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Layer-by-Layer Assembled Enzyme Nanotubes and Mats for Biocatalysis

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**I dedicate this thesis to my beloved family
for their endless support, encouragement and love.**

谨以此博士文献给我挚爱的家人，
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Abstract

Inspired by the biochemical reactions that often occur in confined nanocompartments, *e.g.*, organelles, various artificial biomimetic nanostructures have been synthesized for studying confinement effects in natural biochemical processes. Among these systems, protein-based nanotubes are attracting an increasing interest due to their many potential applications. In this context, the thesis focuses on the construction of tubular enzyme-rich nanocylinders by templated layer-by-layer assembly in nanoporous membranes. After removal of the sacrificial templates, free tubular nanotubes incorporating enzymes are obtained; these can be considered as one step towards artificial biocatalytic organelles.

We first investigate how to synthesize stable enzyme nanotubes of high activity. When prepared in optimized assembly conditions, robust enzyme LbL-nanotubes are obtained with improved resistance towards the detemplating process. Afterwards, we devise a novel collection strategy, namely adjuvant-assisted filtration, able to extract LbL-nanotubes from the template membrane and to redisperse them with minimal aggregation in an aqueous medium. The tubular structure and biological activity of the collected enzyme nanotubes are retained in aqueous dispersion, demonstrating the robust capability of our methodology to recover template-synthesized nanotubes for biological applications.

Finally, the fabrication of a 'paper' mat made of LbL-nanotubes is demonstrated. The structure of the resulting mats is investigated by SEM and AFM, showing the possibility of construction of tailored nanofibrous scaffolds comprising nanotubes incorporating functional components. This methodology thus provides a new and general way to create smart nanofibrillar scaffolds, which offers exciting opportunities for applications in, *e.g.*, tissue engineering, biosensing, and bio-purification.

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List of abbreviations and acronyms

AAO	Anodic aluminum oxide
AFM	Atomic force microscopy
AFs	Aligned fibers
ALG	Alginate
ATR	Attenuated total reflection
bPEI	Branched polyethyleneimine
CA	Cellulose acetate
CAT	Catalase
CHI	Chitosan
CLSM	Confocal laser scanning microscopy
CNTs	Carbon nanotubes
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CVD	Chemical vapor deposition
DD	Degree of deacetylation
DIC	Differential interference contrast
DMF	Dimethyl formamide
DPI	Perylene diimide
ECM	Extracellular matrix
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
FNPs	Fluorescent nanoparticle
FTIR	Fourier transform infrared spectroscopy
GA	Glucoamylase
GOX	Glucose oxidase
HA	Hyaluronic acid
HB	Hemoglobin
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulfonic acid
HRP	Horseradish peroxidase
HSA	Human serum albumin
IRRSA	Infrared reflection-absorption spectroscopy
ITO	Indium tin oxide

LbL	Layer-by-Layer
IPEI	Linear polyethyleneimine
MA	Myristic acid
MES	4-morpholineethanesulfonic acid
MS	Mesoporous silica
PAA	Poly(acrylic acid)
PAH	Poly(allylamine hydrochloride)
PAN	Polyacrylonitrile
PB	Prussian Blue
PBSE	1-pyrenebutanoic acid succinimidyl ester
PC	Polycarbonate
PDADMA	Poly(diallyldimethylammonium chloride)
PDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
PEG	Poly(ethylene glycol)
PET	Poly(ethylene terephthalate)
PI	Isoelectric point
PLA	Poly-L-arginine
PLG	Poly-L-glutamic acid sodium salt
POD	Peroxidase
PS	Polystyrene
PSS	Poly(sodium styrene sulfonate)
QCM	Quartz crystal microbalance
RFs	Random fibers
SEM	Scanning electron microscope
SPR	Surface plasmon resonance
STEM	Scanning transmission electron microscope
TEM	Transmission electron microscope
TPS	Trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane
Znpp	Zinc(II) protoporphyrin IX
α GluD	α -D-glucosidase

Chapter 1

Introduction

Nature created through billions of evolution years unique functional superstructures with sizes from nano- to micro-scale for performing specific tasks. For instance, enzymes are essential components in living beings, consisting of folded peptide chains with sophisticated three-dimensional structures which determine their specific catalytic activity ¹. The catalytic site is embedded within the protein structure next to binding sites which orient the substrates, resulting in high specificity ². The confinement of proteins and enzymes by membrane proteins yields diverse organelles which serve as separated functional entities in cellular compartments defined by a phospholipid bilayer boundary, facilitating the transport of nutrients and signal from the environment and participating in well-organized biochemical reactions to maintain life. This diversity of nature in terms of structure and function has inspired scientists in the design and construction of smart multifunctional assemblies.

Progress in nanoscience and nanotechnology has enabled us to synthesize various well-defined nano/micro supramolecular assemblies, e.g., polymersomes, capsules, wires, fibers and tubes through appropriate strategies ³⁻⁶, mimicking natural systems and design. An efficient approach for the construction of such assemblies is the bottom-up strategy, which even allows uniting synthetic structures with biological elements such as proteins or cells, thereby building artificial analogies having the specificity and functionality inherent to natural systems ^{7,8}. By incorporation of these natural components, the resulting hybrid functional nanomaterials have

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applications as, *e.g.*, biosensors, nanoreactors, targeted nanocarriers, and in tissue engineering. Of particular interest is the combination of enzymes and structural assemblies to generate enzymatic nanoreactors for biocatalysis. These smart bioreactors can be achieved by encapsulation of enzymes within structural boundaries that separate the reaction space from the environment, creating a local ultra-small compartment where enzymes can act, enabling this confinement effect to be studied experimentally with synthetic analogues, such as in polymersomes, polymeric capsules, and other artificial organelles⁹⁻¹¹. A promising and efficient approach to immobilize enzymes is to use them as building blocks, typically combined with polymers to construct functional analogues. The entrapment of biological enzymes in polymer layers can improve the stability of the immobilized enzymes in structure and functionality against changes in the environment¹². These intrinsic properties of nanoreactors combining structural stability with the specificity of biological entities offer new avenues for use in the biomedical field.

So far, most systems studied are spherically symmetric, such as polymersomes or capsules^{5,6,13,14}. Anisotropic tubular structures possess unique channels with opening terminals compared to spherical compartment systems. This intrinsic tubular feature allows the circulation of molecules from one end to the other, with interesting channel applications. In addition, independently tailored functionalities (interior and exterior surfaces) and longer circulation of nanotubular objects *in vivo* distinguish themselves for biomedical applications¹⁵⁻¹⁷.

In this context, the main objective of my thesis was *to construct tubular biocatalytic nanostructures containing enzymes serving as both structural and confined functional components, and nanofibrillar mats of these LbL-nanotubes for biocatalysis*. Beyond their practical interest, the elaboration of enzyme nanotubes and mats as biocatalytic nanomaterials also raises basic concerns. Therefore, our aims in this thesis were *to understand mechanisms at play when enzymes are layer-by-layer assembled in nanoconfined spaces; to develop a robust collection strategy, innocuous to enzymes, to recover and disperse templated-synthesized nanotubes into an aqueous medium for enzymatic reactions; to provide insight on the nature (mechanisms and kinetics) of enzymatic reactions in confinement; and to explore the possibility of using bioactive nanotubes to construct nanoporous materials of practical interest*.

In this regard, enzyme nanotubes were prepared via the well-established layer-by-layer technology (LbL) combined with hard template-synthesis. Track-etched nanoporous polycarbonate membranes (PC) were used as templates in the present work because of their easy dissolution in organic solvent to release the nanotubes, for instance CH_2Cl_2 . Glucose oxidase (GOX) is used as a model enzyme in this work, since it is robust and widely used in industry¹⁸. However, the complexity of protein envelopes makes the assembly of enzymes and polyelectrolytes not always possible^{19,20}. In order to fabricate robust enzyme nanotubes of high activity, counter polyelectrolytes and assembly conditions as well have to be optimized, with respect to the nature of polyelectrolyte and buffer, or pH and ionic strength of assembly solutions. This can be done by evaluating the activity of nanotube-containing PC membranes as well as the structural features of freed enzyme nanotubes. Besides the fabrication of active enzymatic nanotubes, another issue is the recovery of these free soft nanotubes from the dissolved template without structural and biological degradation, and their dispersion without aggregation in aqueous solution, the essential surrounding medium that enzymatic reactions require. In this respect, we developed a promising and general methodology based on adjuvant-aided filtration rather than conventional centrifugation to efficiently collect the soft nanotubes. This collection route was evaluated with respect to the enzymatic efficiency and structure of nanotubes. The influence of the adjuvants on both the structure and activity of liberated nanotubes in aqueous suspension was also examined, confirming that our new methodology is robust and versatile for the collection of well-dispersed soft enzyme nanotubes. The quantitative collection of intact nanotubes allows the comparison of their activity in different settings.

During the development of this collection strategy, we found that the enzyme nanotubes stack up over an adjuvant layer, when filtered, forming clusters of nanotubes. This serendipitous finding inspired us to create self-standing functional mats of LbL-nanotubes by a kind of paper-making process, involving the simple filtration and sedimentation of the nanotubes onto a (possibly sacrificial) filter. As the first step towards the fabrication of self-standing multifunctional mats, we used already synthesized GOX enzyme nanotubes as model building blocks to investigate the filtration process for the production of an entire and nice mat, and subsequently the structure of resulting mats. LbL-nanotubes with different natures of their external shell

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and various rigidities were then used to construct mats in order to understand the mechanism of formation of a cohesive mat, revealing the possibility to create nanoporous scaffold nanomaterials. The activity of enzyme-containing nanotube mats was finally evaluated, demonstrating the applicability of these enzymatic mats for filtration-based purification.

Hence, in the following chapters the content of my work is organized and demonstrated as follows:

Chapter 2 provides a fundamental overview of key technologies utilized in this research work, including the mechanism of layer-by-layer assembly in confined medium, its use to fabricate various functional architectures, in particular nanotubes, and related applications.

Chapter 3 presents the selection of polyelectrolytes and optimization of assembly conditions for the fabrication of robust enzyme nanotubes. The LbL-nanotubes assembled in the optimized conditions are also quantitatively probed by FTIR, providing insight in their internal composition.

Chapter 4 focuses on the development of a novel strategy that is capable of recovering templated LbL-nanotubes and of dispersing them again in aqueous medium without significant loss in structure stability and biological activity of enzyme nanotubes. The activity of aqueous suspensions of free LbL-nanotubes achieved by adjuvant-assisted filtration is also intensively evaluated.

Chapter 5 demonstrates the construction of mats of templated LbL-nanotubes based on filtration with different protocols. Several types of nanotubes with different properties are used as building blocks and the structure of resulting mats is investigated in details by SEM and AFM. The activity of enzyme nanotube mats prepared over a nanoporous PET filter is also examined.

Finally, the thesis is ended by Chapter 6, including a general summary of the results and some perspectives on the multifunctional LbL-nanotubes as vehicles and multilayered mats of functional LbL-nanotubes for various applications.

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Chapter 2

State of the art

This chapter aims to describe the fundamentals of the templated layer-by-layer assembly involved in this research work with particular highlight in fabrication, collection and potential applications of protein nanotubes. Then advances in nanofibrous mats are shortly reviewed, showing the possibility of fabricating LbL-protein nanotube scaffolds for various applications.

2.1 Layer-by-Layer assembly

2.1.1 Basics

The core technology used in this research work is alternating layer-by-layer assembly (LbL), which was first proposed in 1966 by Iler who constructed multilayer assemblies via alternating adsorption of anionic and cationic colloidal particles¹. However, no attention was paid to it until Decher and co-workers introduced this concept to formation of ultrathin multilayer film using bipolar amphiphiles and polyelectrolytes in 1991^{2,3}. Over decades, LbL technology has been extensively studied, and nowadays, it is a well-established method for the construction of nanostructures with tailored properties.

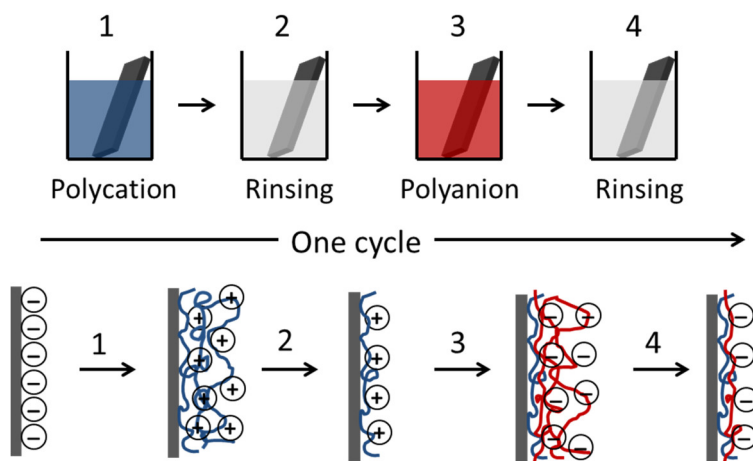


Figure 2.1. Schematic representation of layer-by-layer adsorption processes. The negatively charged substrate, e.g. silicon is immersed in a polycation solution followed by rinsing step to remove loosely adsorbed polyelectrolyte chains. Then the polycation-modified substrate is transferred to a polyanion solution followed by another rinsing step.

The main driving force for LbL assembly is the electrostatic interactions between oppositely charged species. This process consists in consecutively exposing a substrate to polycation and polyanion solutions with intermediate rinsing steps. As illustrated in Figure 2.1, upon immersion of a negatively charged substrate in a polycation solution, adsorption of counterionic

polyelectrolyte chains occurs, followed by rinse step to remove unbounded and loosely attached polymer chains. The deposition of ultrathin polycation layer results in surface charge reversal, as a result of charge overcompensation⁴, facilitating the following deposition of polyanion chains. Identical rinse protocol is applied when the adsorption process of polyanion reaches equilibrium to finalize one cycle, defined as a bilayer. This whole cycle can be repeated until the desired number of bilayers is obtained.

The unique feature of the LbL assembly relies on its ability to control film growth at the nanoscale with respect to the thickness, internal and surface structures, and the related properties by simply adjusting the assembly parameters⁵⁻¹⁴, for instance pH, temperature, ionic strength, and nature of solvent. Whether the sample is dried or not after rinse step is also an influential factor in some cases¹⁵⁻¹⁸.

Besides typical electrostatic interactions, various other types of interactions were also exploited for LbL assembly, including hydrogen-bonding¹⁹⁻²², hydrophobic interactions²³⁻²⁵, charge transfer²⁶⁻²⁹, host-guest interactions³⁰⁻³³, and bio-specific recognition³⁴⁻³⁷, rendering great applicability to a wide range of materials.

2.1.2 Templated synthesis

In LbL assembly, a substrate is required, serving as support for the LbL growth. Typically, flat substrates, such as silicon wafers or glass slides are frequently used in LbL film build-up. Free planar films could be obtained if the films are detached from the flat substrate using appropriate ways. In this sense, LbL assembly is an essential template-synthesis methodology, since the LbL film always replicates the shape and size of the support. The free-standing LbL films have been reported by several groups, although they used different protocols to release the LbL films from flat substrates³⁸⁻⁴¹. Schlenoff *et al.*³⁸ used salt concentration as a delamination stimulus to release stratum poly(styrene sulfonate)/poly(diallyldimethylammonium) multilayers, (PSS/PDADMA)₂₀, by decomposition of a sacrificial 20-layer poly(acrylic acid)(PAA)/PDADMA which was previously built on substrates. PSS/PDADMA multilayers are stable up to a salt concentration of ca. 3.5 M, while PAA/PDADMA films are completely decomposed at salt concentrations greater than 0.6 M. Thus the entire PSS/PDADMA film is quickly released from the substrate upon immersion in 1 M NaCl. Likewise, a thin layer of

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cellulose acetate (CA) was coated on substrates prior to the LbL film construction to facilitate lift-off of the LbL assembly from substrates, followed by dissolution of CA in acetone solvent³⁹. A facile and solvent-free method was proposed by Wagner⁴⁰ who deposited a functional monolayer of trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (TPS) on glass slides to reduce the interactions between LbL film and bare substrate, favoring an easy detachment of LbL film from the substrate (Figure 2.2A). For robust cross-linked LbL films, an intact film can be easily peeled off by tweezers from the virgin glass after a simple sequential immersion in water and ethanol (Figure 2.2B)⁴¹.

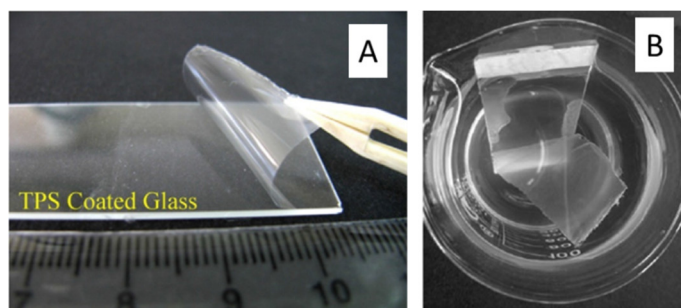


Figure 2.2. Optical photographs of LbL films deposited either on a TPS coated (A) or virgin (B) glass slide. Images extracted from ref. [^{40,41}]

On planar substrates, LbL deposition is not only limited to dip-coating method, but this can be accomplished by using other techniques such as spin⁴² or spray assembly⁴³.

Moreover, the LbL assembly is not only restricted to flat surfaces but can be used for coating any irregular surface that solution can reach. Therefore, by using appropriate substrates (colloids, nanoparticles, nanofibers, and nanoporous matrix)⁴⁴⁻⁵³, a large range of nanoarchitectures can be achieved by LbL coating of either the outer or inner part of the template followed by removal of the sacrificial template. By this way, many different hollow capsules, nanotubes/nanorods and, three-dimensional (3D) networks have already been produced.

Compared to macroscopic templates (planar substrates and porous membranes), performing LbL assembly over colloids or within porous particles suspended in solution, is more tedious, as these 'invisible'

templates need to be alternately dispersed in oppositely polyelectrolyte solutions with rinsing step in between. Typically, the adsorption is carried out in an excess of polyelectrolyte, which should be removed before adding the next complementary polyelectrolyte to avoid formation of complexes in solution. This separation can be achieved either by centrifugation⁴⁵ or filtration⁵⁴. In centrifugation approach, microparticles are deposited from the supernatant solution, facilitating the replacement with rinsing media or polyelectrolyte solution. However, separation of the particles from the solution by centrifugation often results in problems with resuspension in sequential media.

An alternative separation method is filtration where particles are filtered from the excess polyelectrolyte through size-matched filters⁵⁴. The particles are not stressed, since they are only subjected to a slow and adjustable stirring. The liquid level above the filter can be easily controlled by the interplay between the inflow of the reagents and the pressure applied to facilitate the filtration, and the filtrate can be easily recovered. However, for small colloids, the particle-size-matched filters are readily clogged, increasing the particle loss and the fabrication time. Polyelectrolytes can also be deposited on a colloidal template surface based on surface-controlled precipitation as demonstrated by Mohwald⁵⁵. In general, various methods could be used to assist LbL assembly on diverse nanoparticles.

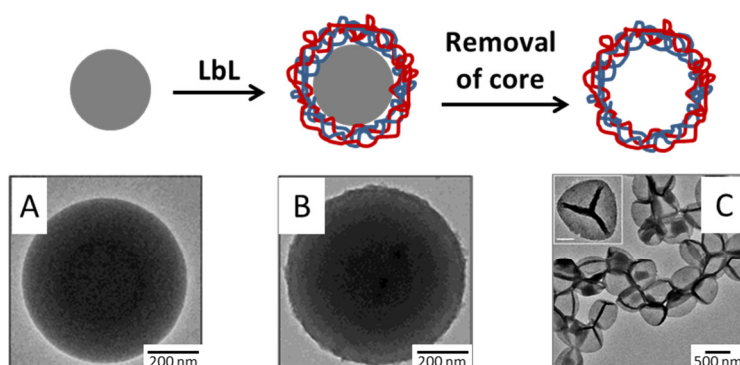


Figure 2.3. Schematic representation of the formation of LbL hollow capsules via templated synthesis. Polyelectrolyte multilayer films were constructed over PS particle (A) as shown in B, followed by the removal of sacrificial templates in tetrahydrofuran, resulting in free nanocapsules (C). Images (A,B,C) extracted from ref. [45]

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In order to achieve intact hollow capsules, the confined colloidal templates have to be completely removed using appropriate methods that do not cause degradation of the hollow structures. Organic solvent can be used to dissolve soluble polymer colloids. For instance, polystyrene (PS) lattices can be dissolved in tetrahydrofuran (Figure 2.3)⁴⁵. The PS chains slowly diffuse out from the capsules; therefore, several wash steps are required to ensure the complete elimination of PS chains. Melamine formaldehyde, CaCO_3 and silica particles are also frequently used as sacrificial core templates for construction of hollow capsules. These core particles are usually eliminated by using acidic solutions⁵⁶⁻⁵⁸.

When using porous templates, the LbL assembly takes place within the channels of the porous substrates. The LbL structures are therefore confined within the porous network and, can be easily liberated by dissolving or decomposing the sacrificial templates, yielding free 3D interconnected networks or nanotubes. Caruso reported LbL uniform coating of mesoporous silicas (MS) with polyelectrolytes followed by removal of silica templates to obtain 3D micrometer-sized nanoporous polyelectrolyte spheres (Figure 2.4)⁵⁹. This approach offers a general route towards the construction of 3D nanoporous polymer networks with tailored composition based on template-LbL assembly.

