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Regular Article

# Layer-by-layer assembly of brushes of vertically-standing enzymatic nanotubes

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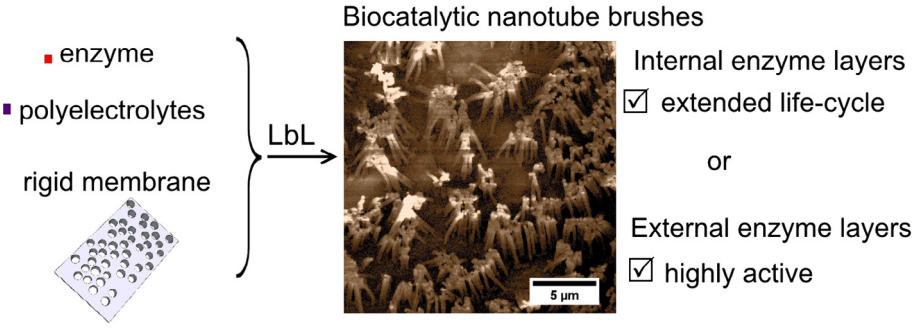
## GRAPHICAL ABSTRACT

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## ABSTRACT

Brushes of vertically-standing enzyme-containing nanotubes are prepared onto planar surfaces by a combination of hard-templating and layer-by-layer assembly. The nanotubes have a core-shell morphology made of two compartments, one for mechanical rigidity, the other containing $\beta$-lactamase for bioactivity. We demonstrate inclusion of the enzymatic component either in the core or in the shell part of the nanotubes. Kinetic studies reveal that both types of systems are bioactive but that the activity is significantly better preserved over long time periods when $\beta$-lactamase is incorporated in the core of the nanotubes.


## 1. Introduction

Nanofinger-shaped protrusions or microvilli can be found at the surface of a large variety of cell types. They offer a larger surface area for the plasma membrane and contribute to diverse biological functions (*i.e.*, absorption, secretion, mechano-transduction and adhesion). Such microvilli are made of a collection of crosslinked actin filaments, packed in a specific way to dictate the binding of other proteins [1]. Motivated by the range of applications of microvilli structures, we show here the potential of layer-by-layer (LbL) assembly to fabricate arrays of bioactive nanotubes standing vertically on a surface. These nanotubes, which have a structure broadly reminiscent of microvilli, contain an enzymatic component and exhibit significant bioactivity against $\beta$-lactam antibiotics.

LbL assembly is one of the most powerful tools to control film properties at nanoscale level, and it has also been used to prepare a variety of biocatalytic thin films, including enzymatic nanotubular structures [2–6]. LbL coatings can be deposited on many different templates; when using sacrificial membranes of submicron

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pore size as templates, polyelectrolyte nanotubes are obtained. Early in the 2000s, the first LbL-assembled nanotubes were made of poly(allylamine hydrochloride) and poly(acrylic acid) [7]. Later on, the same polyelectrolyte pair was used to prepare the first LbL nanotube arrays exhibiting pH-responsive properties [8]. Based on the same principle but varying the polyelectrolyte pairs, the group of Komatsu started the study of protein nanotube arrays containing human serum albumin and poly-L-arginine for biotin capture [9,10]. They also prepared nanotubes loaded with α-D-glucosidase and demonstrated that their rate of reaction was significantly lower compared to the free enzyme in solution (1/38), despite using a polypeptide as co-polyelectrolyte to preserve the bioactivity of the enzyme. Our group recently prepared glucose oxidase-containing nanotubes exhibiting enzymatic activity [5], and used them for the fabrication of enzymatic nanopapers [6].

Herein, we report on the synthesis of brushes of vertically-standing nanotubes containing β-lactamase, and on the bioactivity of the resulting surfaces. The nanotubes are prepared by LbL assembly within polycarbonate membranes, using two different pairs of polyelectrolytes to get a two-compartment core-shell structure, with one compartment providing mechanical rigidity and the second one bioactivity. Then, taking as a starting point the work of Rubner et al. [8], we anchor the LbL nanotubes on a planar surface to produce a microvilli-like surface capable to hydrolyze β-lactams, integrating enzyme-containing layers either inside or outside the nanotubes, leading to a series of efficient biocatalytic surfaces of large relative area.

