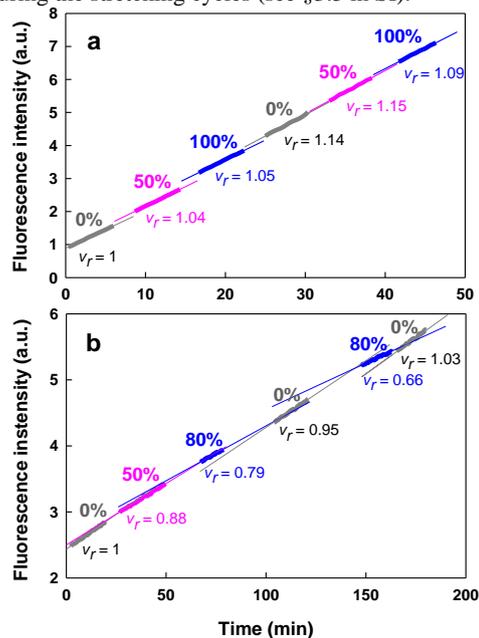


Figure 2. Evolution of the enzymatic activity monitored via production of fluorescence and measured in solution over time during stretching (50%, 80% or 100%) / unstretching (0%) cycles. The fluorescence intensities corresponding to a given strain have been fitted with a linear equation. The coefficients v_r are the ratios of the slopes at each strain to the slope in the initial state (*i.e.* initial curve without stretching, first gray curve). Thin lines are linear regressions and bold lines are raw data. β -Gal-mal, were loaded in the reticulated films; a) no covalent coupling of the enzymes to the film, b) enzymes covalently fixed to the multilayer.

For the second set of experiments, the cross-linked PLL-S-TP/HA films were brought in contact with β -Gal-mal enzymes in the presence of TCEP ((Tris(2-carboxyethyl) phosphine hydrochloride) to deprotect the thiopyridyl moieties (S-TP) of PLL which then react with maleimide groups of β -Gal. β -Gal-mal become thus covalently linked to the film. The deprotection reaction was monitored by measuring the supernatant absorbance at 343 nm (Figure S5 in SI). The increase in absorbance corresponds to the release of the thiopyridone molecules in solution. The reaction takes place during approximately 30 minutes. Beyond this time, no more coupling reaction occurs. The buildup process is represented in figure 1b.

By using a calibration curve, the enzyme concentration in the film was estimated to be of the order of 850 $\mu\text{g}/\text{mL}$ (see details in §3 in SI and Figure S4). These cross-linked PLL/HA films containing covalently attached β -Gal-mal enzymes were then stretched in a stepwise manner up to 80-100%. Figure 2b shows a typical evolution of the fluorescence intensity monitored when a FDG solution is put in contact with the film during the stretching. First in the non-stretched state, the films are enzymatically active indicating that the covalent immobilization of the enzymes within the cross-linked polyelectrolyte network does not affect their activity. Under stretching, the fluorescence production rate monitored in the solution decreased and this diminution was amplified when the strain was

increased. This evolution of the enzymatic activity was different from that observed with non-cross-linked enzymes where the fluorescence production remained almost constant or even increased slightly under stretching. This experiment was repeated several times and results are summarized in Figure 3a and 3b where the fluorescence production rates, averaged over different experiments, are plotted as a function of the strain. The stretching of immobilized enzymes in the PEM film affected its enzymatic activity by about 30% compared to the non-stretched state. It is expected that the decrease of the enzymatic activity is due to a stretching-induced change of the enzyme conformation. This hypothesis is in agreement with our previous reported observations on the stretching of covalently coupling GFP molecules onto an elastomeric substrate: one changes by up to 40% the fluorescence intensity by stretching the substrate by 100%,¹¹ an effect directly related to changes of the conformation of GFP. A 30-40% catalytic decrease by stretching is also in accordance with reductions found by the early experiments of Berezin *et al.*^{6,7}

Next, we investigated the reversibility of the enzymatic activity change. The films were stretched at 80 or 100% and then brought back to the non-stretched state (Fig. 3b).

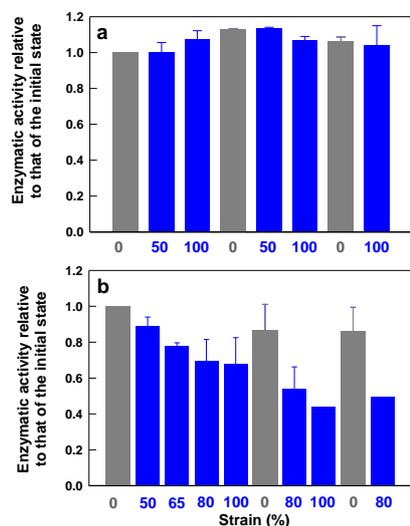


Figure 3. Evolution of the mean enzymatic activity monitored *via* production of fluorescence for different strains. a) Enzymes not covalently linked to the film and b) enzymes covalently linked to the film. The activities have been normalized to the rates measured in the initial, non-stretched state. The rate values correspond to the mean value of 2 experiments in a) and up to 7 experiments in b) and error bars correspond to standard deviations. The Kruskal-Wallis test reveals non-significant influence of the strain in a) ($p = 0.166$) whereas significant influence is suggested in b) ($p = 0.021$) if one refers to risk level of 0.05.

By release of the stretch, an increase of the enzymatic activity is obtained which is about $87 \pm 15\%$ of the initial production rate. When repeated a second time with the same films, the stretching/unstretching process induced again a decrease/increase of the enzymatic activity of the similar amplitude. The stretch-induced change of the enzymatic activity is thus fairly reversible. The fact that during the second stretch the amplitude of the activity decrease is slightly larger than during the first one might be due to some film restructuring and eventually also some additional cross-linking between the enzymes and the film during the first stretch. These results highlight the enzymatic mechano-responsive properties of our designed films.

This original approach constitutes a very general strategy to construct enzymatically mechano-responsive systems. Unlike many

others chemo-mechano-responsive systems reported so far, it is based on very low energy demanding processes, namely conformational changes, instead of covalent bond breaking. This is one of the routes chosen by nature to induce mechano-transduction processes. The next step in this field is now to extend and generalize this approach by using artificial enzymatic systems.

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Electronic Supplementary Information (ESI) available: [Chemical modifications of polymers and enzymes, all experimental methods, build up, characterization and catalytic activity tests of mechano-responsive films]. See DOI: 10.1039/c000000x/

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