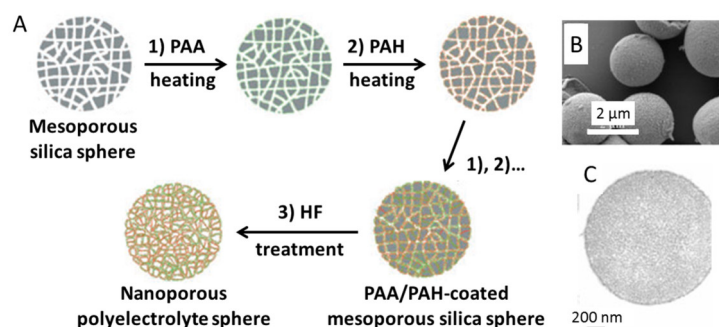


Figure 2.4. Schematic illustration showing the procedures to fabricate 3D polyelectrolyte network (A). Porous MS spheres were LbL coated with PAA and PAH, with the sample heated (160 °C for 2h) after each polyelectrolyte deposition to partially cross-link the layers. The MS template was then dissolved by exposure to hydrogen fluoride (HF), yielding intact nanoporous polyelectrolyte spheres (B). TEM image (C) shows the porosity of the polyelectrolyte spheres. Images adapted from ref. [⁵⁹]

Free LbL nanotubes could also be created by using nanoporous membranes with vertically aligned channels as sacrificial templates^{51,52}. Track-etched porous polycarbonate membranes (PC) and porous anodic aluminum oxide (AAO) plates are commercially available and are commonly used as templates for the fabrication of LbL nanotubes and nanorods (Figure 2.5). Track-etched PC membranes are fabricated by two steps, entailing the bombardment of raw PC membranes with a thickness ranging from a few microns to a few tens of microns with heavy energetic ions and subsequent removal of the degraded polymers in latent tracks by wet-chemical etching, producing the channels inside the PC matrix⁶⁰. Based on this ion-track technology, pore size, shape and density can be varied in a controllable manner by the proper selection of the conditions under which irradiation and post-treatment procedures are carried out. Pore size can be tuned from 10 nanometers to a few micrometers, offering a promising platform for fabricating nano-objects with extremely high aspect ratios. The desirable pore density (within appropriate range) can be obtained by controlling process parameters, however, the highest one is limited by the pore size, owing to their randomly distribution in PC membranes. Typically, the larger the pore size, the lower is the highest pore density that can be reached due to the increasing possibility of interconnection among vertically aligned channels. Typically, for pore size of 200 nm, pore density up to 10^8 cm^{-2} can be reached while, for 1 μm pore size, the higher pore density is limited to 10^6 cm^{-2} .

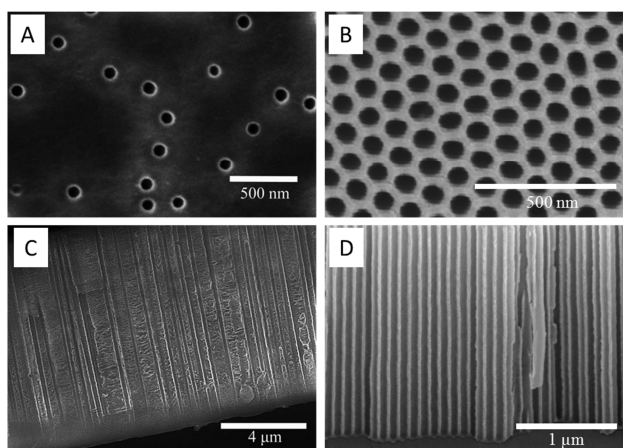


Figure 2.5. Top (A,B) and side-view (C,D) SEM images of nanoporous templates PC (A,C)⁶¹ and AAO (B,D)⁶². Images adapted from ref. [^{61,62}]

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Moreover, intersected nanochannel PC templates can be obtained by a sequential two-step exposure to energetic heavy ions, at different incidence angles with respect to the normal of the polycarbonate (PC) membrane surface. These interconnected nanoporous membranes are promising sacrificial templates for the construction of three-dimensional (3D) networks of highly interconnected nanotubes/wires⁶³⁻⁶⁶.

In contrast, porous AAO plates, fabricated by anodizing high purity Al foils under appropriate voltage, have vertically aligned and well-packed cylindrical nanochannels with diameters ranging from 5 to 400 nm. The pore diameters and interpore distances of AAO template can be adjusted by changing anodic oxidization parameters⁶⁷. Typically, the PC membrane is preferable to the AAO template in the production of LbL nanotubes/rods, because of its easy dissolution in organic solvent, such as CH_2Cl_2 and dimethyl formamide (DMF). The harsh conditions (NaOH or H_3PO_4) required to etch solid AAO templates probably induce deformation in soft LbL tubular structures and degradation in bioactivity of functional components incorporated in nanotubes⁶⁸⁻⁷⁰.

2.1.3 Mechanism of LbL assembly in nanopores

The LbL growth mechanism in confined geometries has been studied by using a well-known polyelectrolytes system: poly(allylamine hydrochloride) PAH/PSS pair⁷¹. This study reveals the existence of two different regimes related to the growth kinetic with a transition point (Δr , defined in Figure 2.6) at around 70%. In the first regime, a growth mechanism similar to the one on planar substrates, including the effect of ionic strength and pH, was observed. As pore size decreases with the build-up of LbL multilayers, the LbL growth enters into the second regime, which is kinetically much slower, due to the formation of polyelectrolyte entangled structures inside the pores. The resulting limitation in the diffusion of polyelectrolytes into pores leads to a slow growth in terms of thickness. The pore size is a significant factor in the LbL buildup. As shown in Figure 2.6A in extremely small pores (about 100 nm), it takes only a few LbL cycles to fill the pores and enters into the slower growth regime (regime 2), while reaching this transition requires more LbL deposition cycles in larger pores.

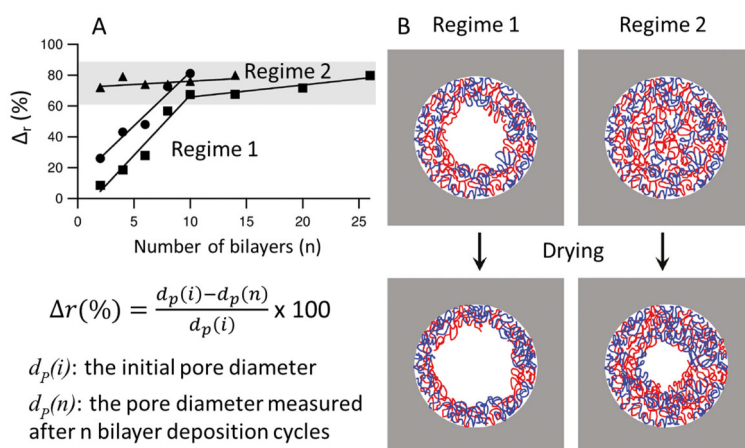


Figure 2.6. LbL growth evolution (Δr) of PAH/PSS multilayers as a function of the number of bilayers (n) in 100 nm (triangles), 200 nm (circles), and 500 nm (squares) pores (A), and the proposed growth mechanism in nanopores consisting of two different two regimes (B), corresponding to A. Images adapted from ref. [71]

Recently, Cho ⁷² investigated the effect of pore size on the formation of tubular structures within AAO templates. Particular attention was paid to the comparison of thickness increment per bilayer between pores of different sizes and flat substrates for LbL assembly carried out under identical conditions. According to the mechanism proposed above (Figure 2.6), in all these cases, the LbL growth is in the regime I, since the value of Δr at 30 bilayers for all AAO templates is around 30%, far below the transition point. In small pores (30-345 nm), the thickness of tube walls was found to be thinner than that of planar film built on silicon substrates under similar conditions (Figure 2.7), regardless of the size of polyelectrolytes as well as the assembly conditions ⁷²⁻⁷⁶. The authors attributed this difference of thickness to the hindered polyelectrolyte chain entrance into the pores, called an entropic entry barrier, as when pore diameter increases and approaches 430 nm, a wall thickness similar to the LbL film on flat substrate was obtained (Figure 2.7b,c) ⁷². However, the smaller the radius of gyration of polyelectrolyte chains (by adding electrolyte salt and adjusting pH), the higher the ratio of nanotubes wall thickness to that of flat film for same small pore size. The value of this ratio increases from 0.33 up to 0.7 in 70 nm nanopores when the pH of PAH solution was adjusted from initial pH (4-5) to

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9.3-9.5. The same phenomenon was observed for all pores presenting a diameter below 100 nm, when the ionic strength was increased. This increase of polycation solution pH- and ionic strength reduces the radius of gyration of pH- and salt-sensitive polyelectrolyte chains, respectively, by reducing the charge density and screening the repulsive interactions between charged units along the chains.

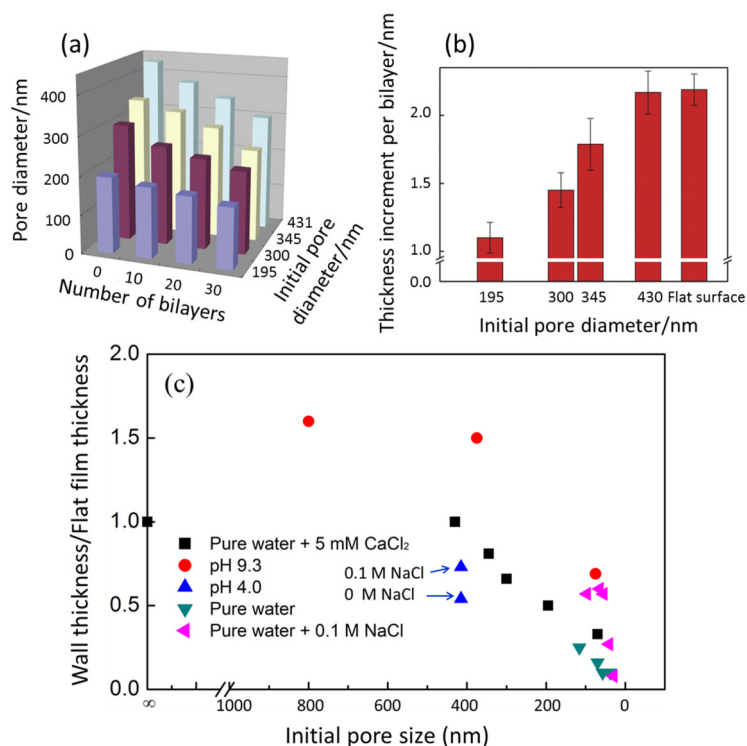


Figure 2.7. Evolution in pore size (a) of AAO template with various initial pore diameters as a function of number of PAH/PSS bilayers (obtained from SEM images), (b) thickness of bilayer (PAH/PSS) assembled in various AAO templates with different pore diameters⁷², which was then divided by the thickness of polyelectrolyte bilayer grown on flat substrates under identical assembly conditions to quantify the difference, shown in (c). The various LbL assembly conditions were used to study the influence of pH, and ionic strength on the growth behavior in nanochannels, summarized in (c). ■^{72,74}, ●^{73,77} and ▲⁷⁶ represent PAH/PSS system, while ▼⁷⁵ and ▲⁷⁵ indicate that 4th generation polyanion and polycation dendrimers were used for LbL assembly. Images (a, b) extracted from ref. [72]

LbL assembly in nanochannels exhibits thus a strong dependence on the pH and ionic strength of polyelectrolyte solutions. For PAH/PSS pair, the ratio with respect to the increment per bilayer in regime 1 evolves from 0.54 to 1.5, in the *ca.* 400 nm nanopores, in response to the increasing of pH and ionic strength. The same trend appears under one specific assembly condition when the initial pore size increases gradually within an appropriate range. However, a LbL growth comparable to what occurs on flat surface is expected when pore size increases up to an enough large extent (point at which the confinement of pores can be ignored). This means that depending on the assembly conditions, there is a critical pore size in which the LbL growth behavior is identical to that on the 'open' planar surface (Figure 2.7c, wall thickness/flat film thickness ratio =1). For instance, this critical pore size is *ca.* 200 nm and 400 nm for LbL growth from solutions at pH 9.0 and pure water with 5 mM CaCl₂, respectively. The presence of this interesting behavior results from a compromise between various effects; either facilitating or hindering the polyelectrolyte adsorption in nanopores, such as incomplete drainage⁷⁷, curvature of pores⁵¹, confinement effect⁷¹⁻⁷³ and, depletion effect⁷⁶.

For these nanoporous templates, apart from the conventional dipping route, pressure or vacuum can be used to assist the adsorption of polyelectrolytes. When using this process for the construction of nanotubes, cationic and anionic polyelectrolyte solutions are alternatively filtered through the nanoporous membranes⁵¹. The filtration-based LbL process has been extensively investigated by Jonas and coworkers⁷⁸ who deposited a pair of strong polyelectrolytes within a series of track-etched nanoporous PC membranes with pore size, ranging from 50 to 850 nm, under various assembly conditions. The results show that when filtration-LbL assembly is performed, the pores are rapidly filled by a dense gel upon limited deposition cycles, depending on the initial pore size. The increment of thickness per cycle of deposition is much larger than that on flat surfaces (increase by a factor of one hundred, in some cases), resulting from the entanglement of the polyelectrolyte chains which are in a concentrated regime when passing in the confined channels.

Regardless of the methodology used for polyelectrolyte adsorption (filtration or immersion), one important issue during the LbL deposition within nanopores is that LbL assembly also occurs on the template top faces, leading to encrusting over the template surfaces. This crust may cover the

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pores causing restriction in LbL growth within the nanochannels when increasing the number of bilayers (Figure 2.8). This surface film must therefore be removed after each or several bilayer depositions when membranes with narrow pores are used as templates. This decrusting treatment is also required at the end of the buildup process in order to separate aligned nanotubes/wires. Various methods have been used to eliminate those LbL films, including plasma etching⁵³, polishing with alumina powder⁷⁹ and scratching with cotton swab^{71,80}.

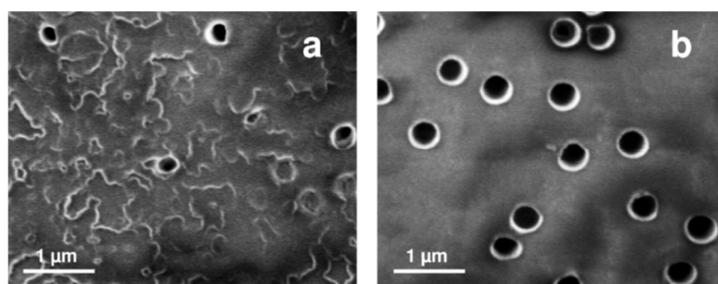


Figure 2.8. SEM images of PC membrane after the deposition of (PAH/PSS)₂₆ (a) before and (b) after removal of surface crust film. Images extracted from ref. [71]

2.2 Proteins

A polypeptide chain is a macromolecule made by condensation of amino acids, forming peptide bonds. 22 types of amino acids bearing different substituents serve as both structural and functional blocks, leading to a great variety of proteins of diverse architectures. Proteins are essential to the specific tasks in living creatures, responsible for transportation of elements, signaling and guiding cells, providing structural scaffolds, and taking part in biochemical reactions as well⁸¹⁻⁸⁶.

These diverse functions of proteins arise from the unique amino acid sequences that make up proteins, called primary structure, shown in Figure 2.9. The favorable folding of a protein chain to a higher level structure (secondary structure) is driven by diverse intrachain interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds; the typical secondary structures are α -Helix, β sheet, polypeptide II helix and hairpin turns. These elements of secondary structures can be arranged to form a

three-dimensional structure (tertiary structure), determined by various interactions involving hydrophobic interactions, hydrogen bonds, ionic and disulfide bonds. This spatial arrangement of tertiary structures results in polypeptide units of global conformation, called 3D domains. Typically, for many proteins, they consist of several polypeptide domains which could be identical or different. The quaternary structure describes how several polypeptide subunits are organized together into a protein, thereby accounting for the functions of formed protein. Sometimes, carbohydrate chains and cofactors are bonded to polypeptide subunits, performing a wide range of functions⁸⁷⁻⁹¹. For example, the carbohydrate chains attached to membrane proteins (glycoproteins) serve as 'recognition' markers to distinguish and communicate between cells⁸⁸. Glucose oxidase (GOX) is a dimeric enzyme composed of two identical polypeptide subunits that are covalently linked together via disulfide bonds⁹², each of which contains one tightly bound but not covalently linked flavin adenine dinucleotide (FAD) moiety as co-factor contributing to the biological activity (Figure 2.10)⁹³⁻⁹⁶.

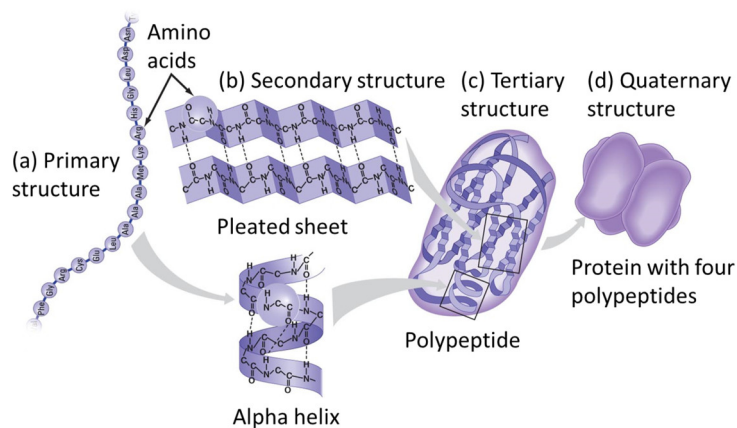


Figure 2.9. Four levels of protein structure. Image from ref. [⁹⁷]

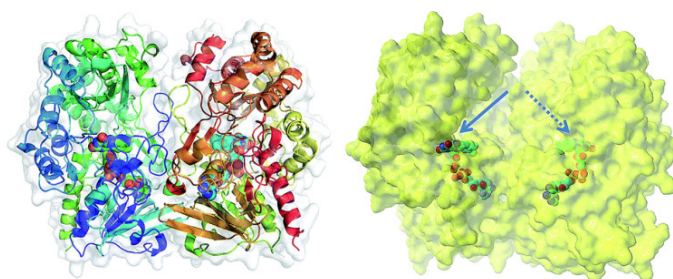


Figure 2.10. The molecular structure of glucose oxidase which is a dimer protein with 2 bounded FAD, the cofactor (flavin adenine dinucleotide indicated by arrow). Images extracted from ref. [⁹⁸]

An important property of proteins is the isoelectric point (pI), originating from the presence of amino and carboxylic acid groups in the amino acid components. The net surface charge of proteins could be either positive or negative, depending on the pH of medium, offering the possibility of using proteins as polycation or polyanion to construct functional protein nanostructures. The pI of proteins can be either theoretically calculated based on their amino acid sequences with reference to pK_a values recorded for ionizable constituents⁹⁹ or determined by experimental methods, such as isoelectric focusing (IEF)¹⁰⁰. Table 2.1 gives the experimentally measured pI of some typical proteins (pI values measured by IEF)^{101,102} including enzymes, showing that it covers a large range from 1.0 to 11.0, related to the composition and structure of proteins.

Table 2.1. Isoelectric point of some proteins

Protein	Isoelectric point	Protein	Isoelectric point
Pepsin	1.0	Catalase	5.6
Glucose oxidase	4.2	hemoglobin	6.8
Human serum albumin	4.8	myoglobin	7.0
Urease	5.1	Lysozyme	11.0

2.3 LbL assembly of proteins

Proteins can be immobilized by complexation with oppositely charged polyelectrolytes/proteins in a layer-by-layer fashion to form functional nanostructures. Besides electrostatic interactions, hydrogen bonds or hydrophobic interactions might also contribute to the assembly driving force. On the one hand, because of the complexity of protein surfaces, the assembly of proteins is sensitive to the external environment¹⁰³, such as pH, ions nature and ionic strength. On the other hand, the pH of enzyme aqueous solutions for assembly should be within a pH range over which enzymes are stable to avoid the free protein denaturation as well as to maintain the activity of immobilized enzymes during film preparation¹⁰⁴⁻¹⁰⁷. Therefore, assembly pH is an essential key in LbL assembly for immobilization of proteins without denaturation. For example, free GOX enzymes can be used as polyanion only when the pH is beyond its pI, 4.2. Fortunately, it is stable over pH 5-7 (Figure 2.11c)¹⁰⁷, thus the assembly pH for GOX as polyanion should be limited in this range.

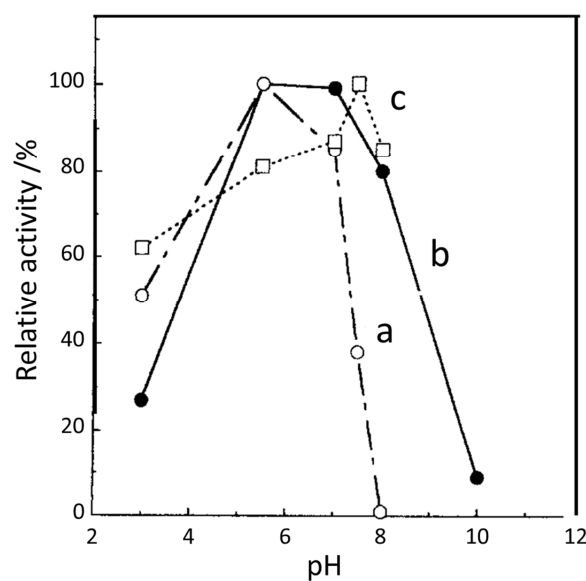


Figure 2.11. pH profile of relative activity of free GOX in water (a, open circles), and immobilized GOX in bPEI/GOX film (b, solid circles) and in silica gel matrix (c, open squares). Image extracted from ref. [107]

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In general, the assembled proteins are in most cases not denatured¹⁰⁶, especially when the adsorption of protein is performed in buffer at optimal pH. The resulting LbL enzyme films can thus preserve their biological activity and can be used for diverse applications such as biosensors, bio-purification and theragnostics. Moreover, the stability of LbL containing proteins can be improved without loss in their biological activity¹⁰⁷. However, it is noteworthy that the immobilized proteins in LbL film could be denatured by incompatible solvent, pH and temperature¹⁰⁷⁻¹⁰⁹.

