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Layer-by-Layer Enzymatic Platform for Stretched-Induced Reactive Release

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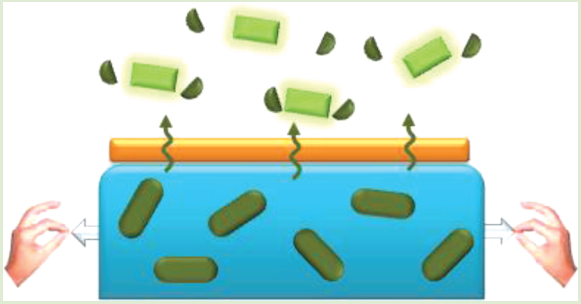
Supporting Information

ABSTRACT: An original "all-in-one" platform combining polymers, enzymes, and enzymatic substrates in a unique film is designed. A polymeric barrier stratum prevents any contact between enzymes adsorbed on top of the film and substrates loaded in an underlying reservoir. Upon stretching of the film, a continuous diffusion of substrates through the barrier is triggered, followed by a catalytic reaction. This leads to the formation of products that are released from the film. This new platform acts as a stretch-induced reactive release system and emerges as an innovative concept in mechano-responsive materials.



The design of responsive materials has emerged as an extremely hot topic. In particular mechanosensitive materials are now thoroughly investigated.[1] Nanosciences and nanotechnologies recently allowed the development of devices and tools to detect a single molecular event that originates from a mechanical stimulus. For example, mechanochromic materials, that are materials whose color changes when they undergoes stresses, have been designed and allow the direct visualization of a mechanochemical reaction.[2−7] Another recent application depicts self-reporting hybrid materials where proteins are used as reporter molecules that sense deformation, strain, or mechanical damage in materials.[8] Such mechanical sensitive architectures mimic the natural mechanism involved in mechanotransduction, where cells convert a mechanical stimulus into a cascade of chemical reactions.[9]

Mimicking nature and designing surfaces that would induce chemical reactions or trigger cellular responses upon stretching the surface is not only of fundamental interest but could also present numerous potentialities from a technological point of view. Such surfaces could be used, for example, to build sensors that become active or are regenerated under stretching, to control reactions in microfluidic devices upon local stretching of the substrate, or to induce specific biological reactions on cells seeded on such devices. A first step, based on polyelectrolyte multilayer films,[10] was recently undertaken by our group to design a "mechanotransductive" surface.[11] For this purpose, a film combining two strata of polyelectrolyte multilayers[12] was built: a first thick and hydrated stratum acting as a reservoir for enzymes was capped by a second thin and dense stratum which plays the role of a barrier. In the unstretched state, enzymes are masked, and the catalytic reaction is "off". Once a critical stretching degree is applied on the system, biocatalysis turns "on" at the film/solution interface due to the unmasking of enzymes. However, this system suffers from one drawback: a feedback inhibition process takes place during the catalysis, and thus only a very short burst of enzymatic activity could be monitored instead of a continuous enzymatic rate. Moreover, it was not possible to control the release of products through mechanical stimuli with this system.

The effect of mechanical stimuli on the release of compounds from polymeric matrices is a subject of major interest which is the focus of numerous studies. Lee et al. described the control of growth factor release from alginate hydrogels with cyclic compression loadings.[13,14] Due to these mechanical stresses, neovascularization in tissue surrounding stimulated hydrogels could be enhanced. More recently, it has been demonstrated that the release of macromolecules from layer-by-layer capsules is strongly improved by compression.[15] However to our knowledge, no mechanoresponsive device releasing products through a chemical process activated by stretching has yet been reported. Herein we address the design of an original "all-in-one" platform combining polymers, enzymes, and enzymatic