## 2. Experimental section

### 2.1. Materials

Chitosan chloride (chit, degree of deacetylation >90%, $M_w \sim 270 \times 10^3$ g/mol, Novamatrix) and poly(allylamine hydrochloride) (PAH, $M_w \sim 15 \times 10^3$ or $58 \times 10^3$ g/mol, Sigma-Aldrich) were used as polycations. β-lactamase (TEM-1 from *Enterobacter cloacae*, Sigma-Aldrich), poly(acrylic acid, sodium salt) (PAA, $M_w \sim 15 \times 10^3$ or $100 \times 10^3$ g/mol, Sigma-Aldrich), and carboxymethylpullulan (CMP, $M_w \sim 220 \times 10^3$ g/mol, degree of sulfonation∼ 97%) were used as polyanions. All polyelectrolytes were dissolved in buffer solution at a concentration of 1 mg/mL, using as a buffer either MES (100 mM, pH 6.5) or sodium acetate (0.1 N, pH 4.6). Crosslinkers, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), were purchased from ThermoScientific and used in aqueous solution at a concentration of 6.0 and 3.5 mM, respectively.

Track-etched polycarbonate membranes PCm1 (200 nm pore diameter, 21 μm thickness, $2 \times 10^8$ cm$^{-2}$ pore density), or PCm2 (300 nm pore diameter and 5 μm thickness, $2 \times 10^8$ cm$^{-2}$ pore density) were kindly provided by it4ip (Belgium) and used as received. Single-side polished silicon wafers (Si, ⟨100⟩ orientation, ACM) were cleaned in piranha solution (2:1 mixture of concentrated sulfuric acid (VWR, Belgium) and 30% hydrogen peroxide solution (VWR, Belgium)) prior to LbL deposition. *Caution! Piranha solution reacts violently with many organic materials and should be used with extreme care.*

### 2.2. Fabrication of free core-shell LbL nanotubes

The nanotubes were prepared by LbL, assembling two different pairs of polyelectrolytes in the pores of a PC membrane. The polyelectrolyte couple that played the role of the shell was adsorbed in a first compartment [shell (+)/shell (−)]$_m$; *e.g.* PAH (15 kg/mol) and PAA (15 kg/mol). Then, the bioactive core made of (chit/β-lactamase)$_n$ layers was deposited in a second compartment. After the release of the nanotubes, the external surface of the nanotubes is thus the first polycation used to build the shell.

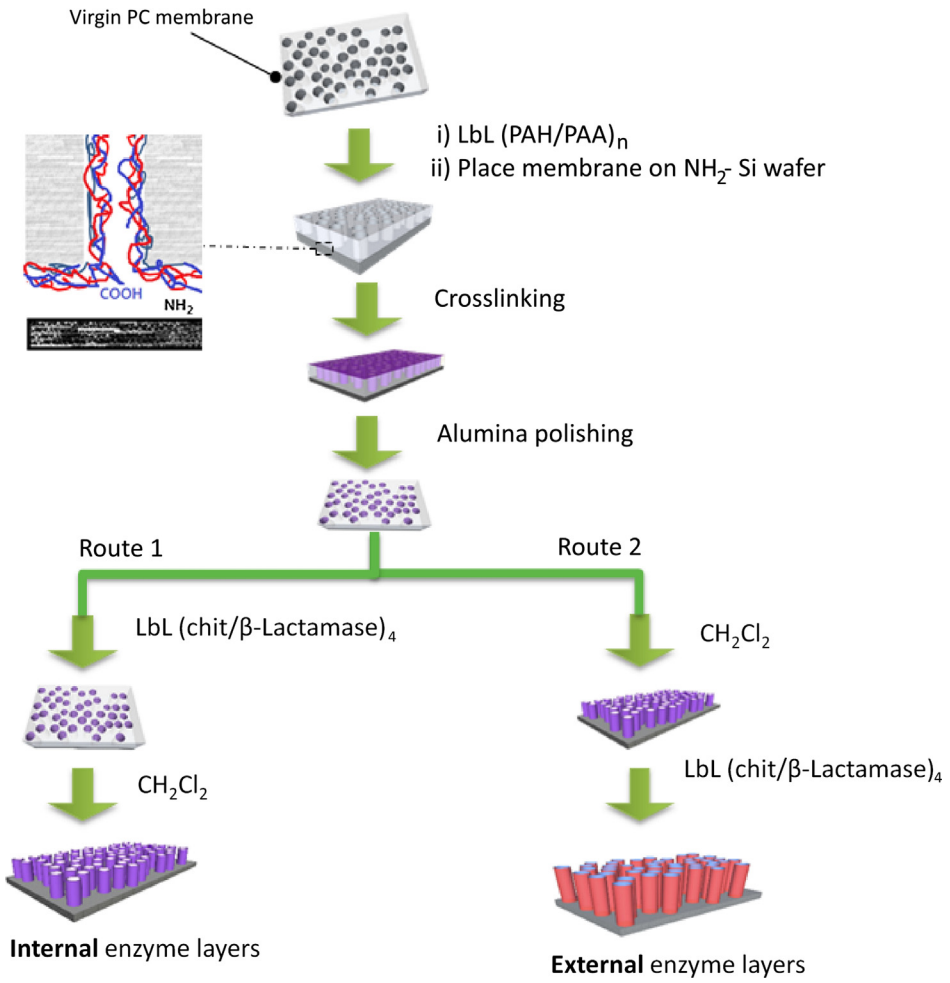
The polyelectrolyte layers were deposited by dipping a PCm1 templating membrane in a polycation solution for 30 min, rinsing it twice in MES buffer solution (100 mM, pH 6.5) and dipping the same template in a polyanion solution for 30 min, followed by two rinsing steps of 2 min in fresh buffer. The process was repeated *n* times to produce (polycation/polyanion)$_n$ films. Since the polyelectrolytes are also adsorbed outside the pores, a decrusting step was performed to remove these layers on both sides of the membrane, after the deposition of the last polyanion layer. Three different decrusting methods were tested: (i) rubbing with a cotton swab with an alkaline solution of high ionic strength (3M NaCl, pH 12), (ii) polishing with a plate covered by alumina powder (1 μm size), or (iii) oxygen plasma etching. The performance of each method varied with the nature of the polyelectrolyte pair that had to be removed, as discussed below. Then the shell compartment was crosslinked by dipping the PCm1 membrane in an EDC/NHS solution for 30 min at room temperature, and rinsed twice for 5 min in fresh buffer. Thus, we proceed to adsorb the core layers, starting by chitosan and then β-lactamase solutions in MES buffer (100 mM, pH 6.5). Decrusting step was also performed after the buildup of the core layers. Finally, the LbL-modified PCm1 was kept in MES buffer solution (100 mM, pH 6.5) at 4 °C. A spare sample of LbL-modified PCm1 was dried for gas-flow porometry measurements, and another spare sample was dried and dissolved in dichloromethane to obtain free nanotubes.