Ferreira¹¹⁰ reported on the preparation of a LbL based glucose biosensor by assembly of GOX and poly(allylamine) hydrochloride (PAH) onto an Prussian Blue (PB) modified indium oxide (ITO) substrate. This ITO/PB/GOX-PAH biosensor has a high sensitivity of $16 \mu\text{A} \cdot (\text{mmol/L})^{-1} \cdot \text{cm}^{-2}$ and a limit of detection of $0.20 \text{ mmol} \cdot \text{L}^{-1}$. These high performances were attributed to the ultrathin nature of the LbL film and the low operating potentials that could be used to detect the H_2O_2 produced in the enzymatic reaction in presence of Prussian Blue. These LbL glucose biosensors were found to be quite stable with a reliable amperometric response and an almost constant sensitivity for a period of approximately 20 days. The introduction of electron mediators to the LbL enzyme films such as methylene blue, carbon nanotubes or polyelectrolytes bearing redox-active groups facilitate the electron transfer between the immobilized enzymes and electrodes, resulting in LbL electrochemical sensors with enhanced properties¹¹¹⁻¹¹³.

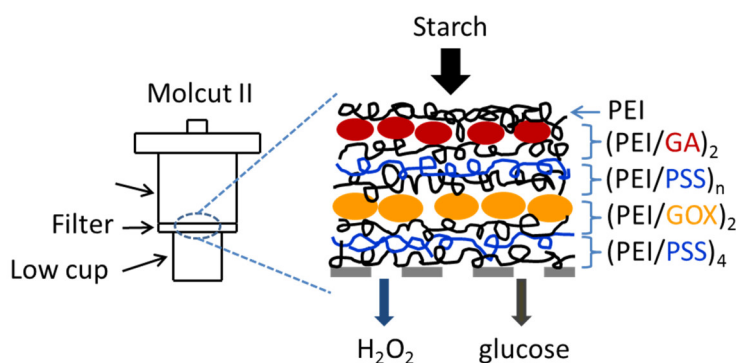


Figure 2.12. Sequential enzymatic process based on the glucoamylase (GA) and glucose oxidase (GOX) and its experimental setup¹¹⁴.

Sequential incorporation of multiple enzymes in a controlled manner within the same films leads to a more advanced innovative structure. The first example is given by Kunitake¹¹⁴ who sequentially deposited poly(ethyleneimine)PEI/GOX and PEI/GA layers on an ultrafilter and examined the enzymatic activities using the experimental setup depicted in Figure 2.12. Hydrolysis of the glycosidic bond in starch by GA produces glucose which is further converted to gluconolactone by GOX film below with H_2O_2 as a co-product. The results showed that the combination of ultrafiltration with the use of a layered protein film allows the separation of the substrate and products without further procedures. Based on this concept of coupled reactions, Shen *et al.* developed a bienzyme maltose sensor¹¹⁵.

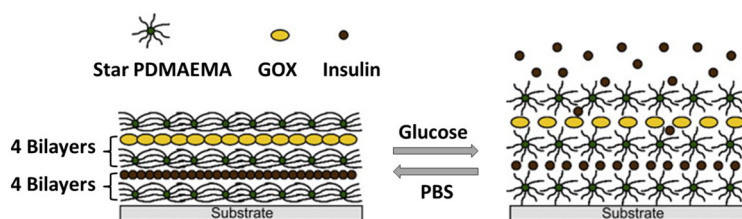


Figure 2.13. Schematic representation of the on-off regulation of insulin release from the glucose-sensitive assembled multilayer film based on star polymer. Images extracted from ref. [¹¹⁶]

Likewise, a glucose-responsive multilayer system was reported by Li¹¹⁶. In this system, a GOX film was built on top of insulin multilayers assembled with 21-arm poly(2-(dimethylamino)ethyl methacrylate) (star PDMAEMA) (Figure 2.13). The outer GOX multilayer is used as a glucose-sensitive layer to control the on-off release of underlying insulin by changing the pH microenvironment within the LbL multilayer film through the enzymatic reaction. The decrease of pH resulting from GOX enzymatic reactions leads to a rearrangement of film morphology induced by the pH-response of star PDMAEMA. The distinctive geometry of PDMAEMA star structure, combined with changes in ionization of the polyelectrolyte, results in an “open” status of the film allowing insulin to be released. Due to its well-controlled structure at the nanoscale level and distinct mechanism of drug controlled release, this novel responsive multilayer film has a great potential as a self-regulated insulin delivery system.

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As described previously, layer-by-layer technology is largely applicable to various supporting substrates (e.g., colloidal particles and porous membranes). Various nanostructures made of proteins can be obtained by using this templated synthesis method and used as nanoreactors or nanocarriers. Among those protein nanostructures, nanoparticles coated with enzyme LbL-shell and enzyme hollow capsules obtained after subsequent removal of sacrificial core, were extensively studied¹¹⁷⁻¹²⁶. When nanoparticles are kept inside enzyme LbL-shells, the properties from core material can be combined, leading to multifunctional core-shell particles. For example, the LbL assembly of enzymes on magnetic-functionalized particles enables to use magnetic properties to recover and re-use these biocatalytic microparticles¹²³. These collectable particle bioreactors can also be achieved by simple incorporation of magnetic nanoparticles in LbL shells^{124,125}. This procedure allows the removal of the core without loss of magnetic properties, resulting in magnetically controlled hollow capsules.

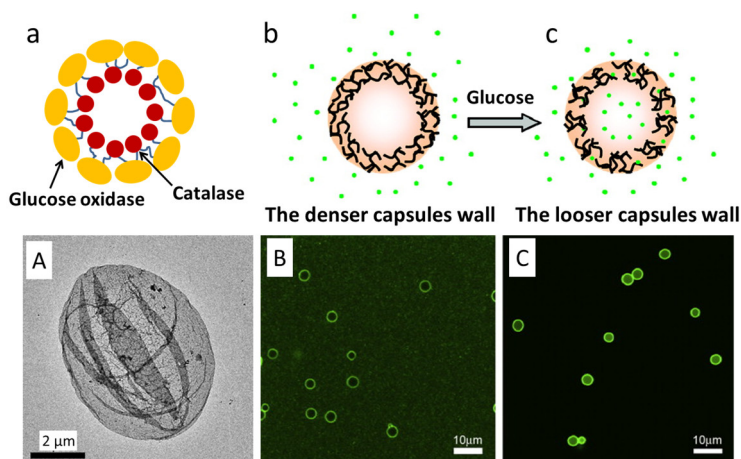


Figure 2.14. Structure (a) of CAT/GOX capsule and relevant TEM (A). The microcapsules were mixed with fluorescein isothiocyanate (FITC) labeled dextran (200 kDa, green dots) without adding glucose (b,B) and with adding glucose for 2 h (c,C)¹²⁶.

Li and coworkers reported on a protein multilayer shell which was fabricated by the alternated assembly of catalase (CAT) and glucose oxidase (GOX) via glutaraldehyde cross-linking (Figure 2.14A)¹²⁶. These resulting capsules are found to be glucose-sensitive (Figure 2.14B,C); this is attributed to the

coupled enzymatic reactions of glucose in the assembled CAT/GOX shell, which leads to the production of H^+ and therefore to a local pH decrease. Under these acidic conditions, part of C=N bonds formed from Schiff base reaction can be broken, leading to an increase of the capsule wall permeability. Using the same method, GOX was also paired with hemoglobin (Hb), leading to similar glucose-sensitive capsules¹²⁷. They further built this glucose-responsive capsule shell over insulin particles and showed that the release of insulin can be tuned by external glucose, providing the possibility of being used as carriers for drugs.

2.3.1 Protein nanotubes

Tubular architectures with tailored properties have attracted a growing attention due to their advantages and wide range of applications¹²⁸⁻¹³⁰. First, nanotubes possess independent inner and outer surfaces, allowing to program desired multiple functions. Second, nanotubes have tubular channels with open ends that may be used as tanks for biomolecules or drug delivery. Third, nanotubes can have long circulation persistence *in vivo*, which was reported by Discher and coworkers¹³¹. They found that filaments remained in the bloodstream of rats up to 1 week, about 10 times longer than spherical particles of the same composition.

The first example of protein nanotubes was reported by Martin who used glutaraldehyde as cross-linking agent to hold protein layers together in a layer by layer route in porous AAO templates⁶⁹. The liberated enzyme nanotubes exhibited higher oxidation activity than that of the nanotubes embedded into the template. However, protein nanotubes constructed on the basis of non-covalent interactions, for instance electrostatic interactions,¹³²⁻¹³⁴ are favorable to avoid significant changes in protein structures, which is the most exciting feature of LbL technology. The resulting smart protein nanotubes retain biological activity, demonstrating their promising biological applications including enzymatic reactions¹³⁵⁻¹³⁸, bioseparation¹³⁹⁻¹⁴³, and biomolecule or drug delivery^{140,144}.

Komatsu¹⁴⁰ reported on the fabrication of LbL protein nanotubes for molecular capture. These nanotubes were constructed via LbL alternated deposition of human serum albumin (HSA) and oppositely charged poly-L-arginine (PLA) into a nanoporous PC membrane, with subsequent dissolution of the template. The building HSA components in walls preserved

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their original ligand binding ability to capture zinc (II) protoporphyrin IX (Znpp), leading to HSA-Znpp complex which can be used as a photosensitizer for hydrogen generation from water ¹⁴⁵. The fluorescence intensity of the Znpp solution incubated PLA/HAS nanotubes with either HSA or PLA as interior layer decreased significantly, demonstrating that the Znpp diffused into the nanotube walls and mainly bound to each HSA layer in nanotubes (Figure 2.15A,C). For practical applications, such as drug delivery and molecular separation, the bound ligands should be released from the nanotubes. The release of bound Znpp to the bulk aqueous solution was achieved by ligand replacement with myristic acid (MA) due to its stronger binding capability for HAS at the same position. Finally, the introduction of magnetic Fe_3O_4 particles into the outer surface of HSA nanotubes enables to efficiently recover these nanotubes (Figure 2.15B).

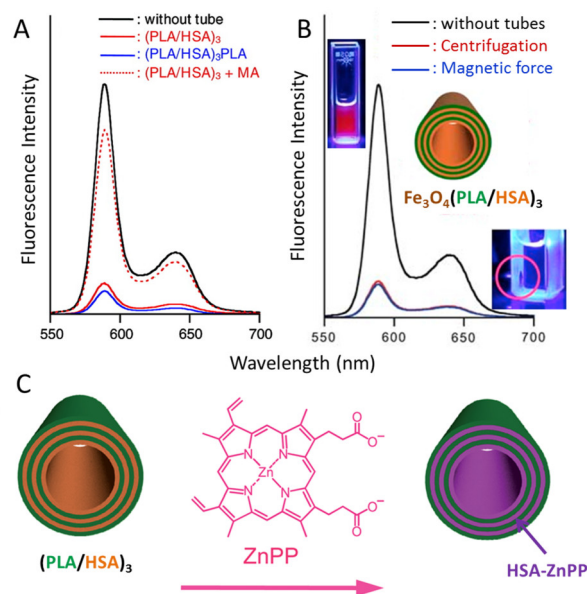


Figure 2.15. Fluorescence spectra of 15% DMSO/PB solution (pH 7.0, 10 mM) of Znpp (0.2 μM) after incubation of either (A) $(\text{PLA}/\text{HSA})_3$ nanotubes with subsequent centrifugation (4000g, 10 min) ¹⁴⁰, or (B) $\text{Fe}_3\text{O}_4(\text{PLA}/\text{HSA})_3$ magnetic protein nanotubes followed by collection using a magnetic field ¹⁴¹. Inserts in B show Znpp solution before adding $\text{Fe}_3\text{O}_4(\text{PLA}/\text{HSA})_3$ nanotubes and after incubation. Znpp-loaded magnetic nanotubes were collected on the cuvette wall using a small Nd magnet.

The elaboration of robust protein microtubes to capture *Escherichia coli* (*E. coli*, 425 nm width) for bioseparation was also reported by Komatsu *et al.*¹⁴². In this case, 9-bilayers deposition of PLA and HSA in 1.2 μm -pore PC membrane yields robust microtubes with *ca.* 1.0 μm outer diameter and a wall thickness of *ca.* 250 nm in aqueous solution, leaving an open channel of *ca.* 500 nm in diameter (Figure 2.16A). Compared to narrower (PLA/HSA)₃ nanotubes (internal pore size, *ca.* 200 nm), these larger (PLA/HSA)₉ microtubes with larger inner pore space present much more efficient trapping capability for *E. coli* (425 nm width) (Figure 2.16B). It is noteworthy that the successful entrapment of *E. coli* into (PLA/HSA)₉ microtubes results from the presence of HSA proteins in the interior layer, as (PLA/HSA)₈/PLA/Poly-L-glutamic acid sodium salt (PLG) and (PLA/HSA)₈/PLA/PSS microtubes with comparable size (outer/inner diameters and length) could not capture *E. coli* (Figure 2.16C). HSA can indeed, bind many bacterial species including *E. coli* by interacting with expressed surface proteins¹⁴⁶.

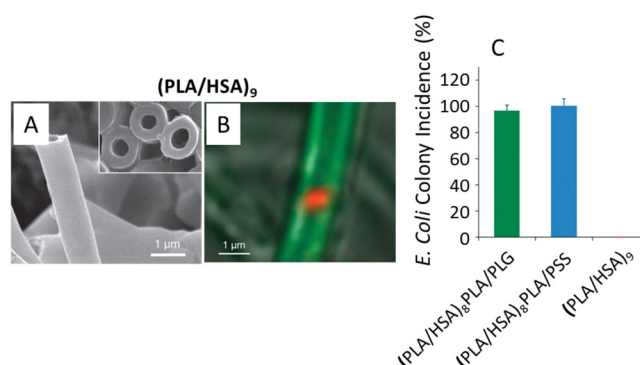


Figure 2.16. (A) SEM image of lyophilized (PLA/HSA)₉ microtubes and (B) overlapping image of DIC and CLSM of fluorescent microtubes capturing 5-cyano-2,3-ditoly tetrazolium chloride (CTC)-*E. coli*. (C) *E. coli* capture capability of protein microtubes with different inner surface layer. The *E. coli* dispersion was mixed with these different protein microtubes and incubated with gentle rotation at 25 °C for 30 min, respectively. Then a part of the mixture was spread on the LB agar plate and cultured at 37 °C for 16 h. The identical protocol was used for *E. coli* dispersion without the microtubes (control). The colonies appearing on the plate were used to compute the colony incidence, $N_C(\text{microtubes})/N_C(\text{control}) \times 100\%$. N_C : number of colony. Images adapted from ref. [142]

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Various functional components can also be deposited as the inner layer of the protein tube wall via LbL, providing a general way to create smart functional tubes and carriers with tailored properties for specific targets. For example, the enzyme, α -D-glucosidase (α GluD), was introduced as the interior layer of protein nanotube walls by electrostatic adsorption, resulting in protein nanotubes with a useful enzyme interior surface for biocatalysis¹³⁶. This type of tubular enzymatic nanoreactors can also be used as bubble-propelled nanomotors¹⁴⁷. Nanotubes bearing an avidin functional interior layer were used as tubular carriers for size-selective capturing biotinylated fluorescent nanoparticles (biotin-FNPs) via the strong avidin-biotin interactions (Figure 2.17)¹⁴⁰. Using the same protocol, a tubular trap for infectious viruses was achieved using antigen-antibody interactions¹⁴³.

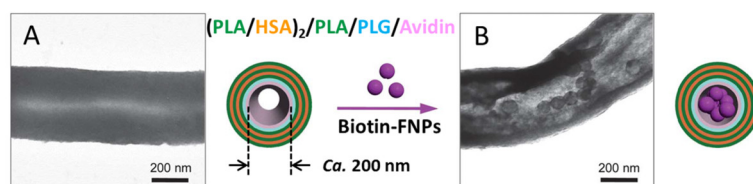


Figure 2.17. TEM images of $(\text{PLA}/\text{HSA})_2/\text{PLA}/\text{PLG}/\text{Avidin}$ nanotubes (A) before and (B) after incorporation of 100 nm biotin-FNPs. Images adapted from ref. [140]

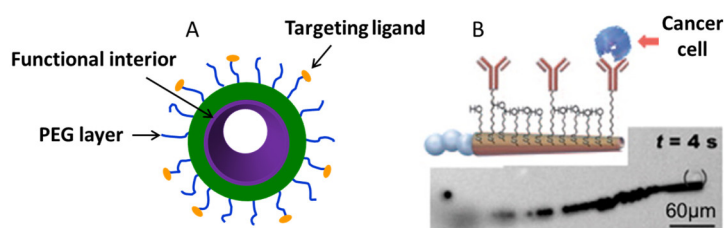


Figure 2.18. Schematic illustration of a typical multifunctional LbL tubes (A), and an example of selective isolation of cancer cells (B)¹⁴⁸. Image B extracted from ref. [149]

The concepts used to engineer the interior surface are also applicable to the outer surfaces, resulting in multifunctional tubes (Figure 2.18A). For instance, fabrication of tubular microrockets through functionalization with targeting ligands or self-assembled monolayers on the exterior surface for highly specific selection and isolation of nucleic acids¹⁵⁰, thrombin¹⁵¹ and cancer cells (Figure 2.18B)¹⁴⁸ was reported. The movement of these microtubes is driven by various forces associated with local catalysts immobilized on inner surface, such as Pt nanoparticles¹⁴⁸ and enzymes¹⁵².

Properties of the outer surface of tubes are particularly important when they are used as nanocarriers *in vivo*, because they determine how the tubes will interact with their surrounding environment. Rubner *et al.* showed that the orientation of a core-shell microtube on a cell surface can be controlled. For that purpose, they built tubes with cell-adhesive/resistant multilayers as either outer or inner layer¹⁵³. Microtubes with a cell-adhesive shell hyaluronic acid/chitosan (HA/CHI) and a cell-resistant PAA/PAH core attached preferentially side-on, while end-on attachment was preferred for microtubes having a reverse sequence of adhesive and resistant layers. This results from the fact that when a cell approaches the end of a tube, the cell-adhesive interior multilayers can be sufficiently exposed to participate in cell adhesion, although they are largely shielded from interaction with cells.

The presence of LbL shell made of natural polyelectrolytes improves the biocompatibility as well as the internalization into cells¹⁵⁴. Surface modification with highly hydrophilic poly(ethylene glycol) (PEG) provides antifouling properties which prevents proteins and serum adsorption on the outer surface of nanocarriers and ultimately facilitates their circulation. Our group has demonstrated the possibility of grafting PEG chains to the outer surface of PAH/PAA nanotubes by using PEG succinimidyl ester reagent¹⁵⁵.

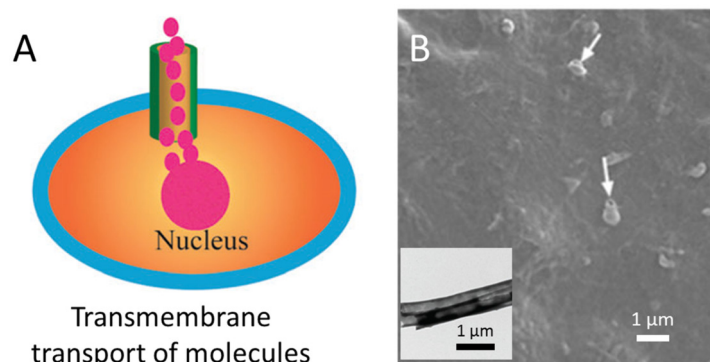


Figure 2.19. Schematic illustration (A) of penetrated protein nanotubes acting as a nanopore for transmembrane transport of molecules, and (B) SEM image of a cell with penetrated protein nanotubes. Arrows in B indicate the location of tubes on the cell surface. Insert in image B shows the tubular structure of obtained protein nanotubes. Images extracted from ref. [¹⁵⁶]

Cell penetration rather than full internalization of high aspect ratio nanotubes provides an elegant way for transmembrane mass transport, since they can act as an artificial nanochannel through cell membrane. Abe and co-workers¹⁵⁶ reported a model system for fabricating protein nanochannels allowing free translocation of molecules into cells (Figure 2.19) and also, offering the possibility of extraction of large protein complexes from cells.

2.3.2 Collection of template-synthesized nanostructures

This versatile templated-LbL assembly provides a general and facile way to fabricate various functional nano-objects. Once released from the template, these nanostructures can be functionalized and used as tanks or vehicles for diverse applications. Compared to the macroscopic flat films that can be simply used on top of 'open' substrates, LbL shell formed over the interior of nanospheres or LbL nanotubes/rods confined within the nanopores should be released by removal of sacrificial templates. For polymer templates (PS colloids or nanoporous PC membranes), they can be easily dissolved in organic solvent. Typically, for nano-objects structure observation by SEM or TEM, the samples can be simply prepared from the organic suspension. However, for their use as functional vehicles or reactors, they have to be recovered from templates and redispersed into an aqueous solution.