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substrates in a unique film. At rest, the catalysis does not occur due to a polymeric barrier that prevents any contact between enzymes and substrates. Upon stretching, embedded substrates diffuse through the barrier. Once reaching the anchored enzymes, a reaction takes place, and the products stemming from the catalytic reaction are released in solution. This approach is strongly innovative in comparison to our previous study devoted to the design of mechanotransductive surfaces:[11] in the previous system, the film was designed as an impermeable catalytic surface converting substrates from an outer solution into products. In the present study, some substrates are embedded in the film, and upon stretch the system converts them in products via enzymatic catalysis and releases them in solution. This new platform provides significant benefits: (i) all of the constituents, that are, substrates and enzymes, are simultaneously present in the film; (ii) mechanical stretches allow to control the release of molecules; (iii) mechanical stimuli trigger a "time-controllable" catalytic reaction with continuous release; (iv) no more feedback inhibition occurs in the catalytic reaction because there is no accumulation of substrates in the vicinity of enzymes. These two last points were not possible with our previous system. We expect the *stretch-induced reactive release* approach to be of interest because of the practical advantages it may provide. A macroscopic stretch is a simple stimulus from a technical point of view, but we demonstrate that it allows to control nanosystems in a precise manner. Moreover, the triggering of chemical reactions inducing release of products compared to a simple release of molecules also brings advantage to avoid storage of these products under their native form within the device but in a precursor form which can protect them from degradation, for example.

In the present study, we used thick and hydrated exponentially growing polyelectrolyte multilayers, namely, poly(L-lysine)/hyaluronic acid (PLL/HA), to embed fluorescein diphosphate (FDP). The FDP molecule is a fluorogenic substrate of alkaline phophatase enzyme, that is, it becomes strongly fluorescent once the enzyme has catalyzed the dephosphorylation reaction of FDP into fluorescein (F). FDP molecules deposited on top of a thick PLL/HA film diffuse inside and are concentrated within it. This film plays the role of reservoir for FDP as displayed by confocal microscopy images (Supporting Information, Figure S1). Following FDP deposition, a capping layer constituted by $(PDADMA/PSS)_{10}$ (10 bilayers of PDADMA: poly(diallyldimethylammonium)/PSS: poly(sodium 4-styrenesulfonate)) strata was added on the top. The thickness was estimated to be around 90 nm from quartz crystal microbalance measurements (QCM; Supporting Information, Figure S2). It is well-known that PDADMA/PSS layers act as a barrier and prevent diffusion of polyelectrolytes[16] or proteins[11] through it. In this study, PDADMA/PSS layers isolate FDP from the alkaline phosphatase enzymes (ALP). To validate the efficiency of this barrier, rhodamine-labeled ALP (ALP^{Rho}, red emission) was adsorbed as a last layer on top of the PDADMA/PSS layers that cap the PLL/HA film containing FDP. Despite the negative charges of the film induced by PSS chains adsorbed as final layer, ALP^{Rho}, also negatively charged, adsorbs significantly on the film according to confocal microscopy image (Supporting Information, Figure S1b′) and quartz crystal microbalance measurements (Supporting Information, Figure S2a). If ALP^{Rho} is deposited on a similar film but with a PDADMA terminating layer instead of PSS, the whole film section displays red fluorescence (Supporting Information, Figure S3). In such a case, the adsorbed amount of ALP is five times larger on PDADMA than on PSS, as checked with QCM (Supporting Information, Figure S2b). However, the PDADMA/PSS capping layer becomes no more efficient as a barrier toward enzymes, and the catalytic reaction occurs directly in the film even in the nonstretched state. This underlines the sensitivity of the barrier to the nature of the last deposited polyelectrolyte.

The deposition of ALP on top of the PSS-terminated film shows no difference in the background level of green fluorescence intensities and demonstrates that no biocatalysis occurs (Supporting Information, Figures S1a and S1b). Moreover, no evolution with time of the green fluorescence within the film and in the supernatant was monitored. This clearly indicates that the enzymatic substrate FDP and the enzyme ALP are both present in the film but in two distinct areas and that this "all-in-one" film $[(PLL/HA)_{30}/PLL/FDP/(PDADMA/PSS)_{10}/ALP^{Rho}]$ depicts no catalytic activity at rest. Finally, the film $(PLL/HA)_{30}/PLL/FDP/(PDADMA/PSS)_{10}/ALP^{Rho}$ constitutes our "enzymatic platform" (Figure 1, step a) that will be selected all along the present study.