### 2.3. Fabrication of surface-anchored arrays of nanotubes

Brushes of nanotubes were prepared following the protocol reported by Chia et al. [8] with some modifications, as schematically presented in Scheme 1. LbL assembly of (PAH/PAA)$_n$ (using $M_w \sim 58$ and 100 kg/mol, respectively) was performed in acetate buffer in PCm2 membranes. The adsorption process started with a polycation layer (PAH) deposition and ended with a polyanion layer (PAA) adsorption. No intermediate decrusting was done until this point, as the layers that cover one of the external membrane surfaces will further be used as anchoring layer. A droplet of EDC/NHS crosslinking solution was placed on top of an aminosilanized silicon wafer ($1 \times 1$ cm$^2$) and a piece of membrane with (PAH/PAA)$_n$-filled pores was laid over it. The stack was then heated at 60 °C for 30–60 min to allow the reaction between the amine groups on the silicon surface and the carboxylic acid groups of the last LbL layer (PAA). The upper side of the membrane was then decrusted by 1 min polishing with alumina microparticles (1 μm) and further rinsed with deionized water in an ultrasonic bath (3 times, 1 min each rinsing step) before drying. Then the process follows either route 1, for nanotube brushes with β-lactamase layers inside the nanotubes or route 2, for β-lactamase layers adsorbed on the external surface of the brushes. Route 1: the supported membrane was alternately dipped in chitosan and β-lactamase solutions in MES buffer to assemble 4 bilayers. The top side of the membrane was decrusted and then, the supported membrane was immersed five times in fresh dichloromethane to dissolve the polycarbonate (10, 10, 2, 2 and 1 min) and reveal the nanostructure array. Route 2: the membrane was first dissolved, and the resulting nanotube array was then alternately dipped in chitosan and β-lactamase solutions in MES buffer to assemble 4 bilayers on top.