Centrifugation is a conventional method allowing the removal of dissolved polymeric templates from organic suspension. The aqueous suspension can be achieved by replacing the pure organic solvent with water when the organic solvent is miscible with water, such as DMF. Large centrifugation forces are, however, required to efficiently sediment nanosized objects. This may provoke the formation of irreversible aggregates. The first achievement of aqueous suspension of nanotubes was reported by Komatsu *et al.*¹⁴⁰ They used centrifugation to separate dissolved PC template from nanotube suspension in DMF, resulting in pure nanotubes/DMF suspension, followed by freeze-drying to yield lyophilized nanotubes aligned in a thin film (Figure 2.20A). These resulting pieces of film made of protein nanotubes can be further redispersed in water, however, to limited extent (nanotube aggregate in water, insert in Figure 2.20B), leading to a homogeneous but slightly turbid dispersion.

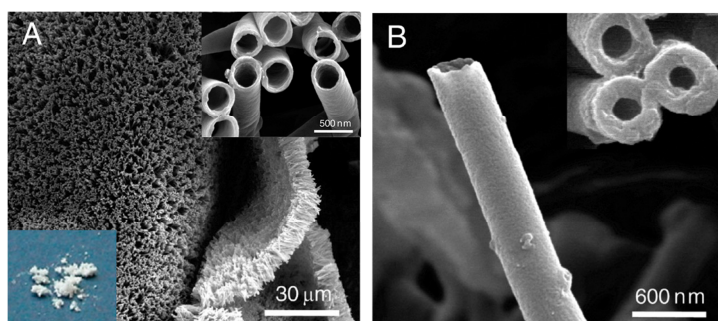


Figure 2.20. SEM images of (A) lyophilized powders (insert in A) comprising numerous arrays of oriented nanotubes and (B) lyophilized sample of aqueous dispersion of nanotubes. The nanotube/membrane was first dissolve in DMF to release embedded nanotubes from PC templates, followed by the separation of dissolved PC from liberated nanotubes in DMF. The obtained pure nanotube/DMF suspension was then freeze-dried *in vacuo*, yielding the lyophilized nanotubes as many small pieces of thin film (A). These small pieces of thin film were then dispersed in aqueous solution, giving a slightly turbid dispersion which was further frozen by liquid nitrogen and dried under a vacuum to reveal nanotube structure in water (B). Images adapted from ref. [¹⁴⁰]

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However, this collection method inevitably involves centrifugation, a process by which the PC polymer can not be completely separated from the nanotubes. Residual PC is still present in lyophilized nanotube powders, which is responsible for the turbidity of the aqueous suspension. Our group could not obtain dispersed PAH/PSS nanotubes aqueous suspension but only insoluble aggregates following this Komatsu's method, due to the presence of residual PC ¹⁵⁵.

The simplest way to collect templated nano-objects from suspension is filtration in which they are collected over membranes followed by sufficient rinsing. However, the critical point of this method is the transfer of nano-objects from the filter to aqueous solution without deformation or rupture. Rubner and coworkers ¹⁵³ used a cell scraper to rub the filter surface and release the filtration-collected microtubes in aqueous solution (Figure 2.21). In this case the short length (5 μm) and sturdy nature of thick microtubes are key features compatible with this approach, but this route is hardly applicable to soft LbL-nanotubes of high aspect ratio, as they are fragile and strongly attached to the surface of filters once collected over the filters ¹⁵⁷. Hence, a promising and universal collection strategy was developed in this work in order to recover soft nanotubes. This method will be discussed in Chapter 4.

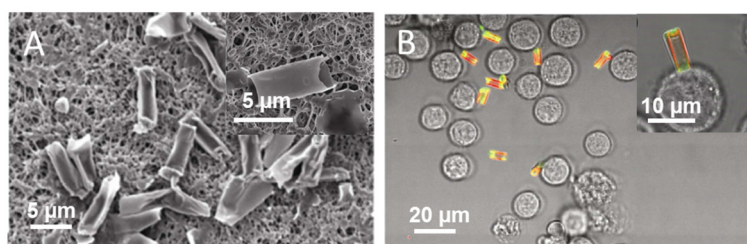


Figure 2.21. SEM images (A) of microtubes with $(\text{PAA}/\text{rhodamine-PAH})_{40}$ outside and $(\text{HA}/\text{FITC-CHI})_{40}$ inside on the surface of a syringe filter. Confocal images (B) of cell-microtube interaction study in aqueous solution after microtubes were transferred to aqueous solution by gently rubbing the filter surface with a cell scraper for 5 minutes in water. Images adapted from ref. [¹⁵³]

2.4 Nanofiber/tube mats

Over the past two decades, the role of nanoscale structural features of the extracellular matrix (ECM) in directing cells and tissues has been increasingly understood, leading to the rapid development of artificial nanomaterials with spatially nanofibrous architectures to mimic biophysical/chemical cues of natural ECM of *e.g.* fibrillar proteins¹⁵⁸. These structural features including fiber/tube composition, diameter, spatial arrangement, porosity and morphology can be manipulated via adjusting process parameters in network scaffold constructions.

Until now, several different strategies were employed to fabricate these nanofibrous frameworks, depending on the way fibers/tubes are formed. Usually, the network is formed during the production of the fibers, such as bacterial cellulose fiber production^{159,160}, spinning¹⁶¹⁻¹⁶⁵ and self-assembly of specific components¹⁶⁶⁻¹⁶⁸, in which cases a mat of fibers/tubes can be obtained directly when the formation of fibers/tubes are completed. However, there are also other routes to form mats of nanofibers/tubes using as-prepared nanofibers/nanotubes as building blocks. That means that nanofibers/tubes are prepared first by alternative methods, followed by the mat construction via an appropriate strategy, such as filtration of carbon nanotube dispersion over a nanoporous filter for a buckypaper¹⁶⁹, immobilization of nanofibers/tubes modified nanoporous templates onto a flat surface followed by the dissolution of templates, yielding a membrane of vertically aligned nanofibers/tubes¹⁷⁰, or layer-by-layer assembly of nanofibers/tubes¹⁷¹.

As a result of availability of various components comprising nanofiber/tube networks, the applications of these nanofiber/tube frameworks have been extended to diverse fields^{159,170,172-174}, *e.g.*, sensing, energy, and purification.

2.4.1 Electrospinning

Electrospinning is currently well established and is a versatile way to produce nanofibers/tubes compatible with a number of synthetic and natural polymers. The electrospinning process involves the extrusion of a polymer solution from an electrically conductive spinneret to which an appropriate voltage is applied. The electric potential within the polymer solution can overcome the surface tension of the droplet formed when the polymer

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solution is ejected from the spinneret. The solvent is evaporated and, the polymer fibers are collected onto the grounded target, resulting in an unweaved fibrous polymer mesh with typical fiber sizes ranging from a hundred nanometers to a few microns in diameter depending on the polymer solution characteristics and electrospinning parameters.

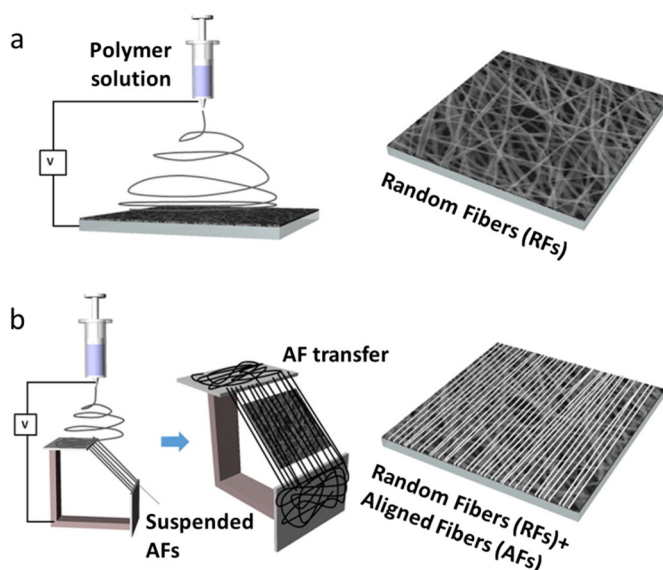


Figure 2.22. Schematic illustration of the scaffold fabrication procedure. (a) Typical electrospinning process to create underlying random fibers. (b) Transfer of aligned fibers onto random fibers. Images extracted from ref. [¹⁷⁵]

Electrospun fibers are often randomly aligned due to the inherent whipping of the fiber jet as the polymer is ejected from the spinneret (Figure 2.22a). To obtain anisotropic films containing well-aligned fibers, different grounded collectors were developed. A highly rotating mandrel is commonly used to align fibers parallel to the direction of rotation¹⁷⁶. The behavior of the oriented fibers towards cells has been revealed in terms of cell morphology, proliferation and cytoskeletal organization, showing a huge potential of this “ideal” scaffold for tissue engineering^{177,178}.

More complex three-dimensional structures were also built by this technique. For example, Mauck and coworkers¹⁷⁹ constructed a bi-lamellar scaffold composed of bilayers of nanofibers presenting different alignment relative to

the long axis of the scaffold. The second aligned nanofiber layer was collected on the previously prepared aligned scaffold which was rotated by 30 degrees with respect to the collected mandrel. These anisotropic scaffolds can direct the deposition of an organized, collagen-rich extracellular matrix that mimicks the angle-ply, multi-lamellar architecture and achieve mechanical parity with native tissue after 10 weeks of *in vitro* culture. Very recently, another comprehensive method of creating hybrid electrospun nanofiber scaffold for tissue engineering was proposed^{175,178}. In this method, a special collector was set up as two separate conductive strips arranged perpendicularly with a void gap. The as-spun nanofibers were suspended between two strip edges and transferred onto random aligned electrospun nanofiber scaffold (Figure 2.22b). The lower random nanofiber layer of hybrid scaffold has mechanical stability, serving as supports, while the top aligned nanofiber layer was used to control the cell alignment and differentiation.

Desirable blending of polymers with biomolecules of interest in the initial formulation can lead to the entrapment of functional components within the entire fibrous architecture. This approach has been used to prepare enzyme nanofibrous membranes for biocatalysis^{180,181} and protein nanofiber scaffolds containing nerve growth factors for controlled release¹⁸². In contrast to the homogeneous distribution of functional biomolecules in co-electrospun fibers, the co-axial electrospinning provides a general way to produce core-shell nanofibers. This technique allows independent control of the central composition and the surface chemistry. The post-grafting of interesting biomolecules can also be used to functionalize electrospun nanofiber scaffolds with correlated improvement in long-term retention of functional components.

However, the formation of bead-free electrospun nanofibers is not always possible, since it requires extensive polymer chain entanglement within the initial polymer solution. Consequently, it is often difficult to electrospin low molecular weight polymers. The choice of adequate solvent is the most important processing parameter to yield nice nanofibers. The solvent must not only dissolve the polymers, but also evaporate before jets arrive at the grounded collector. Therefore, harsh organic solvents are often required, which probably cause structural changes of entrapped biomacromolecules¹⁸³⁻¹⁸⁵.

2.4.2 Buckypaper

Carbon nanotubes (CNTs) have outstanding electronic, thermal and mechanical properties¹⁸⁶, which make them attractive one-dimensional nanomaterials, with promising applications in various fields, *e.g.*, reinforcing composite materials, nanoelectronics^{187,188}, biosensor^{189,190} and biofuel cells¹⁹¹⁻¹⁹³. A thin sheet of compressed CNTs is called buckypaper which has high chemical and electrochemical stability and excellent electrical conductivity, usually serving as conducting nanomaterial for electron transfer. For example, buckypaper containing enzymes can be used as electrodes assembled in a biofuel cell. In this case, CNTs are considered as conducting wires to efficiently transfer electrons from the active site of enzymes and establish persistent current. These bioelectrodes are fabricated by either post-modification of as-prepared pure CNTs buckypaper with enzymes or by using a homogeneous composite of enzymes and CNTs.

Typically, the buckypaper is made on basis of membrane filtration of CNT dispersion where surfactants are required to improve their dispersibility in aqueous solution¹⁹⁴⁻¹⁹⁶. Recently, Tsarfati¹⁹⁷ demonstrated an efficient mean to individualize, disperse, and stabilize CNTs in various media. Perylene diimide (PDI) amphiphiles were non-covalently immobilized onto single wall CNTs via the formation of micellar structures with CNTs at their core, resulting in long-term stable aqueous/organic CNTs dispersion from which a hybrid buckypaper was prepared and used for size-selective separation of gold nanoparticles (Figure 2.23). An alternative approach involves a frit compression process to prepare a buckypaper without the requirement of surfactants or CNT surface modification¹⁹⁸. Enzymes can then be immobilized onto CNT buckypaper by post-modification via either chemical or physical interactions, resulting in mechanically stable biocatalytic electrodes for biofuel cells^{195,199-203}. For example, glucose oxidase and laccase were immobilized onto CNT buckypaper as bioanode and biocathode, respectively, by using a heterobifunctional cross-linker, 1-pyrenebutanoic acid succinimidyl ester (PBSE), which provides covalent binding with amino groups of protein lysine residues through the formation of amide bonds and interacts with CNTs via π - π stacking of the polyaromatic pyrenyl moieties²⁰¹⁻²⁰³.

Besides the post-modification of buckypapers with enzymes, Cosnier and coworkers²⁰⁴ reported the fabrication of three-dimensional bioelectrodes by

mechanical compression of homogeneous composite of enzymes (glucose oxidase or laccase), CNTs and small amount of water. The compression of enzymes and CNTs mixture likely favors a close proximity at the nanoscale level between nanotubes and prosthetic sites of entrapped enzymes, leading to efficient direct electron transfer between enzymes and CNTs matrix, resulting in an excellent biofuel cell.

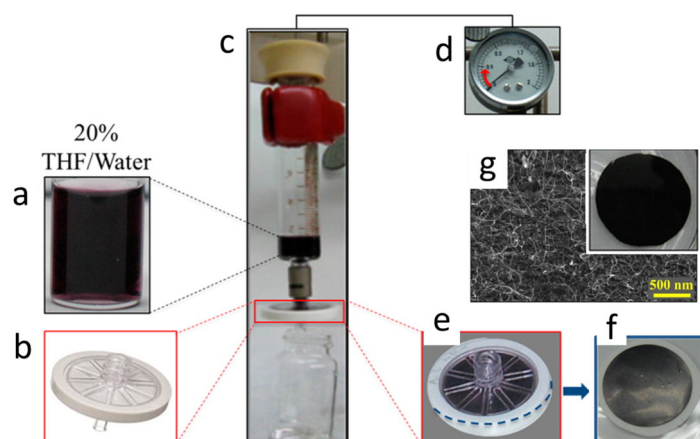


Figure 2.23. Hybrid PDI/CNT buckypaper fabrication. (a) Aqueous dispersion solution of carbon nanotubes is filtered over a cellulose acetate (CA) support (b) with assistance of upstream pressure (c,d) to form a deposited layer on the CA syringe filter (e), which is opened to give rise to CA-supported buckypaper (f). The deposited membrane was released by acetone immersion and then analyzed by SEM (g). Insert in g: self-standing hybrid PDI/CNT buckypaper. Images adapted from ref. [197]

2.4.3 Mats of LbL nanotubes

The architecture built from LbL nanotubes has been demonstrated in terms of nanotube arrays. Tubular LbL-structures shaped by the nanochannels within templates were anchored onto a support, followed by the removal of sacrificial templates, yielding nanotube forests. The first example was given by Chia who constructed a pH-responsive reversibly swellable (PAH/PAA)₂₀ nanotube arrays by immobilization of PAH/PAA modified porous PC membranes on a positively charged glass substrate via electrostatic interactions²⁰⁵. The unattached side of the membrane was then plasma etched in oxygen to selectively remove the crust film deposited on the

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membrane surface prior to dissolution in CH_2Cl_2 . Protein nanotube arrays immobilized on solid substrates were also proposed by Komastu¹³⁹ for molecular trap in aqueous medium. In that case, crust films on both surfaces of hybrid PC membranes were eliminated prior to nanotube immobilization. The PC membrane containing protein nanotubes was glued onto a cover glass using epoxy resin, which was precoated onto the glass top, preventing insertion into the nanotubes. Both immobilization approaches lead to the formation of tube brushes, as shown in Figure 2.24.

However, due to the distance between the pores in the template, the vertically aligned nanotubes on supporting substrate have limited contact with their neighboring nanotubes when the template is dissolved (Figure 2.24). These nanotube arrays form a very fragile film which can not be detached entirely from the supporting substrate. Consequently, no self-standing film of aligned nanotube arrays could be obtained.

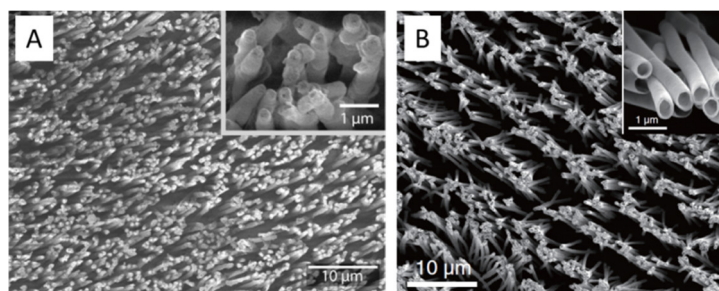


Figure 2.24. SEM images of $(\text{PAH/PAA})_{20}$ (A) and $(\text{PLA/HSA})_2\text{PLA/PLG/Avidin}$ (B) nanotube arrays. Images adapted from ref. [139,205]

Recently, a promising membrane of LbL nanotubes¹⁷⁰ was achieved for ionic separations following similar protocols. In this work, a PC membrane containing nanotubes was immobilized onto NaOH-treated wet porous polyacrylonitrile (PAN) support upon drying in an oven at 80 °C rather than using glue before being immersed in CH_2Cl_2 to dissolve the PC framework. Then, authors went a step further by depositing another type of functional nanotubes onto the first prepared nanotube array, followed by the formation of the sealing layer on top of mixed nanotubes (Figure 2.25). Interestingly, the sequential deposition of nanotubes leads to well-arranged tubular

elements without overlap, producing a densely-packed mixed nanotube arrays.

Due to the high density of aligned nanotubes, contacts occur among neighboring nanotubes, as well as, the LbL on the top to hold them together, possibly leading to an intact free network of nanotubes.

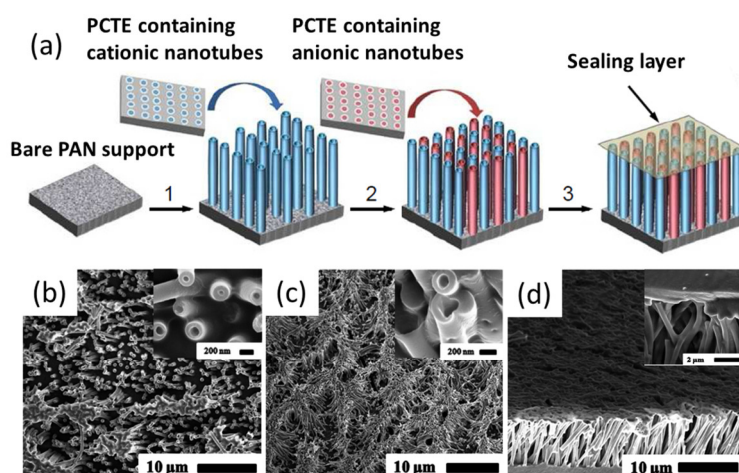


Figure 2.25. (a) Schematic representation of the steps to construct a mixed LbL-nanotube membrane and corresponding SEM images of (PAA/PAH)₁₂PSS nanotubes (b) deposited on PAN support followed by deposition of (PAA/PAH)₁₂PAA/PEI (c) and then final (PAH/PAA)_{3.5} sealing layer (d). Images adapted from ref. [170].

Another way to collect LbL-nanotubes on a substrate to form nanotube biointerfaces was demonstrated by Demoustier and coworkers²⁰⁶⁻²⁰⁸. They used electrophoretic deposition (EPD) to recover nanotubes from suspension after the PC membrane was dissolved in DMF (Figure 2.26), resulting in randomly positioned nanotubes on ITO substrate. However, a full coverage of nanotube on ITO glass was not achieved (Figure 2.26C). The density of collected nanotubes on ITO depends on the concentration of nanotubes in suspension, applied voltage and deposition time. In order to form an intact film of nanotubes on ITO glass, a large amount of nanotubes has to be collected, which requires a high concentration of nanotubes in suspension and a continuous feed of fresh suspension, as well as a high

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applied voltage. This approach might thus have limitations for the fabrication of microscaled nanotube mats.

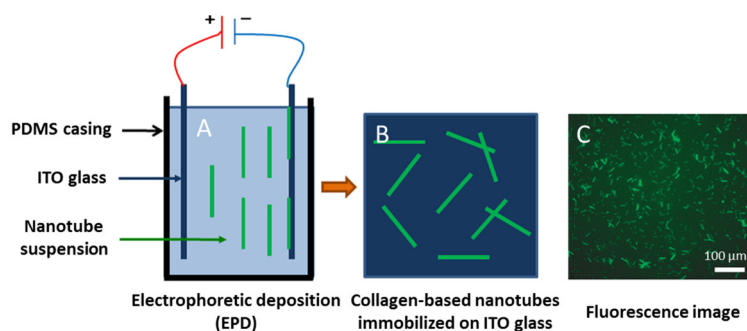


Figure 2.26. Schematic representation of (A) electrophoretic deposition strategy and (B) collected nanotubes on ITO glass from nanotube suspension, and (C) corresponding fluorescence image of collected FITC-tagged protein nanotubes on ITO glass. Images adapted from ref. [206,207]

A serendipitous finding during the collection of nanotubes in Chapter 4 enables us to fabricate mats of LbL nanotubes by 'buckypaper' strategy, involving simple filtration and sedimentation of liberated nanotubes onto a filter, providing a general access to multilayered biofunctional mats of template-synthesized nanostructures. Compared to electrospun fibers, LbL assembly allows the facile incorporation of various biomacromolecules without degradation in large amount. The filtration strategy has extremely high efficiency in collecting nano-objects from suspension. Therefore, the combination of LbL technology and filtration would be an inexpensive and universal method to construct biofunctional nanopapers. The limitation of this methodology perhaps results from the long fabrication process, comprising LbL-nanotube buildup and filtration of nanotube suspension. This work will be presented in Chapter 5.