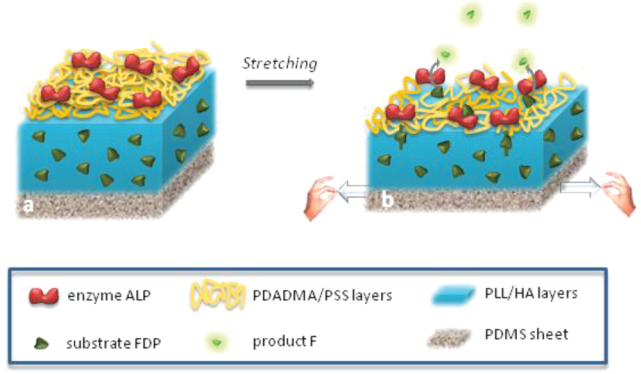

Figure 1. Schematic illustration of the mechanism involved in the enzymatic platform during stretching: (a) before the stretching step and (b) during the stretching step.

To check if a stress applied on the film can trigger the catalytic reaction and release the products of the enzymatic reaction, the evolution with time of the fluorescence intensity in the supernatant was monitored with a confocal microscope for stretching degrees α ranging between 0 and 100% (Figure 2). For α values ranging between 0 and 40%, a slight increase of the fluorescence intensity was monitored, that is, about 0.10 ± 0.05 au s^{1}. This value probably corresponds to a background level as attested by the slope of the same order obtained with a similar film but without enzymes on the top (0.13 au s^{-1}). This rate seems also independent of the stretching degree (Supporting Information, Figure S4).

For α = 60%, the slope strongly increases, reaching about 0.80 ± 0.20 au s^{-1}. This corresponds to a critical stretching degree (α = 60%) at which the production of fluorescein molecules is triggered. For higher stretching degrees, the slope corresponding to the increase of fluorescein solution does not change any more. No significant further increase of the fluorescein production rate takes place beyond.

Inserts in Figure 2 show confocal microscope section images before and after stretching the platform. An increase of fluorescence occurs both in the supernatant and in the film. This indicates that fluorescein molecules produced by the

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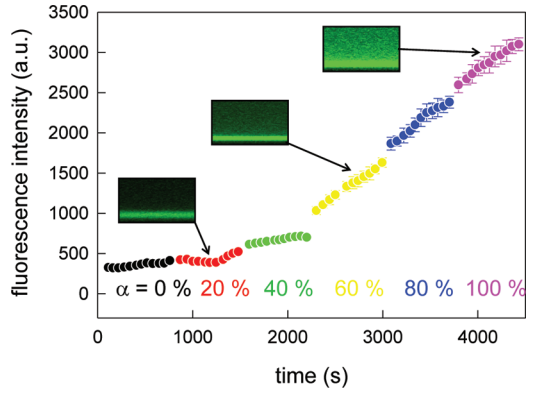

Figure 2. Evolution with time of mean fluorescence intensity of buffer solution in contact with the enzymatic platform. Several stretching degrees α were applied on the film: from α = 0% to α = 100%. The typical stretching time from one stretching degree to the next was of the order of 15 s. The film was then kept in a fixed stretched state (α = 20%, 40%, 60%, 80%, or 100%) for more than 1500 s. The time steps between two consecutive fluorescence measurements was 60 s. The inserts correspond to confocal microscope section images x,z (130 × 76 μm^2) acquired at t = 1200 s, t = 2800 s, and t = 4300 s. The error bars correspond to standard deviations calculated from image analyses.

enzymes on top of the film diffuse within the underlying PLL/HA reservoir and are also continuously released into the supernatant. It should be noted that we never observed delamination or surface defects in the stretched films.

At this stage, the enzymatic reaction rate is constant with time over at least 30 min; thus no feedback inhibition of the enzyme takes place, in contrary to the previous designed systems.[11] In the present study, the products of the reaction and more particularly phosphate ions are probably released in the supernatant and are not confined close to the enzymes.