### 2.4. Characterization

The pore diameter of the PC membranes was determined by gas-flow porometry measurements on air-dried samples, before




**Scheme 1.** Preparation of nanotube brushes by LbL and crosslinking the template on a wafer. Depending on the order of template dissolution and (chit/$\beta$-lactamase) LbL we can adsorb the enzyme inside the nanotubes (route 1) or onto the external surface (route 2).

and after LbL assembly, using the method reported by Roy et al. [11]. Briefly, a piece of membrane was held perpendicularly to a nitrogen flux with a known pressure ranging between $10^4 - 10^5$ Pa, then the flux downstream from the sample was measured with a flowmeter (Agilent). At least ten flow measurements were averaged and the pore size was calculated based on Knudsen diffusion and Hagen-Poiseuille flow. The thickness of LbL layers adsorbed within the pores of the membranes was then calculated from the difference between starting and final pore radii (before and after LbL).

Freed LbL nanotubes were collected on a copper grid and imaged with a LEO 922 TEM microscope at 200 kV. Brushes and films anchored on Si wafers were air-dried and imaged with a field-effect gun digital scanning electron microscope FE-SEM (either DSM 982 Gemini from LEO operated at 1 kV, or JEOL 7600F at 5 kV).

The activity of enzymatic $\beta$-lactamase-containing nanotubes (embedded in PC membranes, or in arrays of surface-anchored nanotubes) was followed by measuring the hydrolysis of nitrocefin by UV/Vis spectrophotometry (Agilent Cary 50) using a previously-reported method [12]. A nitrocefin solution (2 mL, 50 µm in 100 mM MES buffer at pH 6.5) was added over a 1 $cm^2$ sample of either a pore-filled PC membrane, or of a nanotube brush anchored on a Si wafer. The samples were stirred at 150 rpm and aliquots were taken at different time intervals. The absorbance of the hydrolyzed nitrocefin was detected at 485 nm in standard cuvettes of 1 cm path length, and converted to molar concentration using the Lambert-Beer law and an extinction coefficient $\epsilon_{485} = 16,700\ M^{-1}\ cm^{-1}$. A single value of activity represents the average of triplicates, and all the samples were prepared and analyzed in parallel to decrease variability.

## 3. Results and discussion

### 3.1. Fabrication of rigid core-shell LbL nanotubes

Polyelectrolyte nanotubes made of proteins can be extremely soft and flexible, which is an issue for the fabrication of brushes of vertically-standing tubes. Therefore, we first investigated whether a core-shell architecture could solve the issue, with a core compartment containing the enzyme, and a shell compartment providing sturdiness and protecting the enzyme from external aggressions. Two different pairs of polyelectrolytes were tested for the rigidifying shell (Fig. 1: TOP scheme): a synthetic (PAH/PAA) pair and a polysaccharide (chit/CMP) pair. For this part of the work, a membrane of large thickness (*ca.* 21 µm, PCm1) was used to obtain long tubes of which the rigidity is easier to assess.

Three bilayers of polyelectrolytes (either PAH/PAA or chit/CMP) were thus adsorbed within the pores of PCm1 templates to fabricate a shell, which was crosslinked before starting the adsorption of three core bilayers of chitosan and $\beta$-lactamase, as described