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Chapter 3

Fabrication of robust enzyme nanotubes by layer-by-layer assembly

In this chapter, we investigated the construction of enzyme layer-by-layer (LbL) films by alternated deposition of polycation with glucose oxidase (GOX) enzyme, on planar substrates and on the nanochannel surfaces within polycarbonate (PC) membranes. The assembly conditions were optimized in order to obtain free sturdy enzyme-based nanotubes. The catalytic activity of those intact free nanotubes was then compared to membrane-embedded nanotubes. Moreover, a quantitative FTIR analysis of these nanotubes was also performed, providing insight into the internal composition of LbL films assembled in confined medium.

3.1 Introduction

In natural biochemical processes, enzymatic reactions often occur in the spatially confined environment of extremely small compartments, resulting in an improved specificity and activity¹. This inspired the synthesis of artificial nanostructures for performing reactions in confinement; different morphologies were developed, varying from spherical to tubular, such as nanoporous organic crystalline hosts², micelles and vesicles, or nanocapsules³⁻¹⁰ and nanotubes¹¹⁻¹⁵, enabling confinement effects to be experimentally studied with these synthetic analogues.

Among different possible building components¹⁶⁻²², biomacromolecules are especially interesting for inclusion in such biomimetic nanostructures. In particular, protein-based nanotubes, which present several advantages over nanospheres²³, are attracting an increasing interest because of their potential applications in various fields, for instance as drug nanocarriers²⁴, as enzyme-powered micromotors for water-quality testing¹¹, as biochannels¹³ or bioreactors¹⁴, or as bioanodes for biofuel cells²⁵. Compared to free enzymes, nanotubes may offer a protective shell to stabilize enzyme, and may substantially ease the recovery of enzymes after use. Thanks to the mild adsorption conditions of layer-by-layer (LbL) assembly^{23,26}, various protein-based tubular nanocylinders have been achieved via the assembly of two different oppositely charged proteins and/or counter-polyelectrolytes²⁷⁻³⁴ in nanoporous membranes, such as anodic aluminum oxide (AAO) or track-etched polycarbonate (PC) membranes, since the first example of enzyme nanotubes was reported by Martin *et al.* in 2005¹⁵, in which glucose oxidase (GOX) was used to be self-assembled with an interlayer cross-linker, glutaraldehyde, in AAO templates. After dissolution of the membrane template, nanotubes are obtained, somehow reminiscent of natural protein cages or viral capsids³⁵. Most importantly, proteins complexed with polyelectrolytes retain their secondary structure, and consequently exhibit high biological activity. Among these protein-containing LbL nanotubes, enzyme-based nanotubes are expected to be promising artificial agents for biocatalysis.

Glucose oxidase is a robust enzyme with a readily available substrate, hence a good model candidate to explore. However, owing to the complexity of protein structure and the patchy nature of their surface charge distribution, the assembly of proteins with oppositely charged polyelectrolytes, which

utilizes electrostatic interactions, is not always possible^{13,36,37}. For instance, only branched polyethyleneimine (bPEI) or poly-L-arginine (PLA) could be combined with human serum albumin (HSA) to produce stable nanotubes by LbL deposition in PC templates²⁹, while chitosan (CHI) works well when paired with β -lactamase³⁸. For GOX enzyme, some attempts regarding construction of GOX multilayer films have been conducted on flat surfaces or on colloid particles^{9,37,39-43}, in which various polycations were tested. Kunitake^{37,42} investigated the assembly of GOX with polycations in pure water by quartz crystal microbalance (QCM) and found that bPEI/GOX films grew much faster than films where GOX was paired with poly(dimethyldiallylammonium chloride) (PDDA) in which case the previously adsorbed GOX was moderately removed by following PDDA deposition. Similarly, heavy release of immobilized GOX by subsequent poly(allylamine hydrochloride) (PAH) was observed by Caruso⁹. Chitosan, a naturally derived polycation, has been commonly employed in the assembly of LbL films owing to its excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties, etc. Li⁴⁴ has described the construction of Biodegradable nanotubes through alternated adsorption of alginate (ALG) and chitosan (CHI) onto the inner pores of polycarbonate template. Moreover, LbL self-assembly of CHI/GOX nanolayered film has been reported⁴⁵. Hence, chitosan may be an attractive candidate as polycation for generating biocompatible LbL enzyme nanotubes for bio-applications.

Therefore, in the present study, chitosan and branched polyethyleneimine were explored as polycations to be assembled with GOX. Herein, buffer was used in GOX depositions to minimize the degradation of enzyme during the long-time buildup process rather than pure water always used in most previous publications. A variety of assembly conditions such as nature, concentration and pH of buffer, as well as, the medium used in rinsing steps (pure water or buffer) were first tested for LbL assembly on flat surfaces, providing useful fundamental information for the further fabrication of enzyme nanotubes in nanoporous PC template. In this way, robust enzyme nanotubes of high enzymatic activity, able to survive detemplating process were successfully synthesized.

3.2 Experimental section

3.2.1 Materials

Glucose oxidase (GOX from *Aspergillus niger*, type x-s, lyophilized powder), horseradish peroxidase (HRP, type VI, lyophilized powder), branched polyethyleneimine (bPEI, M_n 60,000 g·mol⁻¹, M_w ca. 750,000 g·mol⁻¹, 50 wt. % in H₂O), *o*-dianisidinedihydrochloride, 4-morpholineethanesulfonic acid (MES), acetate acid, acetate sodium (AC) and glucose were purchased from Sigma-Aldrich. Linear polyethyleneimine (lPEI, M_w 100,000 g·mol⁻¹) and chitosan (CHI, degree of deacetylation (DD) > 90%, M_w ca. 270,000 g·mol⁻¹) were obtained from Polysciences and Novamatrix, respectively. 2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulfonic acid (HEPES free acid), potassium bromide (FT-IR grade, ≥ 99%) and dichloromethane (99.8%, for analysis) were purchased from Acros Organics.

All polyelectrolytes and GOX solutions were freshly prepared at a concentration of 1.0 mg/ml either in water or in buffer at desired pH (pH range, 5.0-10.0). In these conditions, GOX has a negative charge given an isoelectric point of 4.2^{37,39,40}, and PEI is positively charged^{46,47}. The pH of chitosan solution is kept below 6.5, since precipitation occurs when pH exceeding 6.5 due to its low solubility dependent on the charge degree of lateral amine group⁴⁸. The molecular structures of branched polyethyleneimine and chitosan are shown in Figure 3.1. Ultrapure Milli-Q water (Millipore) with 18.2 MΩ·cm resistivity was used in all experiments. Single-side polished silicon wafers and track-etched polycarbonate (PC) membranes of 300 nm average pore diameter, 25 μm thickness and 1×10⁸ cm⁻² pore density, were used as templates, for LbL deposition, whereas poly(ethylene terephthalate) (PET) membranes of 200 nm average pore size, 23 μm thickness and 5×10⁸ cm⁻² pore density, were used as filters to collect nanotubes for scanning electron microscopy (SEM); both types of membranes were provided by the it4ip company, Louvain-la-Neuve, Belgium (<http://www.it4ip.be>).

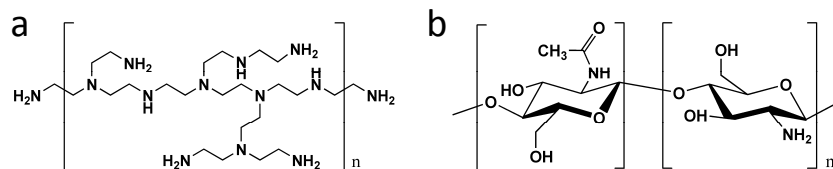
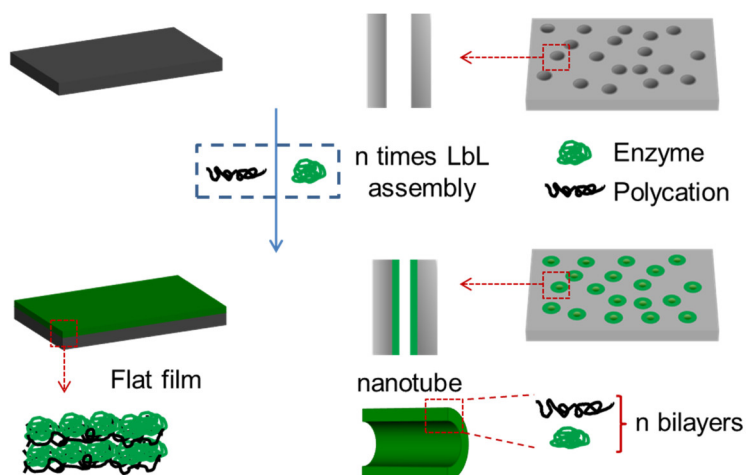


Figure 3.1. Molecular structures of branched polyethyleneimine (a) and chitosan (b) used in this chapter. Chitosan is produced by partial deacetylation of chitin; m is the degree of deacetylation (DD).

3.2.2 LbL assembly

Herein, silicon and nanoporous polycarbonate membranes were used as templates for planar and tubular structures, respectively. The representative buildup is shown in Scheme 3.1.

Briefly, for flat substrates, prior to alternated adsorption of GOX and polycation, the silicon was cleaned by immersion in a freshly prepared piranha solution [2:1 mixture of concentrated sulfuric acid (VWR BDH Prolabo, Leuven, Belgium) and 30% hydrogen peroxide solution (VWR BDH Prolabo, Leuven, Belgium)] for 30 min to remove contaminants as well as to maximize the negative charge density on surfaces, followed by rinsing with water and then blown dry by Ar gas. (Caution: “piranha” solution reacts violently with many organic materials and should be used with extreme care). The silicon surface was slightly negatively charged after treatment. The multilayer films were assembled manually through the repeated sequential dipping of silicon substrates into polyanion and polycation solutions with rinsing between polyelectrolyte depositions. In this hand-dipping procedure, the silicon substrates were firstly placed in a polycation solution for 15 min, followed by two rinses of 2 min each using the buffer or water which was used for adsorption solutions. Substrates were then transferred into the GOX solution following the same protocol used for the assembling of the previous layer to finalized one cycle, defined as one bilayer. At the end of the buildup process (when the desired number of bilayers was reached), the multilayered films were washed by pure water to remove the buffer salt entrapped in film when buffer was used for the polyelectrolyte deposition, followed by drying under compressed nitrogen flow.



Scheme 3.1. Illustration of the fabrication process of flat LbL films and tubular nanostructures by using silicon substrates and nanoporous PC templates, respectively.

For track-etched nanoporous polycarbonate (PC) templates, a piece of PC membrane was first dipped in a polycation solution (track-etched nanoporous PC membrane has negative surface charges because of negatively charged carboxyl groups created on the pore surface during etching process; in addition, hydrophobic interactions also contribute to the adsorption of the first layer), immediately sonicated for 15 min to ease transportation of polycation into the pores, then allowed 30 min for adsorption; this was followed by two rinses of 3 min each in the same but polyelectrolyte-free aqueous solution as used for polyelectrolyte adsorption. The polycation-modified porous PC membrane was then immersed in the GOX solution following the same protocol as used for adsorbing the polycation layer. After each GOX deposition, the polyelectrolyte crust simultaneously deposited on the top and bottom surfaces of the membrane was removed mechanically with a cotton swab. When buffer was used throughout the buildup process, the nanotube-modified membrane was rinsed at the end of entire process in pure water for 1 min to remove the buffer salt, followed by a final decrusting on the top and bottom surfaces with a cotton swab to make sure the surface films were eliminated completely. An identical final decrusting treatment was carried out for the samples with intervening rinses in water. All depositions were performed manually at

ambient temperature. No drying step was performed during the whole procedure until the desired number of bilayers was obtained. The prepared samples were stored at 4 °C in the dry state in a fridge, and were used within 2 days for activity measurements.

3.2.3 Spectroscopic ellipsometry

The growth evolution of LbL planar films on silicon substrates was monitored by spectroscopic ellipsometry (Uvisel, Horiba-Jobin-Yvon) via measuring the thickness in dry state. The measurement was performed at an incident angle of 67.2° in a wavelength ranging from 300 to 800 nm at 4 nm resolution. At least three measurements were performed on each sample to give an average thickness.

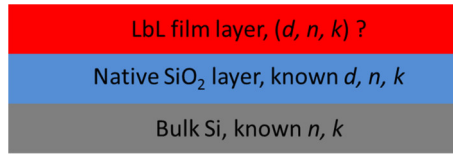


Figure 3.2. A three-layer optical model used in ellipsometry data analysis.

The collected data were analyzed by using the built-in DeltaPsi 2 software with a three-layer model (Figure 3.2). In this model, the complex index of refraction ($n+ik$) of bulk Si and SiO₂ were taken from the build-in database provide by the manufacturer. The thickness (d) of the native SiO₂ was measured firstly by ellipsometry on the bare wafers just after piranha solution treatment. 1.5 nm was determined by analyzing the exclusive parameter d_{SiO_2} , on the basis of two layered optical model (without polymer layer on top). The SiO₂ thickness, d_{SiO_2} was fixed at 1.5 nm during the fitting of LbL film layer in three-layer model where the LbL film layer is assumed to be a sole unknown, but homogenous and isotropic layer. The complex index of refraction and the thickness of LbL film were then modeled by using the Cauchy dispersion expressed as follow:

$$n(\lambda) = A + \frac{B 10^4}{\lambda^2} + \frac{C 10^9}{\lambda^4}, k(\lambda) = D 10^{-5} + \frac{E 10^4}{\lambda^2} + \frac{F 10^9}{\lambda^4} \quad (3.1)$$

Where n , k are the refractive index and extinction coefficient, respectively, and A, B, C, E, D, F, G are Cauchy coefficients that can be determined by

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fitting the functions to measured complex indices of refraction at each wavelength (λ). In most cases, dielectric materials are transparent in visible spectral wavelengths and become absorbing in the higher UV range. Therefore, a simple Cauchy transparent model was used in visible range (560 nm to 800 nm) by assuming the $k(\lambda) = 0$.

During the analysis of ellipsometric data, the refractive index of LbL polymeric film at 623.8 nm ($n_{623.8\text{ nm}}$, 1.45-1.60⁴⁹⁻⁵³) was monitored and used to determine the validity of the fitting result. Sometimes, the false thickness from fitting was obtained, since the refractive index at 623.8 nm from fitting was far away from the typical value 1.45-1.60. In such a case, the refractive index of LbL film was assumed to be constant within 1.45-1.60 (this value was selected according to the successful fitting case) during the fitting to validate the resultant thickness.

3.2.4 Atomic force microscopy (AFM)

The topography and thickness measurements of LbL films were performed using a commercial AFM instrument (NanoScope VIII MultiMode AFM, Bruker). The enzyme LbL film is essentially soft; hence, images were recorded using peak force tapping mode (PeakForce QNM) with controlled force. It is performed with ScanAsyst-air probe (Bruker) under 1-2 nN of force at a scan rate of 0.5 Hz in air at room temperature (20 °C). For thickness measurement, a scratch line was made by prep blades to uncover the silicon substrate. Both film and exposed silicon substrate were revealed in one image; the thickness of film was calculated by using histogram drawn over 20x20 μm^2 images after silicon substrate area flattening (film area excluded) using 1st order polynomials in a line-by-line type in X direction. Root-mean-square (RMS) roughness was reported for 10x10 μm^2 images after flattening the whole image by using zero order polynomials in a line-by-line type in X direction. All acquired data were analyzed by home-made program.

3.2.5 Gas flow porometry measurements

LbL growth on nanopore walls was monitored by measuring the evolution of the average pore diameter with the number of deposited bilayers. This was performed by using gas flow porometry at room temperature on air-dried

samples. Prior to measurements on samples, tests on the virgin membrane were conducted, and the flow value was used as reference for normalization, assuming a pore diameter of *ca.* 300 nm (reported by *it4ip*). The measured pore diameter for each sample was an average of at least 9 measurements on different spots. Details regarding the setup and analysis were reported in a previous publication⁵⁴. Briefly, nitrogen gas with a known pressure (10^4 - 2×10^5 Pa) was applied on the upside of membrane with an effective area of 0.138 cm^2 . The gas flow (mL/min) passing through the sample was then recorded by a flowmeter (Agilent), giving the average pore diameter computed by using Knudsen diffusion flux, J_{diff} , $\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (3.2) combined with Hagen-Poiseuille flow, J_{visc} , $\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (3.3), which together model a steady laminar flow of an incompressible fluid through a rigid-walled tube of constant radius.

$$J_{diff} = \frac{4d}{3l} \frac{(P_{up} - P_{down})}{\sqrt{2\pi MRT}} \quad (3.2)$$

$$J_{visc} = \frac{d^2}{l} \frac{(P_{up}^2 - P_{down}^2)}{6 \mu RT} \quad (3.3)$$

Where d is the pore diameter (m), l is the thickness of the membrane (m), P_{up} and P_{down} are the pressure (Pa) upstream and downstream of the membrane, respectively, M is the gas molar mass weight ($\text{kg} \cdot \text{mol}^{-1}$), μ is the dynamic viscosity of the gas ($\text{kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$), R is the ideal gas constant ($\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$) and T is the gas temperature (K). The total volume flow rate Φ ($\text{m}^3 \cdot \text{s}^{-1}$) from equation 3.2 and 3.3 are expressed by equation 3.4, where S is the effective contact area of the membrane (m^2), P_{atm} is the atmospheric pressure (Pa) and P is the transparency of the membrane, defined by equation 3.5, in which N is the pore density (m^{-2}).

$$\Phi = SP \frac{RT}{P_{atm}} (J_{diff} + J_{visc}) \quad (3.4)$$

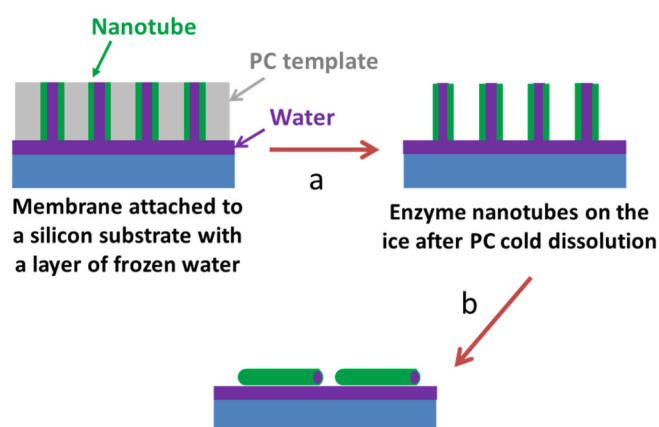
$$P = N \frac{\pi d^2}{4} \quad (3.5)$$

3.2.6 SEM and STEM characterization of enzyme nanotubes

The morphology of the enzyme nanotubes was evaluated with a field emission scanning electron microscope (JSM-7600F, JEOL Ltd.), equipped with a transmission detector, operated at 15 and 30 kV for the scanning mode (SEM) and for the scanning transmission mode (STEM), respectively.

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To observe the nanotubes collected from PC/CH₂Cl₂, the samples for SEM were prepared by filtration of the nanotube suspension in PC/CH₂Cl₂ through PET membranes covered by 20 nm of gold (Cressington Sputter Coater 208HR), and then rinsed several times by pure CH₂Cl₂ prior to observation; for STEM, carbon-coated copper grids (200 mesh, Electron Microscopy Sciences) were placed in the nanotube suspension of PC/CH₂Cl₂ and agitated gently for 1 min, then washed by fresh CH₂Cl₂ and air-dried at room temperature. In another approach to prepare samples for STEM observation (Scheme 3.2), the nanotube-filled PC template was floated over a thin layer of water cast on a Si wafer, left to impregnate for 30 min, then placed at -20 °C until water crystallized. Subsequent dissolution by CH₂Cl₂ of the PC membrane (three immersions in cold CH₂Cl₂ at -20 °C) followed by ice melting, yielded a thin film of nanotubes floating over water, which was transferred to a carbon-coated copper grid and dried at ambient temperature.

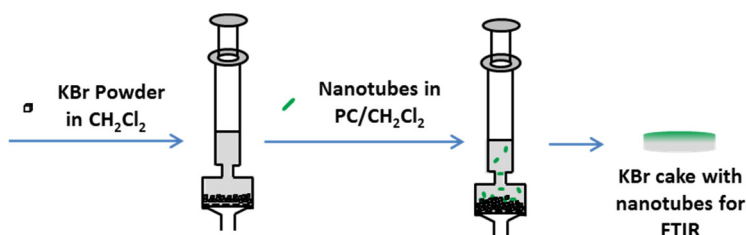


Scheme 3.2. Schematic illustration of the liberation process of enzyme nanotubes from a PC template for STEM observation, using an ice collection route. (a) Cold dissolution of the PC framework in CH₂Cl₂. (b) Ice melting at room temperature. The tubes are subsequently transferred to a TEM grid.