To test the reversibility of the system, the enzymatic platform was stretched at α = 100% and brought back to α = 0% (Supporting Information, Figure S5). No change in the rate of fluorescein production was observed; the same slope was maintained once the system was brought back to rest. Thus, the "opening" of the barrier which occurs at stretch is not reversible. This permeability could originate from pores appearing in the barrier layer. However, no pores were visualized on top of the film in the stretched state as demonstrated by AFM images (Supporting Information, Figure S6). We characterized the electric surface potential before and after the stretching step, and no strong variations were monitored (−2.44 ± 0.13 mV and −4.18 ± 0.59 mV, respectively). Moreover, no desorption of enzymes was monitored during the whole experiments, the red fluorescence corresponding to ALP^{Rho} adsorbed on top of the film remains unchanged with time, whatever the stretching degree α. Finally, during the stretching step, the barrier probably becomes less dense, permitting the FDP molecules to pass through it. When the film is brought back at rest, the barrier on top of the reservoir should be less structured compared to its initial state, and efficiency toward diffusion of small molecules is lost.

A thicker barrier constituted with 20 PDADMA/PSS bilayers instead of 10 bilayers was also tested. This barrier remained tight when the system was stretched up to 140%, indicating that no enzymatic reaction occurs (Supporting Information, Figure S7). FDP molecules were in this case not able to cross the barrier, whatever the mechanical state of the film. For thinner barriers (2 or 6 PDADMA/PSS bilayers) the catalysis already occurs when the film is at rest (data not shown). This demonstrates that the barrier is too thin and not tight enough. Finally, the enzymatic platform constituted by 10 (PDAMDA/PSS) bilayers corresponds to an optimal design for our purpose, to trigger a reactive release through a mechanical stimulus.

To elucidate the mechanism involved in the product release, the supernatants above stretched films with and without ALP adsorbed onto the barrier were analyzed by fluorometry. The fluorescence intensity of the supernatant of the enzymatic platform $[(PLL/HA)_{30}/PLL/FDP/(PDADMA/PSS)_{10}/ALP$ film] was measured 40 min after stretching the film at 100% (Figure 3, blue bar on the foreground). A control experiment

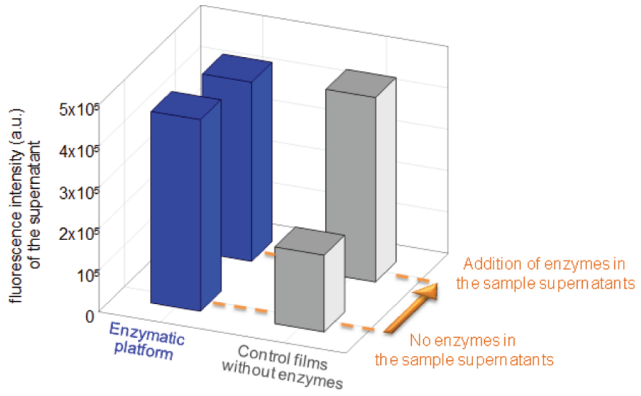

Figure 3. Fluorescence intensities of supernatants which were in contact with the enzymatic platform (blue) and a control film without enzyme. The supernatants were sampled after 40 min in the stretched state (α = 100%), and fluorescence were measured with a fluorometer. Bars on the foreground correspond to a direct measurement of fluorescence and bars on the background to a measurement of the fluorescence after an addition of a large excess of ALP in the sample solution. The standard deviations for these measurements are below 5 × 10^4 a.u.

with similar conditions but for a film without enzyme on top was also performed (Figure 3, gray bar on the foreground). As expected, the fluorescence of the supernatant for the control system is much lower compared to that of the enzymatic platform where the enzymes catalyze the production of a large amount of fluorescein molecules which are afterward released in the supernatant. The nonzero fluorescence monitored for the control system is attributed to the few F molecules initially present in the commercial product and which are probably slowly released from the film upon stretching. In a further experiment, we added a large excess of ALP solution to both of these sampled supernatants (without any film in contact) and performed a second series of measurements (Figure 3, bars on the background). For our enzymatic platform, same fluorescence intensities were measured with or without addition of enzyme in the supernatant showing that no FDP but only the F molecules were released after stretching. This strongly suggests that all FDP molecules diffusing out of the film are dephosphorylated by the enzymes on top of the film and are released as F molecules (Figure 2, step b). Thus, the rate of diffusion of FDP molecules out of the film is lower than the enzymatic reaction rate. For the control system, the fluorescence intensity of the supernatant strongly increases after enzyme addition, and this intensity became identical to the enzymatic platform. This clearly demonstrates that, when the film was stretched in the absence of adsorbed enzyme on the

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top, FDP was released from the film to the supernatant. This film being not coated with an enzyme layer, no dephosphorylation of FDP took place. We hypothesize that the barrier should undergo some reorganization under stretching which makes the barrier permeable toward FDP.