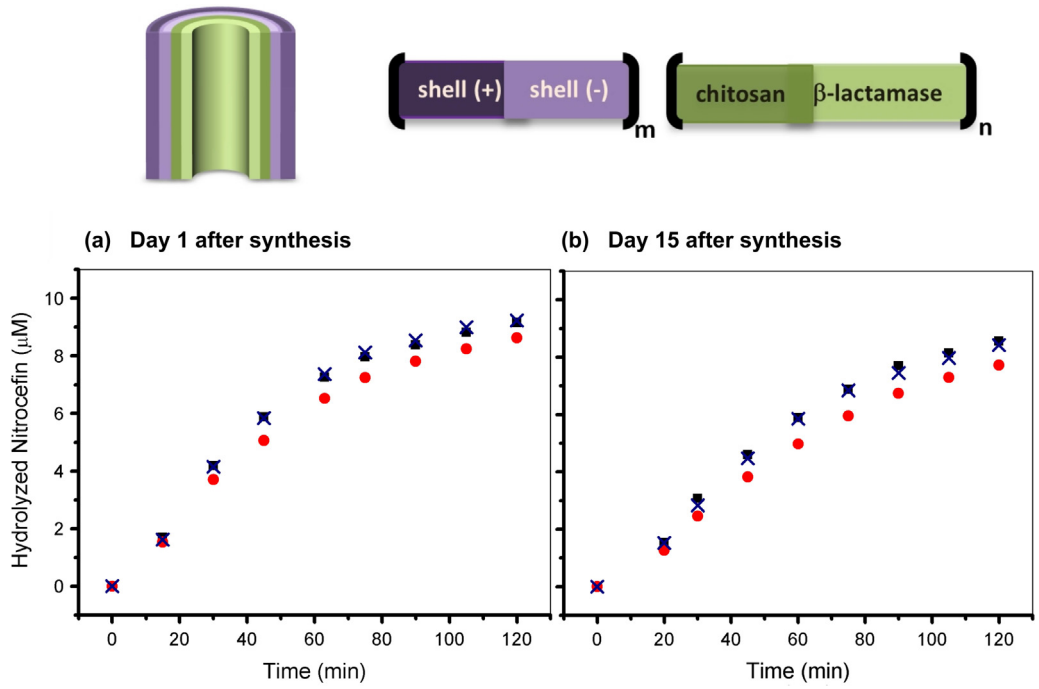



**Fig. 1.** TOP: Schematic representation of core-shell nanotubes. The polyelectrolytes of the shell compartment are either PAH and PAA, or chit and CMP. BOTTOM: Activity assay in filled pores: $(PAH/PAA)_3/(chit/\beta\text{-lactamase})_3$: ■, $(chit/CMP)_3/(chit/\beta\text{-lactamase})_3$: ●, $(chit/\beta\text{-lactamase})_3$: ×.

in the Experimental Section. At the end of the assembly, the top and bottom layers deposited on the membrane were removed by rubbing it with a cotton swab impregnated with an alkaline solution (3M NaCl, pH 12). The core-shell LbL assemblies were also compared to single-compartment tubes, namely $(chit/\beta\text{-lactamase})_3$. Table 1 summarizes the wall thicknesses of the nanotubes at different stages of their construction, as evaluated by gas-flow porometry. For nanotubes with a (PAH/PAA)-based shell, the $(chit/\beta\text{-lactamase})_3$ core compartment is of a thickness comparable to the one obtained when directly adsorbing these polyelectrolytes in the pores of the pristine PC membrane. However, a thinner $(chit/\beta\text{-lactamase})_3$ shell compartment is obtained when built over a $(chit/CMP)_3$ shell. This is due to a combination of adverse factors, such as the lower linear charge density of the polysaccharides compared to the synthetic polyelectrolytes and the much higher degree of swelling of their LbL assemblies resulting from the hydrophilic nature of their backbones [13,14].

Activity assays (nitrocefin hydrolysis) were conducted while the tubes were still contained in the membrane. Fig. 1 shows that the activity of the three systems is very close, although the shell made of chitosan and CMP is slightly less active (*ca.* 10% of loss). This observation is valid on the day of samples preparation, as well as two weeks later, demonstrating that the tubes properly protect the enzyme against slow denaturation. Considering the much smaller thickness of the enzyme-containing compartment in nanotubes having a $(chit/CMP)_3$ shell (Table 1), it might seem surprising that the enzymatic activity remains high for these tubes. This actually results from the penetration of the enzyme in the chit/CMP layer during tube fabrication, similarly to what was demonstrated by us before on flat multilayers [15]; this leads to the blurring of the core-shell architecture, and the impossibility to evaluate the enzyme content by simple means.

To determine if the core performs its rigidifying function, the nanotubes with and without shell layers were imaged by TEM after membrane dissolution in dichloromethane (Fig. 2). Although the nanotubes with a $(PAH/PAA)_3$ shell appear flattened under vacuum, they only bend moderately. In contrast, the tubes made only of $(chit/\beta\text{-lactamase})_3$ have smaller radii of bending, similarly to the nanotubes with a supplementary $(chit/CMP)_3$ shell. This clearly indicates that a compartment made of highly-charged synthetic polyelectrolytes is best for our purposes.

**Table 1**
Thickness of core-shell adsorbed layers in the PCm1 pores, calculated from gas flow porometry measurements.

| Sample | Shell (nm)* | Core + Shell (nm)* |
|---|---|---|
| $(PAH/PAA)_3/(chit/\beta\text{-lactamase})_3$ | 33 ± 3 | 59 ± 2 |
| $(chit/CMP)_3/(chit/\beta\text{-lactamase})_3$ | 21 ± 3 | 34 ± 2 |
| $(chit/\beta\text{-lactamase})_3$ | – | 23 ± 3 |

* Error corresponds to the range of wall thickness calculated for two independent experiments.

### 3.2. Fabrication of brushes of vertically-standing bioactive nanotubes

As shown in Scheme 1, brushes of vertically-standing nanotubes are made by anchoring the tubes on a flat surface before dissolving the membrane. Our previous results led us to select PAH/PAA as the structurally-rigidifying component of the nanotubes in brushes; in addition, to reduce bending further, we switched to thinner membranes of 5 μm thickness (PCm2) and 300 nm pore diameter. The number of (PAH/PAA) bilayers was set to 12, leading to tubes of 300 nm external diameter and *ca.* 180 nm internal diameter if no decrusting is performed during the deposition.