3.2.7 FTIR measurements and analysis

Fourier transform infrared (FTIR) spectra were collected at 8 cm⁻¹ resolution (128 passes) on a FTIR spectrometer (Nicolet Nexus 870, Thermo Scientific). For free glucose oxidase, polyethyleneimine and their mixtures, and the tube thin mat obtained by ice-immobilization approach, a standard KBr disk was

prepared, except when the product was a liquid, in which case it was simply deposited over an IR-transparent support (ZnSe crystal), recorded by Continuum microscope (Nicolet) under transmission model. For (bPEI/GOX)_n films or GOX adsorbed on silicon substrates, the spectra were recorded directly in transmission model with freshly prepared bare silicon as background, whereas Infrared reflection-absorption spectroscopy (IRRAS) was exploited to detect the layer of bPEI or GOX adsorbed on the Au-coated silicon with the help of smart SAGA accessory. For template-synthesized nanotubes, the samples were prepared by filtration on a PET membrane as follows (Scheme 3.3). A finely-crushed KBr powder suspended in CH₂Cl₂ was first filtered on the nanoporous PET membrane. Then the nanotube suspension in PC/CH₂Cl₂ was filtered gently over previously generated KBr cake to separate the PC from the nanotubes. The KBr powder with entrapped nanotubes was collected after rinsing with pure CH₂Cl₂ and dried at 60 °C overnight, and used to prepare a KBr disk containing nanotubes for FTIR measurement.



Scheme 3.3. Collection of nanotubes for FTIR by KBr- assisted filtration.

For the quantitative analysis of bPEI/GOX nanotubes, the raw spectra of pure bPEI and GOX were first recorded, and used to fit the spectra of a series of mixtures of bPEI and GOX prepared with a known ratio by weight, according to

$$A_{\text{mixture}} = F_{\text{bPEI}}A_{\text{bPEI}} + F_{\text{GOX}}A_{\text{GOX}} + F_{\text{KBr}}A_{\text{KBr}} + a + bk + ck^2 + dk^3,$$

where A_{mixture} , A_{GOX} , A_{bPEI} and A_{KBr} are the absorbances of the mixture, pure bPEI, GOX and KBr, respectively, F_{GOX} , F_{bPEI} and F_{KBr} are the corresponding scale factors, and the polynomial expression of third order in the wavenumber (k) is the baseline.

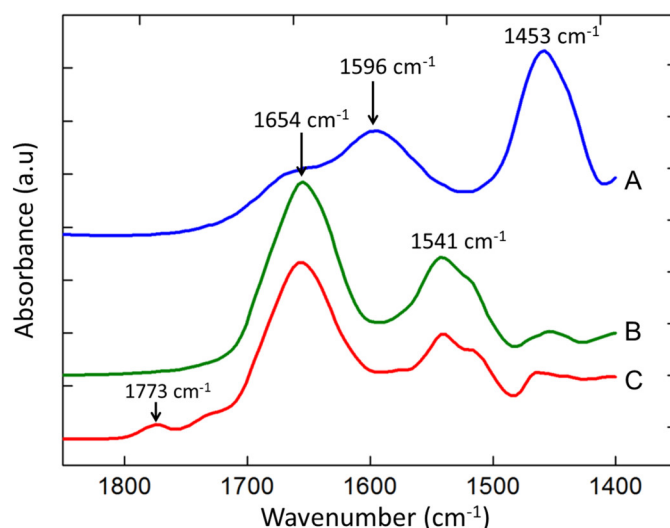


Figure 3.3. FTIR spectra of pure bPEI (A), GOX (B), and (bPEI/GOX)₆ nanotubes (C) assembled in 10 mM HEPES buffer (pH 7.0). The preparation of nanotube sample for FTIR follows the protocol described in Scheme 3.3. The spectra are shifted vertically for clarity.

For bPEI, peaks at 1596 cm^{-1} (N-H bending vibration) and 1456 cm^{-1} (C-H stretching vibration) were prominent (Figure 3.3A). For GOX, the characteristic amide bands were observed in the $1410\text{--}1780\text{ cm}^{-1}$ range (two strong peaks at 1654 and 1541 cm^{-1}), as well as a C-H vibration at 1453 cm^{-1} (Figure 3.3B). For nanotubes, a weaker but apparent peak was also observed at 1773 cm^{-1} , corresponding to the O=C-O stretching vibration (Figure 3.3C); this band was absent in the spectra of bPEI/GOX mixtures (Figure S3.1), and was ascribed to residual traces of PC in the nanotube/KBr pellets (Figure S3.2). The fit was performed from 1410 to 1780 cm^{-1} , in a region presenting well-separated peaks of the two components, excluding the $1504\text{--}1569\text{ cm}^{-1}$ spectral region in which the spectra of the mixtures could not be represented as linear combinations of the spectra of the pure components, probably due to specific interactions. The fit of the mixtures allowed us to draw a calibration curve reporting $F_{\text{bPEI}}/F_{\text{GOX}}$ versus the known weight ratio of the samples, $W_{\text{bPEI}}/W_{\text{GOX}}$ (Table S3.1, Figure 3.4). The nanotube spectra were analyzed in the same way, with the spectrum of a pure PC membrane included to take into account traces of improperly washed PC on KBr (Figure S3.2). The ratio by weight of bPEI to GOX in the

nanotubes, computed from the values of F_{bPEI}/F_{GOX} obtained by the fit and from the calibration curve, are summarized in Table S3.2 for all tested samples.

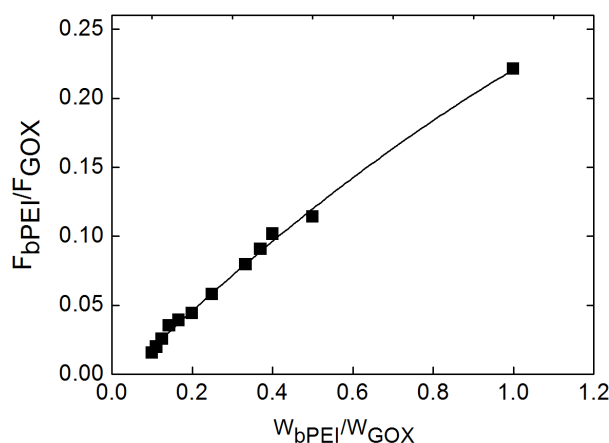
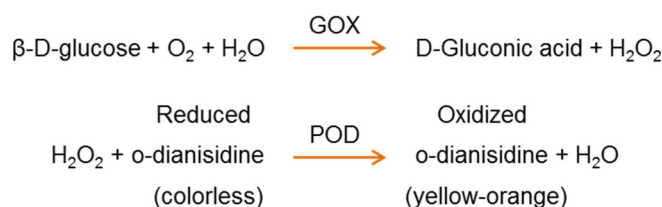


Figure 3.4. FTIR calibration curve drawn from Table S3.1; the line is: $F_{bPEI}/F_{GOX} = -0.05689 x^2 + 0.28712 x - 0.00939$, where $x = W_{bPEI}/W_{GOX}$ (w:w).

3.2.8 Enzyme activity

The activity of enzyme was measured spectrophotometrically (Agilent Cary 50) by monitoring the production of soluble oxidized o-dianisidine (yellow-orange), which is generated by the oxidation of reduced o-dianisidine (colorless) in the presence of peroxidase (POD) and hydrogen peroxide (H_2O_2). The H_2O_2 is the product of oxidation of glucose by oxygen catalyzed by glucose oxidase. The couple enzymatic reactions are described here below:



For free enzyme, the activity was tested following a published protocol ⁵⁵. Briefly, 1.92 mL of 0.21 mM o-diansidinedihydrochloride (reduced) solution

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in 50 mM sodium acetate buffer (pH 5.1, optimal pH for free GOX enzyme, Figure 2.11a), 0.4 mL of a β -D-glucose solution with different concentrations range from 4 mM to 222 mM, and 0.08 mL of a HRP solution (containing approximately 60 units/mL) were mixed in a cuvette (light path, 1 cm) and air-equilibrated until the absorbance at 500 nm was constant. 0.08 mL of a GOX enzyme solution (2 μ g/mL) was then added to the mixture, and the increase in absorbance at 500 nm ($A_{500\text{ nm}}$) was recorded immediately at the interval of 0.2 min for at least 6 min after mixing, as shown in Figure 3.5A. The maximum linear increase in absorbance at 500 nm, $\Delta A_{500\text{ nm}}/\text{min}$, was obtained by fitting in minimum period of initial 2.0 minutes (Figure 3.5B), and subsequently the initial reaction rate of GOX towards glucose at different concentration was determined. The enzyme activity towards glucose was evaluated using the classical Michaelis-Menten kinetics⁵⁶ which is expressed as:

$$v = \frac{v_{max}[S]}{K_M + [S]}$$

Where $[S]$ is the substrate concentration (mM), v_{max} is the maximum reaction rate (mM min^{-1}) reached at infinite substrate concentration, and K_M is the Michaelis constant (mM).

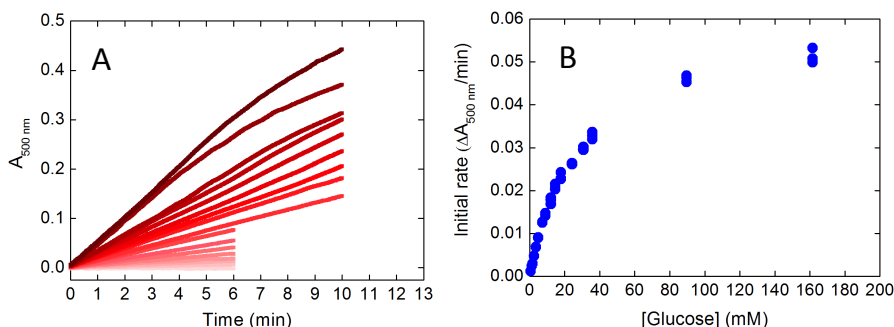


Figure 3.5. Typical plots of GOX reaction kinetics (A) and derived initial rate (B), $\Delta A_{500\text{ nm}}/\text{min}$, under different glucose concentrations.

To measure the activity of nanotubes embedded in membranes, disks of 10 mm diameter were cut from the filled membranes (possibly partially); then, 2.00 mL of a 0.3 mM o-dianisidine dihydrochloride solution in 50 mM Sodium acetate buffer (pH 5.5, optimal pH for GOX enzyme LbL films, Figure 2.11b),

and 0.20 mL of a horseradish peroxidase (HRP) solution (containing approximately 60 units/mL) were mixed in a well (12 well cell culture plate) with the nanotube-containing membrane, and air-equilibrated. 0.50 mL of a 3.0 mM glucose solution was then added to the well and the increase in absorbance at 460 nm (maximum absorption) of the solution was recorded at room temperature every two minutes immediately after mixing (Figure 3.6A), for 20 min during which continuous shaking was applied (KS-15 shaker, Edmund Bühler GmbH). The glucose concentration of 3 mM used here was optimal for activity measurement, since this glucose concentration only results in a moderate increase in absorbance at 460 nm as a function of time (Figure 3.6A), which facilitates the calculation of initial rates (Figure 3.6B). The activity of planar LbL films grown on flat silicon squares (11 mm x 11 mm) was measured similarly, with rough surface glued to base of well using double-sided tape to exclude the contribution of deposited enzyme film on rough surface to the measured activity. The enzyme activity was here defined as the initial rate of the coupled reaction, *i.e.*, the initial increase per min of the absorbance at 460 nm, $\Delta A_{460 \text{ nm}}/\text{min}$ (Figure 3.6B).

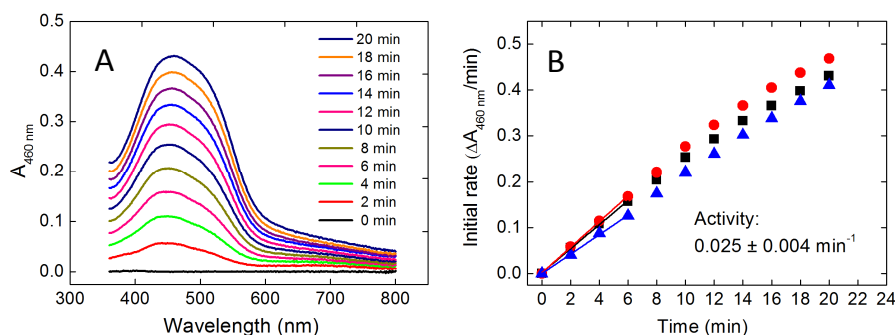


Figure 3.6. The evolution of coupled enzymatic reaction (A) occurred in membrane-embedded nanotubes (6 bilayers in this case) and the increment of absorbance at 460 nm against reaction time (B). In this case, the activity was calculated to be *ca.* 0.025 min^{-1} from linear fits (solid lines) at an initial period of 6 min. Three independent samples were tested here to give an average value (shown in B).

3.3 Results and discussion

3.3.1 Free GOX activity

Upon receiving the GOX product, we first evaluated the activity of enzyme with respect to the substrate affinity (K_M), catalytic rate (k_{cat}) and activity (U/mg) to ensure that the GOX used to fabricate films or nanotubes has high activity.

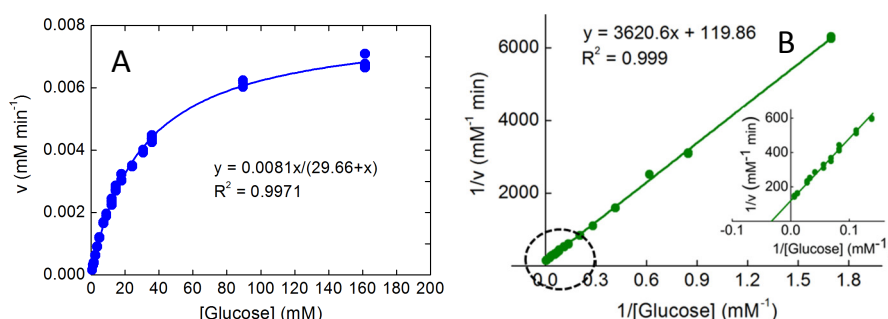


Figure 3.7. Activity kinetics of GOX enzyme activity, analyzed by (A) Michaelis–Menten plot and (B) Lineweaver–Burk double reciprocal plot, giving rise to identical kinetic parameters. The insert in B clearly revealed the intercept, related to v_{max} .

In order to compute the K_M , the initial rate ($\Delta A_{500 \text{ nm}}/\text{min}$) has to be converted into the increment in concentration per min by using the millimolar extinction coefficient of oxidized o-dianisidine⁵⁷, $\epsilon_{500 \text{ nm}}$, $7.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. As presented in Figure 3.7, K_M and v_{max} were calculated to be *ca.* 30 mM and $0.0082 \text{ mM} \cdot \text{min}^{-1}$, from which k_{cat} and specificity constant (k_{cat}/K_M) can be derived with a value of 20336 min^{-1} and $677.9 \text{ mM}^{-1} \cdot \text{min}^{-1}$, respectively. These values are in good agreement with those reported in previous publications^{58–60} for GOX extracted from the species *Aspergillus niger*, demonstrating that the enzyme product has comparable performance.

We also evaluated the quality of GOX product following the method described by Sigma-Aldrich, in terms of unit definition. One unit of enzyme is capable of oxidizing 1.0 micromole of β -D-glucose to D-gluconic acid and hydrogen peroxide per minute at pH 5.1 and at 35 °C. The activity (U/mg) of GOX product was determined to be *ca.* 100 U/mg which is superior to that of

typical product produced using *Aspergillus niger*⁶¹. However, it is lower than the value (ca. 200 U/mg) announced by the manufacturer, probably due to the lower test temperature in our case (room temperature, 25 °C) and to the enzyme degradation occurring during storage.

3.3.2 Enzyme multilayer assembled on flat silicon substrate

The LbL self-assembly on the basis of weak interactions between polyelectrolytes is not always feasible^{62,63}, depending on the nature of polyelectrolytes and the assembly conditions, typically pH, and ionic strength of polyelectrolyte solutions, and use or not of intermediate drying steps^{64,65}. In particular, the buildup of protein multilayer films via assembly of various proteins with oppositely charged polyelectrolytes is really a challenge^{9,13,36,37}, owing to the complexity of their structure and the patchy nature of their distribution of surface charge. As reported previously^{29,38,39}, several polycations, for instance, chitosan (CHI), and branched polyethyleneimine (bPEI), work well when pairing with enzymes. Herein, the buildup of GOX alternating with chitosan or branched polyethyleneimine (bPEI) films on planar surface in various assembly conditions was investigated by spectroscopic ellipsometry monitoring the thickness evolution of the multilayer films, providing preliminary insight into growth behavior of protein films. To make sure that GOX enzyme exhibits negative charge in solution, the pH of GOX solution was always adjusted at a value higher than its isoelectric point of 4.2^{37,39,40}. For chitosan, with a pKa = 6.0-6.5^{66,67}, pH beyond 6.5 of the solution reduces sharply the positive charge density along chitosan chains due to the deprotonation and, consequently decreases its solubility, resulting in flocculation. To avoid precipitation, the pH of chitosan solution is limited to the range from 5 to 6.5 and the assembly of CHI with GOX was always performed in this pH range. However, for bPEI/GOX films, the assembly was performed at a pH beyond 6.5 to maximize the surface negative charge density of globular GOX enzymes and strengthen the electrostatic interactions.

Under all tested conditions, similar fluctuation trends in the thickness of CHI/GOX films were observed (Figure 3.8A). The thickness fluctuation is also shown to depend on the nature of the outermost layer (GOX or CHI), demonstrating that the previously adsorbed GOX on chitosan layer was heavily released. Low buffer concentration and high pH facilitate the

adsorption of GOX on the chitosan layer: for instance, beyond 3 bilayers, almost a GOX monolayer (thickness increment, average 8 nm, identical to the spherical diameter of GOX, 60-80 Å^{37,68,69}) is adsorbed on CHI layer in 10 mM MES buffer (pH 6.5). However, it can not prevent heavy release of adsorbed GOX by subsequent CHI deposition, regardless of the amount of previously adsorbed GOX on CHI layer. Similar release phenomenon has been reported for proteins before^{13,40}.

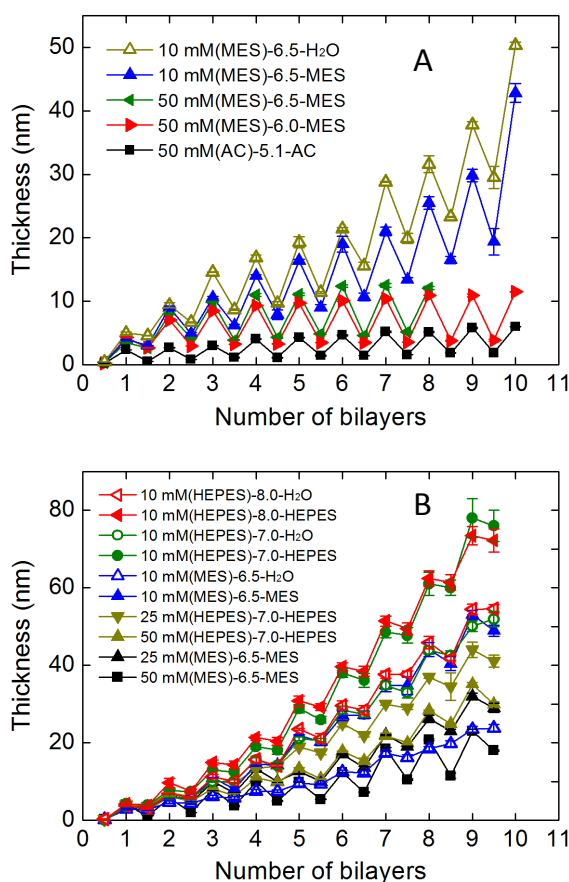


Figure 3.8. LbL growth evolutions determined by ellipsometry versus the number of bilayer deposited on flat silicon substrates for CHI/GOX (A) and bPEI/GOX (B) films under various deposition conditions. Acetate buffer (pH 5.1), MES buffer (pH 6.5) and HEPES buffer (pH 7.0, or 8.0) were used for the LbL assembly. The sample denomination is (concentration and solvent used for the deposition)-(solvent pH)-(solvent used for rinsing).

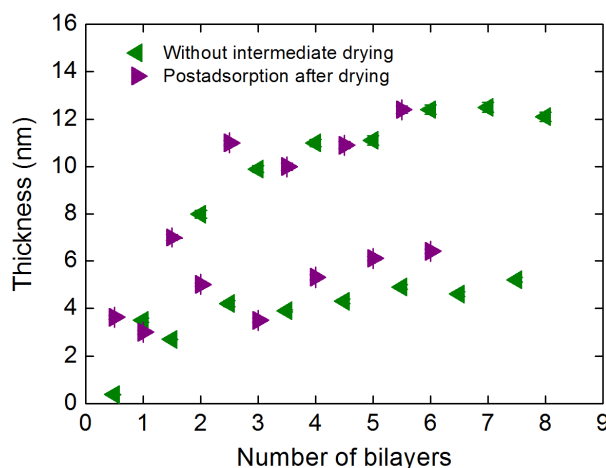


Figure 3.9. Thickness of $(\text{CHI/GOX})_n$ film prepared using 50 mM MES buffer pH 6.5 in terms of number of bilayers, and $(\text{CHI/GOX})_n$ films after post-adsorption in GOX or CHI solution of 50 mM MES buffer pH 6.5. During the build-up of $(\text{CHI/GOX})_n$ films, no drying step was applied until the desired number of bilayers was reached. Then, dried GOX-terminated film was placed into chitosan solution, followed by buffer rinse, while GOX solution allowed post-adsorption of GOX on dried $(\text{CHI/GOX})_n/\text{CHI}$ films. All samples were dried prior to ellipsometric measurement.

To check the possibility of applying intermediate drying step to improve the stability of enzyme layer against the deposition of subsequent chitosan layer, as well as, increasing the amount of adsorbed GOX on dried chitosan layer, a series of samples with different number of $(\text{CHI/GOX})_n$ bilayers were dried and further immersed into polyelectrolyte solution (CHI or GOX) which pairs with the outermost layer of samples. So, GOX and CHI-terminated films were incubated in CHI and GOX solution, respectively. A similar release and adsorption of enzymes were observed on the samples which experienced drying history (Figure 3.9), revealing that drying neither enhanced the stability of the enzyme layer nor increased enzyme adsorption.