A new type of experiment was then designed to clarify the underlying mechanism: (i) stretching the enzymatic platform at α = 100% for 15 min, (ii) rinsing it for 5 min, and (iii) monitoring again fluorescence evolution of a fresh buffer solution brought in contact with the platform maintained in the stretched state (Figure 4a). After rinsing step iii, the film being

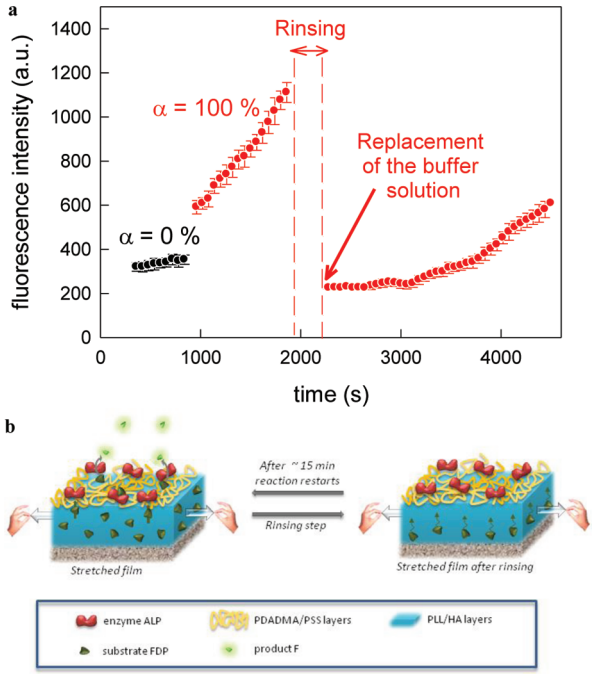

Figure 4. (a) Evolution with time of mean fluorescence intensity of buffer solution in contact with the enzymatic platform. First, the enzymatic platform is at rest (α = 0%, black discs), and then a stretching is applied above the critical stretching degree (α = 100%, red discs). After ca. 15 min, a rinsing step of 5 min is performed, and supernatant is replaced by a fresh buffer solution. (b) Schematic illustration of the mechanism involved in the enzymatic platform during stretching and after a rinsing step. The error bars correspond to standard deviations calculated from image analyses.

maintained at α = 100%, the fluorescence intensity recovered a constant background level during ca. 15 min. After this period, the release of fluorescein molecules started again progressively, and the fluorescence intensity rose up again with a high rate. The rinsing step at α = 100% has probably induced a fast release of the FDP molecules located in the upper part of the reservoir (under the barrier) (Figure 4b). Thus, the remaining FDP molecules located more deeply in the film probably diffuse in the upper part and then are dephosphorylated by enzymes before released as fluorescein molecules in the supernatant. This mechanism is certainly fully diffusion-controlled.

Finally, Figure 1 overviews the concept of *stretch-induced reactive release*. The designed and so-called "all in one enzymatic platform" allows to release catalyzed products from the film to the solution through a mechanical stimulus. Both enzymes and substrates are initially embedded in the polymeric architecture with no reaction occurring, and a mechanical stress applied to the system is necessary to initiate the catalysis. This reaction takes place without any feedback inhibition process and leads to a release of the catalyzed products according to a linear profile with time. The mechanism behind this behavior corresponds to the diffusion of the substrate through the stretched film followed by its conversion into products by the adsorbed enzymes on top of the film. The products then diffuse in the solution.

Finally, the designed platform represents a new concept in mechanotransductive materials which could find numerous applications in many fields and in particular in prodrug delivery. These prodrugs could be stored in a reservoir and converted through enzymatic reaction into drugs while crossing the enzymatic barrier. Activation of this reactive release could be obtained by a local mechanical stimulus.

■ ASSOCIATED CONTENT

Supporting Information

Details of materials, experimental procedure, methods of characterization (confocal laser scanning microscope, quartz crystal microbalance, fluorometry, atomic force microscope, zeta potential measurements), confocal microscope, QCM and zeta potential control experiments, and AFM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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