One possible strategy to fix the tubes onto a solid support is the use of a superglue [16]. However, it has been shown that the glue can be easily adsorbed by capillarity into the pores, which could be detrimental to enzymatic activity. Therefore, we used a method based on chemical crosslinking, similar to the one proposed by Rubner et al. [8]. For this purpose, $(PAH/PAA)_{12}$ was assembled into the pores of the membrane and on its top and bottom surfaces. The membrane was then placed over a silicon wafer pre-silanized with an amino-silane, and the system was chemically crosslinked,

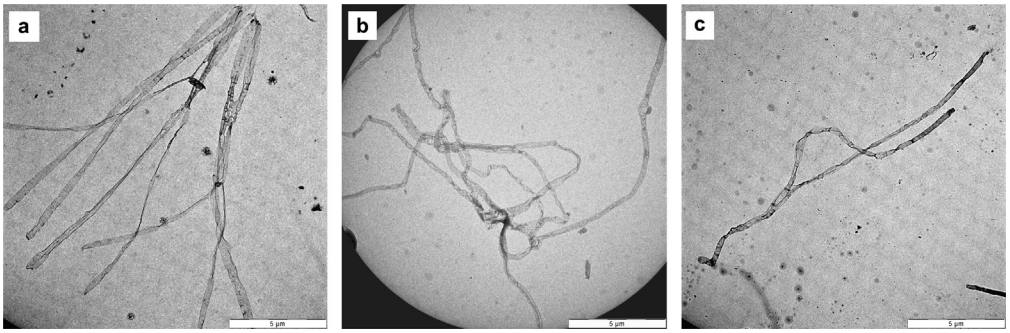



**Fig. 2.** TEM images of freed nanotubes with and without shell layers: (a) $(PAH/PAA)_3/(chit/\beta\text{-lactamase})_3$, (b) $(chit/CMP)_3/(chit/\beta\text{-lactamase})_3$, (c) $(chit/\beta\text{-lactamase})_3$. The scale bar is 5 μm.

resulting in the simultaneous anchoring of the membrane on the wafer through reaction of the carboxylic acids of the LbL film present at the bottom surface of the membrane with the amine groups of the Si wafer. The crosslinked LbL film atop the polycarbonate template was then removed before dissolving the PC template in dichloromethane and observing the samples by SEM. This removal is an important step to obtain nice nanotube brushes of a large surface-area. Initially, we tried to decrust the pores by oxygen plasma as described by Rubner et al. [8]; however, the plasma produced highly heterogeneous surfaces (Fig. 3a), having islands of uncovered nanotubes and regions still covered by the top polyelectrolyte layer. Increasing the time of plasma did not solve the issue but strongly damaged the areas that were already uncovered at the beginning of the process. We also tried decrusting with a basic solution as used for the fabrication of free tubes (Fig. 3b), but found that this process softened the nanotubes, leading to tangled nanostructures.

Finally, we used a mechanical decrusting method, polishing the membrane with 1 μm alumina particles (too large to penetrate the 300 nm pores), leading to much better results even though some defects were sporadically found due to the random nature of the polishing process (Fig. 3c). The $(PAH/PAA)_{12}$ nanotubes have a length in the expected range (5 μm) and stand straight on the surface, even though they tend to sag against each other in small clusters in the dry environment of the SEM.

To prepare bioactive nanotube brushes while minimizing damage to the enzyme, the $(chit/\beta\text{-lactamase})$ layers were deposited after crosslinking and decrusting of the top side of the membrane. Two different systems were made: nanotube brushes with enzyme inside the tubes, or arrays of nanotubes with external enzyme layers (Scheme 1). In both cases, four bilayers of chitosan and β-lactamase were deposited, since this number of bilayers was shown by our group before to provide high enzymatic activities when deposited in pores of 180 nm diameter [12]. To incorporate the enzyme within the nanotubes, the membrane template was kept after crosslinking the $(PAH/PAA)_{12}$ compartment, and the whole assembly was dipped alternatively to build the $(chitosan/\beta\text{-lactamase})_4$ core compartment in the tubes; the template was only then dissolved in fresh dichloromethane (route 1 in Scheme 1). Conversely, to fabricate nanotube arrays with an