For bPEI/GOX films, the situation is quite different. In most tested conditions, general growth evolutions were observed, characterized by an increase in film thickness (Figure 3.8B). Depending on the assembly conditions, a tiny decrease in thickness (evidenced by AFM, Figure S3.3) was however detected due to slight desorption of GOX (evidenced by FTIR, Figure S3.4)

when bPEI was deposited on GOX layer. Higher buffer concentration and lower assembly pH gave rise to a larger enzyme release, indicating that lower ionic strength and higher pH facilitate the buildup of LbL enzyme films, resulting in a stable LbL growth. At low ionic strength, unlike CHI/GOX pairs, rinsing medium plays a signification role in bPEI/GOX film growth. Using a buffer medium instead of pure water for intermediate washing steps yielded thicker enzyme LbL films. The poorer growth when pure water was used in intermediate rinsing steps might be attributed to the reorganization of previously adsorbed chitosan layer upon exposure to media of different pH, consequently affecting subsequent GOX accumulation. No significant difference was observed between LbL assembly at pH 7.0 and 8.0 when 10 mM HEPES buffer was used for buildup, suggesting further increase in solution pH made minor contribution.

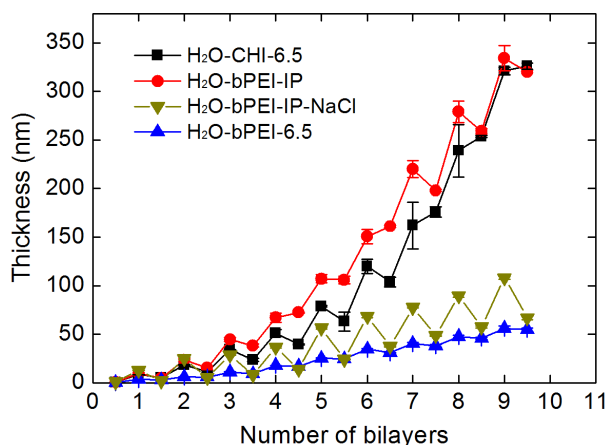


Figure 3.10. Evolution of thickness of CHI/GOX and bPEI/GOX films, fabricated by deposition of polyelectrolytes in water and subsequent rinse by pure water. The pH of all polyelectrolyte solutions was adjusted to 6.5 using HCl or NaOH, except for “H₂O-bPEI-IP” and “H₂O-bPEI-IP-NaCl” in which cases the initial pH (IP) of polyelectrolyte solutions was used, pH 7.2 for GOX solution and 10.2 for bPEI solution, while for latter 9 mM of NaCl was added only in bPEI solution for bPEI deposition.

The buildup process of alternated deposition of GOX and polycations (CHI, bPEI) in water was investigated as well. Figure 3.10 shows that a thickness of ca. 300 nm could be reached for 9.5-bilayer films, regardless of polycation

used. Although, a slight removal of adsorbed enzyme occasionally occurred during polycation deposition, indicating that pure water (low ionic strength) not only promotes GOX deposition on previous polycation layer but also prevents the dissolution of GOX-polycation complexes. Apart from the first few initial adsorption steps, the thickness of a single GOX adsorption layer was found to be approximately 50 nm, higher than the spherical diameter of the enzyme, revealing that a single GOX adsorption layer consists in approximately 6 monolayers of GOX.

However, when the pH of GOX (initial pH, 7.2) and bPEI (initial pH, 10) solutions were adjusted to 6.5, the growth evolution (rinsed in pure water without NaCl) was totally different, exhibiting much less increment in thickness per bilayer. This is due to the primarily moderate amount of GOX adsorbed on previous bPEI layer, 10 nm of GOX layer compared to 50 nm (when the pH of GOX and bPEI solutions were 7.2 and 10.2, respectively); although major adsorbed enzymes were preserved when exposed to bPEI solution, pH 6.5. We attributed this deposition feature in terms of amount of adsorbed GOX primarily to the properties of previous bPEI layer which depends on the pH and ionic strength of bPEI solution, since the properties of underlying layer was found to be crucial for sequential adsorption of oppositely charged polyelectrolytes. One should be aware of the fact that, apart from the pH change by addition of HCl, the increase in ionic strength of bPEI solution, resulting from the pH adjustment may also play important role in LbL film growth. Moderate amount of HCl was required to decrease the pH of bPEI solution from 10.2 to 6.5, resulting in NaCl concentration of approximately 9 mM in bPEI solution, while the ionic strength caused by tiny additional HCl could be ignored for GOX solution. The amount of generated NaCl in bPEI solution was estimated by assuming that it is identical to the amount of HCl used for the neutralization reaction. Significant difference was observed upon addition of 9 mM NaCl in bPEI solution (pH 10.2) shown in Figure 3.10. The presence of 9 mM NaCl in bPEI solution has no adverse influence on the sequential adsorption of GOX layer. However, it causes a large release of adsorbed GOX due to the salt-induced effect during the bPEI layer deposition in PEI solution compared to the condition “H₂O-bPEI-IP”, leading to a slower growth with pronounced odd-even growth pattern different from “H₂O-bPEI-6.5”. These observations demonstrated that high pH and low ionic strength facilitated the LbL film growth.

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The high release of adsorbed GOX enzymes following CHI adsorption was observed in all tested buffer conditions, probably due to the weak interactions between GOX and adsorbed chitosan layer resulting from the low charge density along the chains and the high hydrophilicity of the polysaccharide. This leads to very poor LbL growths in buffer solution, which is not desirable, although a good LbL growth is obtained in pure water. For bPEI, a stable LbL growth is achieved in 10 mM HEPES buffer at pH 7.0, perhaps due to the highly branched arms along the chains which are efficient for holding adsorbed GOX enzymes. The buffer solution is preferred to maintain the activity of GOX enzymes rather than just pure water. Therefore, bPEI seems better than CHI to be assembled with GOX enzymes and was therefore selected as polycation for further LbL buildup.

In order to check if denaturation of GOX occurs or not upon the formation of films by complexation with bPEI and under high pH assembly conditions, the activity of GOX planar films assembled in 10 mM HEPES buffer at pH 7.0 was measured (Figure 3.11). A regular increase in the activity of films with varying number of bilayers was observed, revealing the accumulation of enzyme amount on the silicon in a layer by layer fashion, as evidenced by the thickness evolution; though, there exists a transition in activity at around 4 bilayers. At this point, the film enters into a stable growth region (Figure 3.8B, and 3.11). This transition phenomenon occurring during growth was commonly observed, because the film usually takes several bilayers to fully eliminate the influence of the substrate and establish the stable growth⁷⁰.

We noticed that the activity increased somehow linearly in both regions, regardless of the GOX or bPEI as outermost layer; however, the GOX content (in terms of GOX IR absorbance at 1654 cm^{-1}) of the GOX-terminated films was reduced slightly by the sequential deposition of bPEI layer owing to the tiny desorption of previously adsorbed GOX, which is in accordance with thickness evolution.

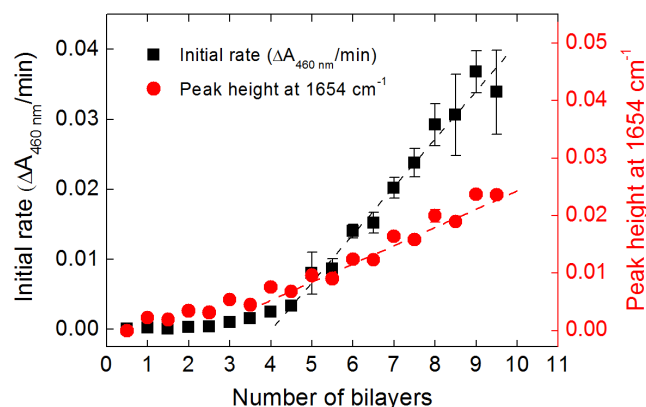


Figure 3.11. Enzymatic activity and GOX absorbance intensity at 1654 cm^{-1} in FTIR spectra of planar films fabricated by using 10 mM HEPES buffer, pH 7.0 throughout the buildup process; The content of GOX in planar films is supposed to be proportional to the absorbance of GOX at 1654 cm^{-1} (amide I) measured by FTIR transmission on the basis of Beer-Lambert law. The peak height at 1654 cm^{-1} was derived from Figure S3.4. Error bars in activity are taken from three independent experiments. Dashed lines are drawn to guide the eye.

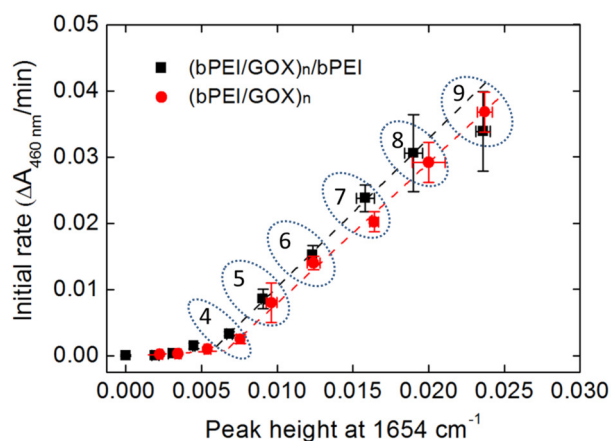


Figure 3.12. Plots of activity against GOX content for $(\text{bPEI/GOX})_n$ films. The red filled circles represent the films $(\text{bPEI/GOX})_n$ with GOX as ending layer, while black solid squares denote the $(\text{bPEI/GOX})_n/\text{bPEI}$ films as a results of bPEI deposition on $(\text{bPEI/GOX})_n$ films. The number, n is indicated in dotted ellipses. Error bars are derived from three independent experiments. Dashed lines are drawn to guide the eye.

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To make it clearer, we plotted the activity against the content of GOX in flat films. In Figure 3.12, the tiny increment in activity due to the deposition of bPEI layer was observed, however, could not be detected precisely because of the large variation in the activity measurement. This minor effect presumably resulted from the structural organization of enzyme film either upon exposure to bPEI solution, yet without significant removal of adsorbed enzymes, or during activity measurements in 50 mM acetate buffer (pH, 5.5) due to the change of pH and ionic strength; this structural reorganization may influence the measured activity of enzyme films, contributing to a slightly higher activity of bPEI-terminated enzyme films. The structural reorganization in terms of surface morphology was probed by AFM (Figure S3.5); this leads to a smoother surface verified by the low RMS roughness (Figure S3.6)

Of more interest is the low activity of films below 4 bilayers of thickness no more than 13 nm (3-bilayer film) (Figure 3.12). For instance, as for (bPEI/GOX)₂ film, a monolayer of GOX (complexed with bPEI) was expected to present on the silicon substrate, since its thickness reached around 7.7 nm, approximately identical to the GOX enzyme size^{68,69}. Moreover, by comparison of AFM images collected on bare silicon, 1 and 2-bilayer films (Figure S3.7), it seems that the surface of silicon substrate is almost completely covered by the 2-bilayer enzyme film. This one GOX monolayer yielded 0.00029 of increment in activity, which is at least 100 times lower than that estimated from second linear region. This slower increment in activity per amount of GOX could probably be ascribed to the denaturation of adsorbed enzyme closed to substrate at the initial stage of buildup process.

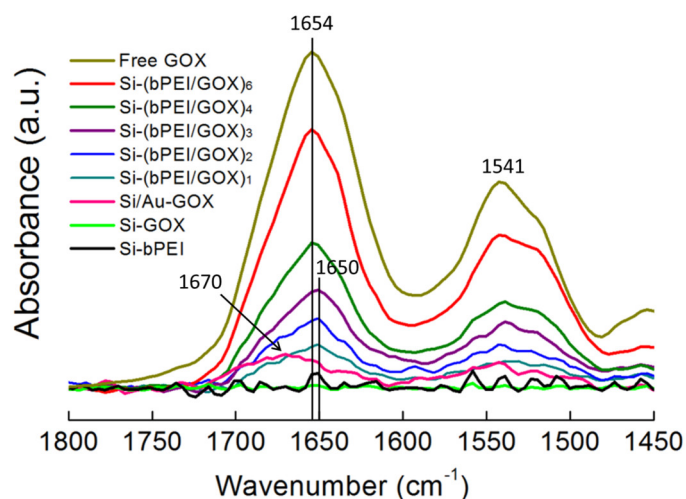


Figure 3.13. FTIR spectra of free GOX enzyme, and GOX, 1-bilayer, 2-bilayer, 3-bilayer, 4-bilayer and 6-bilayer films deposited on substrates. For GOX and LbL-films deposited on silicon substrate (Si), the measurements were carried out in transmission model with bare silicon as background, while GOX adsorbed on Au coated silicon (Si/Au) was recorded by IRRAS-FTIR. GOX on silicon or Au-coated silicon was prepared by immersion of substrate in GOX solution, followed by washing and drying.

Herein, IR spectrum was used to detect any change of the protein secondary structure, which is essentially important for enzymatic activity⁷¹⁻⁷³. Intense bands in the amide I and II bands were observed in all the spectra in Figure 3.13. Comparing IR spectrum of free GOX, specifically the amide I and II bands at about 1654 and 1541 cm⁻¹, no significant changes in either position or band shape were observed for 4 and 6-bilayer films, suggesting that the GOX component retains its native secondary structure. However, a slight red shift of amide I band and pronounced change in amide II band shape for 1, 2 and 3-bilayer films implied that the incorporated GOX closed to silicon was denatured by substrates, consequently leading to a low activity of several-nanometer thin films. Unfortunately, GOX was not successfully adsorbed onto the bare silicon substrate by just immersion of silicon substrate in GOX solution because of the pretty weak interaction, verified by the absence of GOX feature in Figure 3.13 (Si-GOX). However, the appearance of characteristic peaks of enzyme confirmed the presence of GOX enzyme on

the Au-coated silicon somehow. A blue shift of amide I band and change in shape of amide II was detected in the spectrum of GOX adsorbed on Au-coated silicon compared to the free GOX. These observations verified that the substrate indeed plays a significant role on the denaturation of protein. Moreover, the films were primarily dominated by GOX component rather than bPEI, since no pronounced characteristic peaks of bPEI but noise were present in the spectrum of a single bPEI layer on silicon wafer because of the trace of adsorbed bPEI, several Angstroms in thickness.

3.3.3 Enzyme multilayer assembled in confined nanopores

The growth mechanism of LbL multilayers in nanopores has been investigated by our group ⁵⁴, suggesting a transition for pore filled by approximately 70 % (in the dry state) in the evolution of wall thickness with number of bilayers. When pore filled lower than this threshold, the LbL growth in nanopores is comparable to that observed on the flat substrates including the influence of ionic strength, allowing us to pre-optimize the assembly condition for synthesis of nanotubes on the basis of preliminary investigation of LbL enzyme films on planar substrates. With these essential information in hand, we could narrow the assembly condition down for fabrication of robust nanotubes, with respect to the pH, buffer concentration, and ionic strength, as well as the polycation. To obtain the stable nanotubes, we decided to use bPEI as polycation to be assembled with GOX because of heavy release of adsorbed GOX caused by the chitosan deposition. Buffer was also strongly recommended to use in bPEI and GOX adsorptions to prevent enzyme degradation during the long-time LbL buildup process.

Aiming at the synthesis of robust enzyme nanotubes of high activity capable of surviving detemplating, herein, the assembly conditions were optimized as well for alternated deposition of bPEI with GOX into nanoporous PC membranes, varying pH from 6.5 to 8.0, ionic strength (either 0 or 0.1 M), and rinse with Milli-Q water or buffer (HEPES or MES). Similar thicknesses of the nanotube walls were obtained within 25% for most tested conditions (Figure 3.14A), with the LbL growth characterized by a regular diminution of dry pore diameters, demonstrating that the assembly of PEI and GOX occurs well in the nanopores. However, two conditions led to a poor LbL growth, namely in the presence of 0.1 M NaCl (in bPEI solution), and when using linear PEI (IPEI). This indicates that lower ionic strengths and

branches on the PEI facilitate the buildup of enzyme nanotubes, yielding thicker nanotube walls of presumably improved structural stability.

To eliminate the films covering the surface of the membranes, a decrusting protocol using a cotton swab to clean the membrane was used as reported previously^{29,30,54,74}, rather than other harder methodologies such as plasma etching⁷⁵ or polishing with alumina powder^{44,76}; in our case, however, the cotton swab was saturated with the buffer solution used during the rinsing process. Thereby, the encrusting film on the membrane surfaces could be readily and completely wiped off as revealed by SEM (Figure 3.15). In addition, SEM clearly shows the deposition of a LbL layer over the inner walls of the nanopores, although the nanotube lumen fluctuates somewhat from tube to tube (Figure 3.15); in contrast, gas-flow porometry provides an average pore diameter (Figure 3.14A) that probably better represents the average filling of the pores.

With the enzyme film growing on the inner surface of the pores, the enzymatic activity of the nanotube-implanted membranes increased rapidly towards a high value within 3 to 4 bilayers, followed by a decrease or plateau upon additional deposition of enzyme layers (Figure 3.14B). This is most probably due to a reduced diffusion rate of the glucose substrate in the more filled pores as already reported before¹⁵ or in pores having a clogged end as can be observed in Figure 3.15B. Hence, the restricted diffusion of the substrate in the filled pores dominates the activity of the partially-filled membranes for 3 or more bilayers as a result of decreased lumen size; it should be reminded here that the pore sizes reported in Figure 3.14A are average values for systems in the dry state, whereas the activity of the membranes is measured in an aqueous solution which swells the LbL and consequently closes further the pores³⁰.

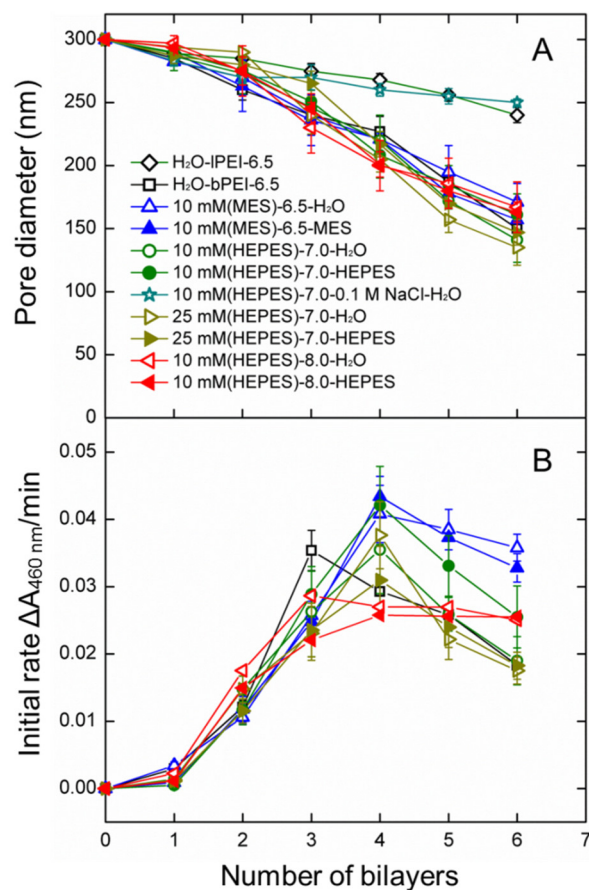


Figure 3.14. LbL growth of nanotubes in nanopores (of 300 nm initial diameter) determined by measuring the dry pore diameters by gas-flow porometry for different deposition conditions (panel A), and enzymatic activity ($\Delta A_{460 \text{ nm}}/\text{min}$) of the corresponding membranes with embedded nanotubes (panel B), as a function of the number of deposited bilayers. The sample denomination is (solvent used for the deposition)-(solvent pH)-(solvent used for rinsing) where branched poly(ethyleneimine) (bPEI) was used for deposition, except for two samples "H₂O-xPEI-6.5" which were fabricated by deposition of either linear (IPEI) or branched (bPEI) poly(ethyleneimine) in water (pH=6.5) and subsequent rinse in Milli-Q water. The same symbols are used in panel B as in panel A.

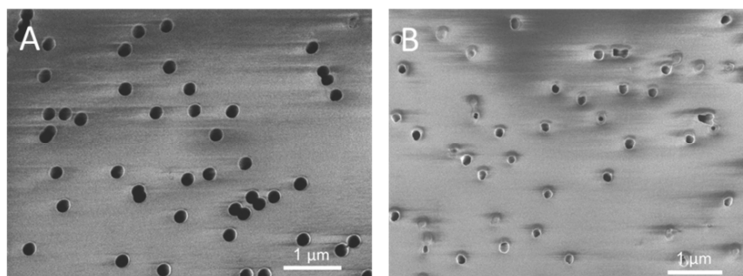


Figure 3.15. SEM micrographs (A) of a virgin track-etched polycarbonate membrane as used in this work; (B) of a membrane containing (bPEI/GOX)₆ nanotubes, after surface decrusting.