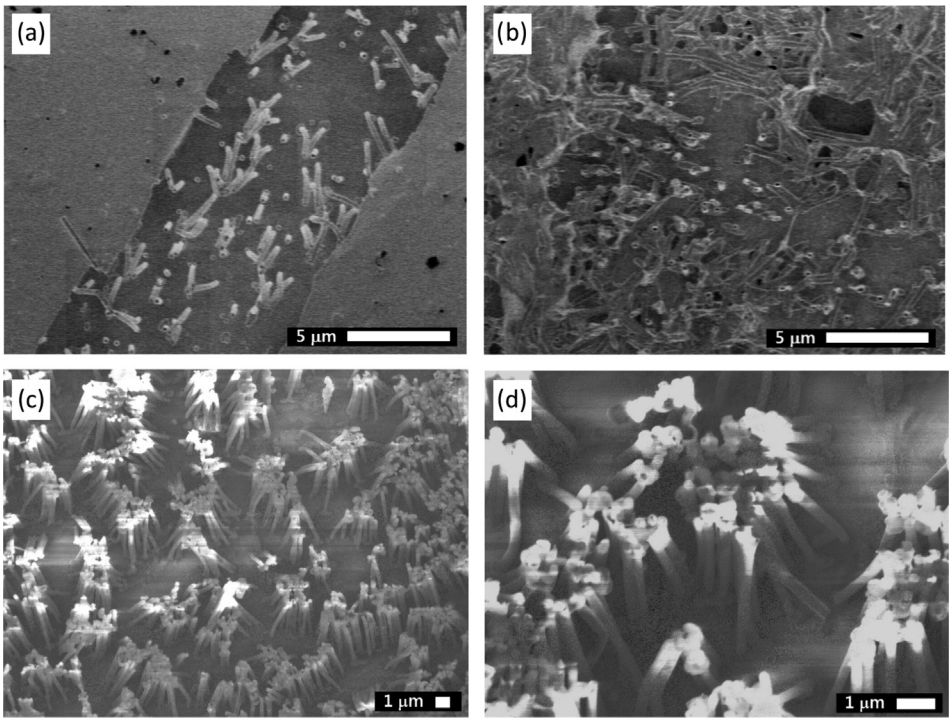



**Fig. 3.** SEM pictures of brushes of $(PAH/PAA)_{12}$ nanotubes, decrusted by: (a) oxygen plasma treatment, (b) basic solution, (c) polishing with alumina powder.

external enzymatic compartment (route 2 in Scheme 1), the templating membrane was dissolved prior to the LbL deposition of four (chit/β-lactamase) bilayers (this has to be performed very carefully to avoid damaging the nanostructures).

The activities of the two different β-lactamase-containing nanotube brushes were compared by following the hydrolysis of a β-lactam containing dye, nitrocefin; as reference, we used a $(chit/\beta\text{-lactamase})_4$ flat LbL adsorbed onto a clean Si wafer. Fig. 4 shows that the faster rate of nitrocefin hydrolysis is obtained when β-lactamase is present in an external compartment deposited over the $(PAH/PAA)_{12}$ tube array. The nanotubes coated with the enzymatic compartment are *ca.* twice faster than the flat reference surface. This is lower than expected based on the increase of surface area: indeed, a sample of 1 $cm^2$ presenting $2 \times 10^8$ vertically-aligned nanotubes of 5 μm length would provide an increase of the surface area by a factor of *ca.* 10. The less-than-expected increase of activity is probably due to the clustering of the nanotubes as shown in Fig. 3, and to defects arising from the decrusting treatment. Contrarily, when the enzyme is in a core compartment within the tubes, the enzymatic activity is much lower than the one of the flat $(chit/\beta\text{-lactamase})_4$ film. This results from the limited inward diffusion of nitrocefin and outward diffusion of hydrolyzed products within/from the nanotubes, as well as to the contact of the enzyme with dichloromethane during membrane dissolution (Supporting Information).

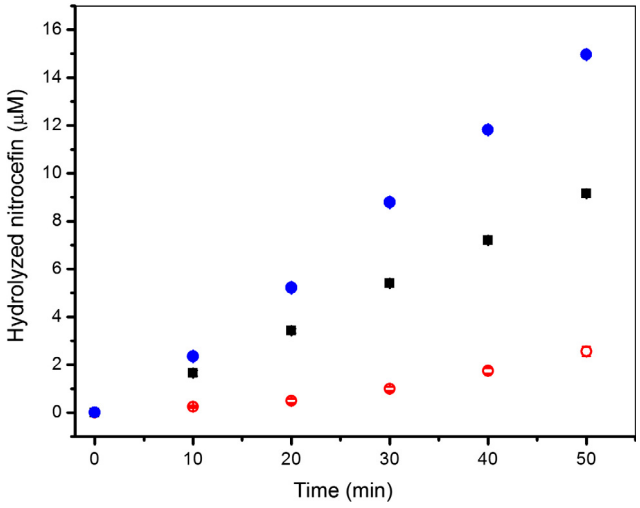



**Fig. 4.** Nitrocefin hydrolysis by the brushes of $(PAH/PAA)_{12}$ nanotubes with a $(chit/\beta\text{-lactamase})_4$ core compartment within the tubes (○) or a $(chit/\beta\text{-lactamase})_4$ shell compartment on the tubes (●), compared to a $(chit/\beta\text{-lactamase})_4$ LbL flat film on a Si wafer(■).

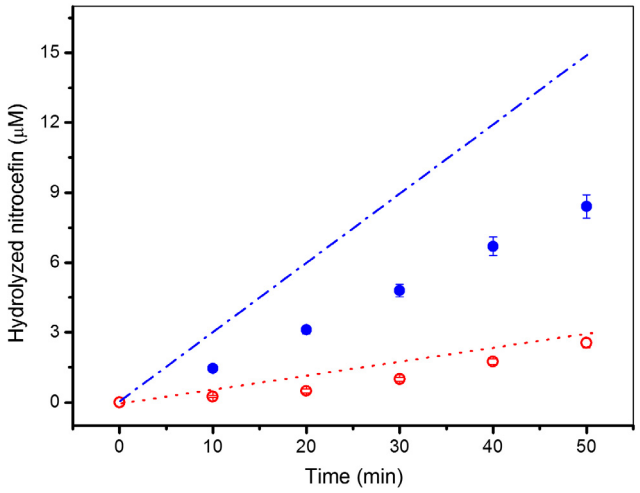



**Fig. 5.** Nitrocefin hydrolysis by the brushes of $(PAH/PAA)_{12}$ nanotubes with $(chit/\beta\text{-lactamase})_4$ layers within the tubes (○) or over the tubes (●), one month after their preparation. The dashed and dotted lines show the original activity, measured one day after the brushes were prepared.

Most important, we found that the brushes stored over a month at 4 °C in MES buffer (100 mM, pH 6.5) suffered activity loss to a different extent. If the enzyme was located in the core of the brushes, we detected a negligible loss of bioactivity; whereas a *ca.* 30% loss of enzyme activity was observed in same conditions for the nanotube array with exposed enzyme layers (Fig. 5). Thus, despite the lower activities of the brushes of nanotubes with biocatalytic core, they are of interest due to a better retention of enzyme activity after storage. This finding is in agreement with previous reports pointing out that LbL confinement can improve enzyme properties, enhancing stability under operation and in stock [17–19].

## 4. Conclusions

Bioactive brushes of nanotubes were prepared onto planar surfaces by a combination of hard-templating and layer-by-layer assembly. A core-shell strategy was developed to obtain enzymatic nanotubes able to stand vertically. Synthetic polyelectrolytes nanotubes are first grown and crosslinked onto a surface; alumina polishing is then used to remove the overlayer resting on the top side of the membrane with minimum physical damage. The crosslinked synthetic polyelectrolytes nanotubes are rigid enough to allow the further deposition of -lactamase-containing layers, (i) within the nanotubes to favor activity retention for long periods of time at the expense of global activity, or (ii) over the polyelectrolytes nanotubes to promote faster short-time activity.

Whereas previous work already showed the possibility to fabricate arrays of nanotubes [8–10], our results developed further these results towards the fabrication of bioactive structures of high-surface area. Thereby, we made one more step towards microvilli-like surfaces, and showed how the sequence of deposition also allows us to tune enzymatic activity and lifetime. In this respect, it is important to point out the stabilizing effect of polyelectrolyte multilayers, which are capable to preserve enzymes from denaturation for extended periods of time, especially when shielded into the inner part of nanotubes.

Although not performed here, our methodology could be easily extended towards more complex systems encompassing a cascade of enzymatic reactions, systems combining enzymes and cofactors, or systems having more LbL compartments for increased functionality. We are now especially investigating how these structures can be provided with a responsive component, so that enzymatic activity can be controlled when applying an external stimulus, thereby bringing our arrays of nanotubes even closer to natural systems.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcis.2017.12.063.

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