Although a similar LbL growth evolution in nanopores was observed for most tested conditions, significant differences in enzymatic activity are nevertheless observed between the systems. Membranes modified in 10 mM MES buffer (pH 6.5) regardless of the type of rinsing (buffer or pure water), or in 10 mM HEPES (pH 7.0) rinsed in HEPES buffer, have a higher activity (ca. 0.04 min^{-1}) at 4 bilayers, compared to other preparation conditions. In contrast, the lower activity (ca. 0.026 min^{-1}) is obtained for membranes prepared in 10 mM HEPES (pH 8.0) irrespective of the rinsing process. After 4 bilayers (or 3 bilayers only in H₂O at pH 6.5), the activity decreases rapidly except for 10 mM HEPES (pH 8.0) for which the activity only slightly decreases or even keeps constant. These complex effects arise from different enzyme loadings and variations in the ability of the substrate to access the enzyme, which is itself related to the amount and distribution of adsorbed enzyme, residual pore size and degree of complexation between the enzyme and PEI in different assembly conditions. The structural reorganization of nanotubes may occur during activity measurements because of the different pH and ionic strength of 50 mM acetate buffer solution (pH 5.5) from assembly conditions. A tiny release of enzymes from nanotubes was found when immersing them in 50 mM acetate buffer solution (pH 5.5); this might be the consequence of a structural reorganization (discussed in Chapter 4), also contributing to these complex effects.

As a rule of thumb, it appears better to keep LbL films at a constant pH during the whole buildup process, using the same medium for intermediate washing steps; indeed, the ionization of adsorbed chains can change upon

exposure to media of different pH, inducing the reorganization of previously adsorbed layers, resulting in a film of different properties⁷⁷. From these results, 10 mM MES buffer (pH = 6.5) or 10 mM HEPES (pH = 7.0) buffer appear as most suitable for the construction of GOX-based nanotubes of high activity, in agreement with conclusion summarized from planar film section; hence, these conditions will be taken in the sequel. In addition, only branched PEI will be used in what follows.

3.3.4 Morphology of the nanotubes

The morphology of nanotubes built in our two optimized conditions was observed by SEM and STEM. Samples for SEM were prepared by collecting the nanotubes by dissolution of the membrane and direct filtration from the resulting suspension in CH_2Cl_2 (Figure 3.16). Well-defined cylindrical structures were obtained for both preparation conditions; however, nanotubes prepared in 10 mM HEPES buffer (pH 7.0) tend to be more rigid and less deformed. Intact nanotubes of ca. 25 μm length, well replicating the nanopores, were generally obtained, suggesting that the inner surfaces of the nanopores could be coated all along their length during LbL assembly. Their outer diameter is ca. 400 nm (Figure 3.16B,D), moderately larger than the virgin pore size (300 nm), which is due to the flattening of the liberated nanotubes on the filters upon drying. This flattening often makes the observation of the inner cylindrical open channel of the nanotubes difficult. However, STEM allowed in many cases to observe the presence of this open channel (Figure S3.8). In addition, individual nanotubes without aggregation were generally observed, confirming the efficient and complete removal of the crust layers on the flat surfaces of the templates.

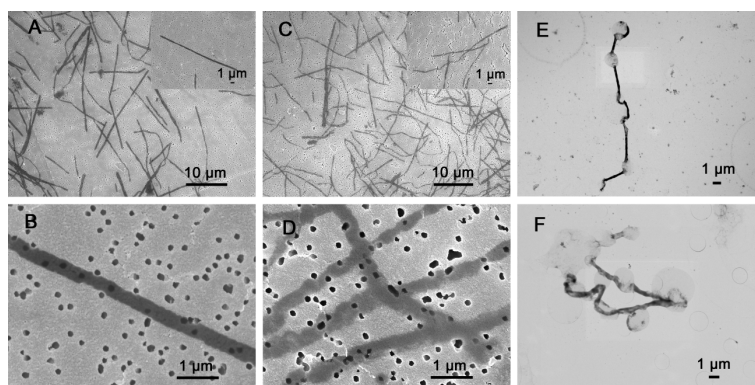


Figure 3.16. SEM (A-D) and STEM (E,F) images of (bPEI/GOX)₆ nanotubes fabricated in 10 mM HEPES buffer (pH 7.0) throughout (A,B,E) and 10 mM MES buffer (pH 6.5) throughout (C,D,F). The nanotubes were collected by filtration from CH₂Cl₂ solutions over PET membranes, or by adsorption over TEM grids, just after dissolution of the PC membrane.

STEM images (Figure 3.16E,F) provided comparable information regarding the nanotube morphology. However, the nanotubes appear to have burst at specific locations along their length, which was not seen in SEM images. This phenomenon is due to the capillary forces exerted on the tubes upon CH₂Cl₂ drying, combined with a different interaction strength between the nanotubes on either the PET filter or the carbon-coated TEM grid. To confirm this contention, the specimens for STEM were prepared by another methodology, involving immobilization of the membrane on a layer of ice (Scheme 3.2), followed by PC dissolution in CH₂Cl₂ with the nanotubes anchored on the ice, leaving a bare nanotube array. With this methodology, intact nanotubes without structural defects could be transferred to a carbon-coated TEM grid (Figure 3.17), showing that the replacement of CH₂Cl₂ by water in the drying step solved the issue. Unfortunately, the lower temperature also leads to a decreased solubility of PC in CH₂Cl₂, which complicates the complete removal of PC from the tubes, as evidenced by FTIR (Figure S3.9A); moreover, the liberated nanotubes tend to pack irreversibly in thick bundles when using this methodology (Figure S3.9B).

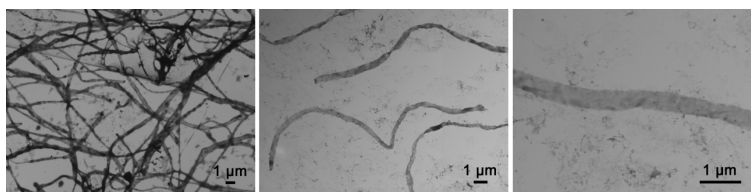


Figure 3.17. STEM images of free nanotubes fabricated in 10 mM HEPES (pH 7.0) buffer throughout. The nanotubes were extracted after immobilization over an ice layer (Scheme 3.2) followed by dissolution of the PC template containing frozen nanotubes, to avoid the bursting of portions of nanotubes during drying.

Based on their more sturdy morphology, nanotubes fabricated in 10 mM HEPES buffer (pH 7.0) throughout were chosen for exploring confined biocatalysis in membrane-supported and liberated nanotubes in following chapters.

3.3.5 Quantification of the amount of proteins in the nanotubes

Attenuated total internal reflectance infrared spectroscopy (ATR-FTIR) is well established for probing in-situ the internal structure of flat LbL films⁷⁸⁻⁸⁰, including water content, adsorbed mass of polyelectrolyte layers, ion pairing, and ratio between anionic and cationic monomer units. In contrast to planar films, the LbL growth of nanotubes occurs over the inner walls of nanopores within membranes, leading to the impracticability of conventional monitoring technologies, commonly used for flat films, such as ATR-FTIR, quartz crystal microbalance (QCM)^{42,81} or surface plasmon resonance (SPR)⁸². So far, the growth of LbL films in cylindrical pores of PC membranes was merely quantified with respect to the variations of layer thickness^{83,84}, with multilayers grown in confined spaces being generally different from those fabricated on an 'open' planar surface under identical build-up conditions. However, few report discusses the internal composition of LbL films assembled in confinement³⁸.

Herein, the nanotube wall composition was studied by FTIR measurements in transmission mode, after extraction of the nanotubes from the PC membranes. Comparing the IR spectra of free GOX and enzyme nanotubes,

no significant difference in the amide I and II bands at *ca.* 1654 cm^{-1} and 1541 cm^{-1} was found for 3-9 bilayers (Figure 3.3 and S3.10), suggesting that the GOX component preserves its native secondary structure after complexation with bPEI in the nanotubes, a prerequisite for the preservation of its enzymatic activity. However, for 1 or 2-bilayer nanotubes, the signal of remnant PC appears obvious in the spectra because of the low content of nanotubes in KBr disk (Figure S3.10).

The ratio by weight of bPEI to GOX in the nanotubes assembled in 10 mM HEPES pH 7.0 buffer was obtained from a fit of the IR spectra, as detailed in the experimental section. As shown in Figure S3.10, the fitting curves matched the experimental IR data very well except for one and two-bilayer nanotubes. The results of the fits are collected in Table S3.2, from which the proportion between the enzyme and bPEI was obtained for various numbers of bilayers. The bPEI:GOX weight ratio was almost constant at *ca.* 0.20 from 4 to 9 bilayers (for smaller numbers of bilayers, the signal was dominated by the traces of PC). The slight differences between samples is most likely due to the limited precision of the FTIR method for GOX-rich systems (as is the case here), to slight variations in the protocol, and possible evolution of the enzyme. These experiments show that GOX is largely dominating in the nanotubes, with an estimated weight fraction of *ca.* 80%, and that there is little variation depending on the number of bilayers. The large content in GOX of the nanotubes is evident from Figure 3.3, which shows almost identical spectra for the nanotubes and pure GOX. Recently, the content of the enzymes in LbL assemblies was also investigated by our group³⁸. ^1H nuclear magnetic resonance (^1H NMR) spectra of chitosan/ β -lactamase LbL nanotubes and flat films were analyzed by assuming that the ^1H NMR signal of the LbL assemblies is solely the sum of chitosan and β -lactamase pure spectra. It was found that in the nanotubes, the enzyme weight fraction was *ca.* 90%, irrespective of the number of bilayers from 6 to 12. In contrast, the content of enzyme in LbL films exhibits a strong dependence on the number of bilayers, with weight fraction decreasing from *ca.* 90 % to *ca.* 70% from 6 to 12 bilayers. As for bPEI/GOX flat film assembled in 10 mM HEPES buffer (pH 7.0) in our case, GOX enzymes are believed to dominate the bPEI/GOX flat film. In this condition, a large amount of GOX is adsorbed on bPEI layers for each GOX adsorption step after 4 bilayers (confirmed by the large thickness increment shown in Figure 3.8B, and the large increase in peak height at 1654 cm^{-1} in Figure 3.11). More importantly, a tiny release of

adsorbed GOX and a negligible decrease in film thickness is caused by the following bPEI deposition in 10 mM HEPES buffer pH 7.0 (Figure 3.11 and 3.8B), implying that an extremely limited amount of bPEI adsorbs on a stable GOX layer in this condition (Figure 3.18). Moreover, the content of enzymes in bPEI/GOX flat film assembled in 10 mM HEPES buffer pH 7.0 was roughly estimated to be constant with the number of bilayers beyond 4, because of the almost linear film growth in that region.

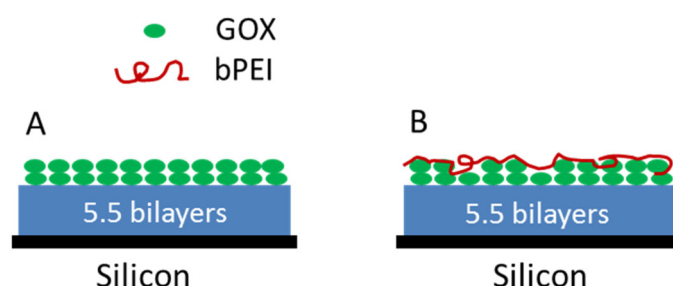


Figure 3.18. Schematic illustrations of (A) 6th adsorption of GOX on 5.5-bilayer bPEI/GOX film and (B) following bPEI deposition on A. The assembly is performed in 10 mM HEPES buffer (pH 7.0).

By knowing the weight fraction of GOX in the nanotubes, the ratio of the number of repeating units of bPEI ($\text{CH}_2\text{CH}_2\text{NH}$) per GOX macromolecule was calculated to be *ca.* 750. In addition, we can also analyze quantitatively the activity of enzyme entrapped in the nanotubes and discuss the confined effect after the release of nanotubes from PC template in Chapter 4.

3.4 Conclusions

In this chapter, the assembly of GOX with either chitosan or branched polyethyleneimine on flat surfaces was firstly investigated intensively by monitoring the thickness evolution. The results showed that for both CHI/GOX and bPEI/GOX systems, the ionic strength and pH of polyelectrolyte solution play a significant role in LbL film growth. Specifically, for CHI/GOX pair, ionic strength dominated the adsorption of polyelectrolytes. Apart from the only case in which regular growth of CHI/GOX film was observed in pure water (pH 6.5), other conditions (MES buffer used) lead to a very slow growth involving the heavy release of previously adsorbed GOX by subsequent deposition of chitosan, suggesting that this polyelectrolytes pair is not suitable for the synthesis of robust enzyme nanotubes. While, for bPEI/GOX system, in most cases (buffer used) regular increase in thickness was observed; more important is that the deposition of bPEI caused negligible release of adsorbed GOX, demonstrating the possibility of using bPEI and GOX to build stable enzyme nanotubes of high activity.

Then the construction of bPEI/GOX films on the nanochannel surfaces in various conditions was investigated as well. Likewise, lower ionic strength facilitates the buildup of enzyme nanotubes. Among all the tested conditions, 10 mM HEPES buffer (pH 7.0) was found to yield nice tubular structure of high activity; thus this condition was chosen for template-synthesis of enzyme nanotubes. The FTIR results offered insight into the internal composition of nanotubes prepared in 10 mM HEPES (pH 7.0), revealing that the enzyme dominates the nanotubes by weight, with a content of ca. 80% independent of the number of bilayers, and that the enzyme retains its protein secondary structure in the nanotubes after complexation with bPEI.

Supporting Information

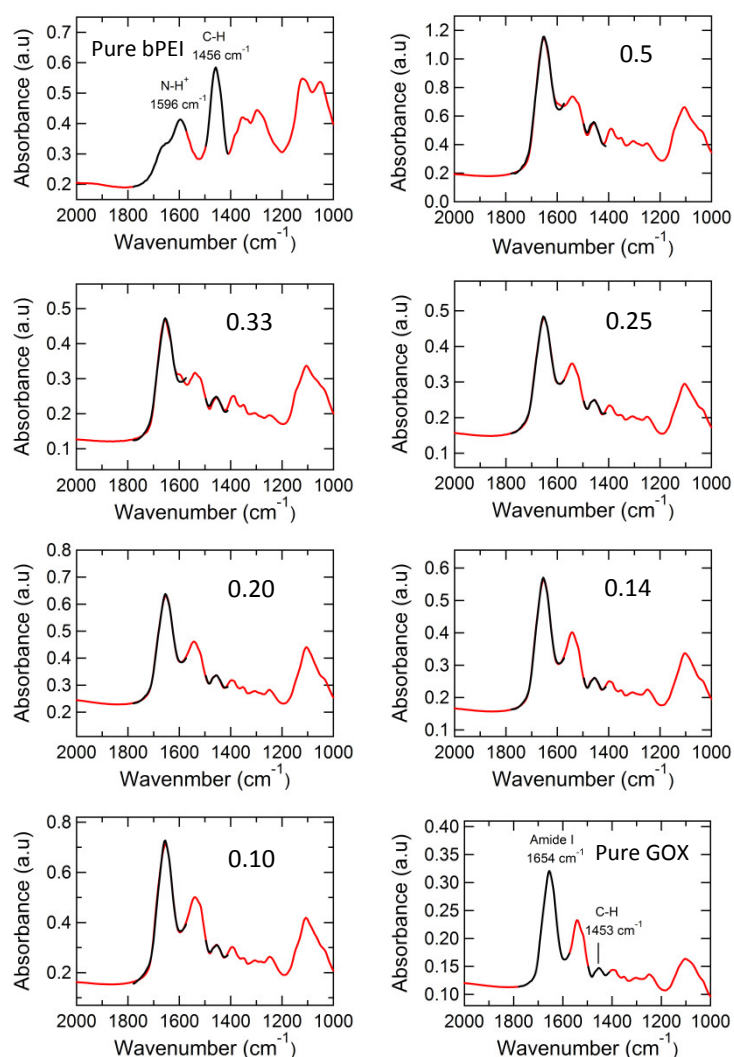


Figure S3.1. Fits (black lines) of the raw spectra of bPEI/GOX mixtures (red lines) of precisely known compositions. These analyses were performed in order to obtain a calibration curve relating the composition by weight of the mixtures to the relative intensity of the components of their FTIR spectra. The fits were restricted to spectral regions in which the spectra vary linearly with respect to the contents in both bPEI and GOX. The ratio ($W_{\text{bPEI}}:W_{\text{GOX}}$) is indicated in each graph.

Table S3.1. Fitting results from the FTIR spectra of bPEI and GOX mixtures measured for the calibration.

Mixtures W_{bPEI}/W_{GOX}	1	0.5	0.4	0.37	0.333	0.25	0.2	0.167	0.143	0.125	0.111	0.1
F_{bPEI}	0.496	0.195	0.142	0.164	0.107	0.074	0.074	0.076	0.062	0.044	0.042	0.038
F_{GOX}	2.241	1.71	1.398	1.807	1.346	1.298	1.685	1.956	1.768	1.74	2.133	2.468
F_{bPEI}/F_{GOX}	0.221	0.114	0.102	0.091	0.079	0.058	0.044	0.039	0.035	0.026	0.020	0.016

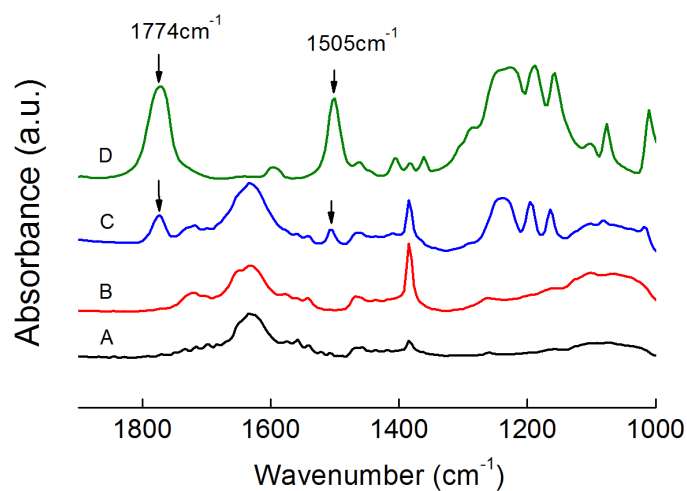


Figure S3.2. FTIR spectra of (A) pure KBr, (B) KBr powder after filtration of CH_2Cl_2 , (C) KBr powder after filtration of pure PC in CH_2Cl_2 , and (D) pure PC; these spectra demonstrate the trend of PC to adsorb on KBr powders from CH_2Cl_2 . Therefore, the signal from residual PC should be considered in the fitting of nanotube spectra.

Table S3.2. Fitting results and calculated ratio by weight of bPEI to GOX in nanotubes prepared in 10 mM HEPES buffer (pH 7.0).

Number of bilayers	F_{bPEI}	F_{GOX}	$F_{\text{bPEI}}/F_{\text{GOX}}$	$W_{\text{bPEI}}/W_{\text{GOX}}$
1	0.102	0.021	0.208	0.93
2	0.175	0.021	0.121	0.51
3	0.289	0.024	0.084	0.35
4	0.131	0.0077	0.059	0.25
5	0.178	0.0064	0.036	0.16
6	0.192	0.0092	0.048	0.21
7	0.118	0.0060	0.051	0.22
8	0.105	0.0041	0.040	0.18
9	0.140	0.0071	0.051	0.22

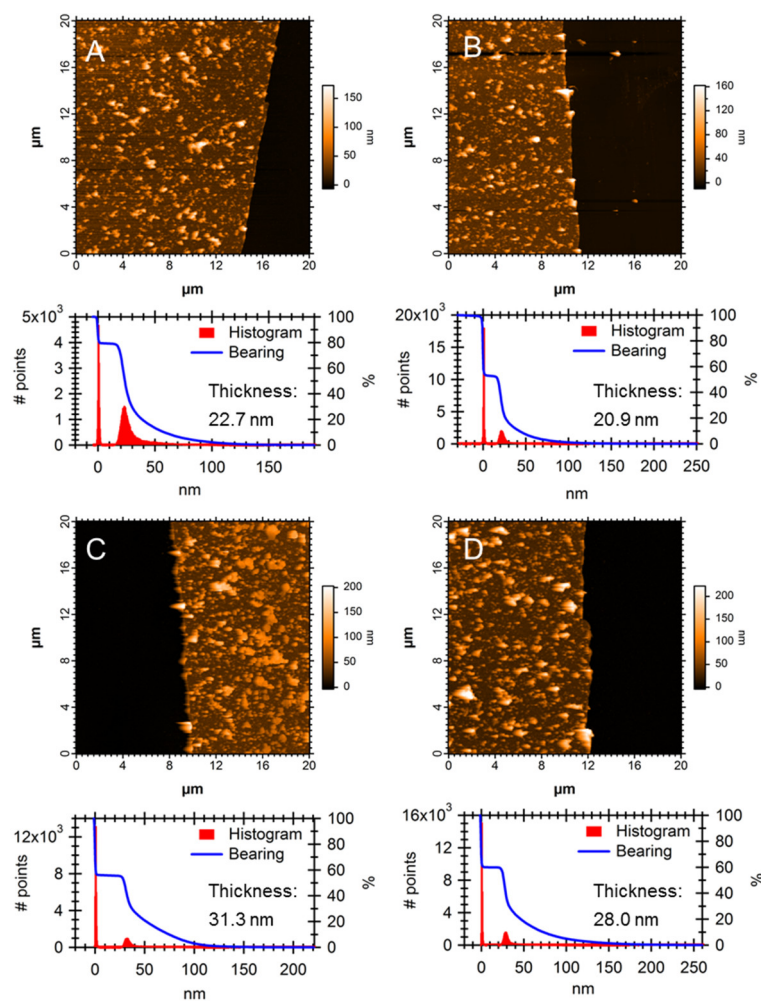


Figure S3.3. Dry thickness of bPEI/GOX films of 6 (A), 6.5 (B), 7.0 (C) and 7.5 (D) bilayers prepared in 10 mM HEPES (pH 7.0) determined by AFM. The thickness, d was obtained by statistics analysis (beneath each AFM image), shown in histogram graphs. These thicknesses measured by AFM are in agreement with those obtained by ellipsometry measurements, showing the decrease in film thickness by bPEI deposition.

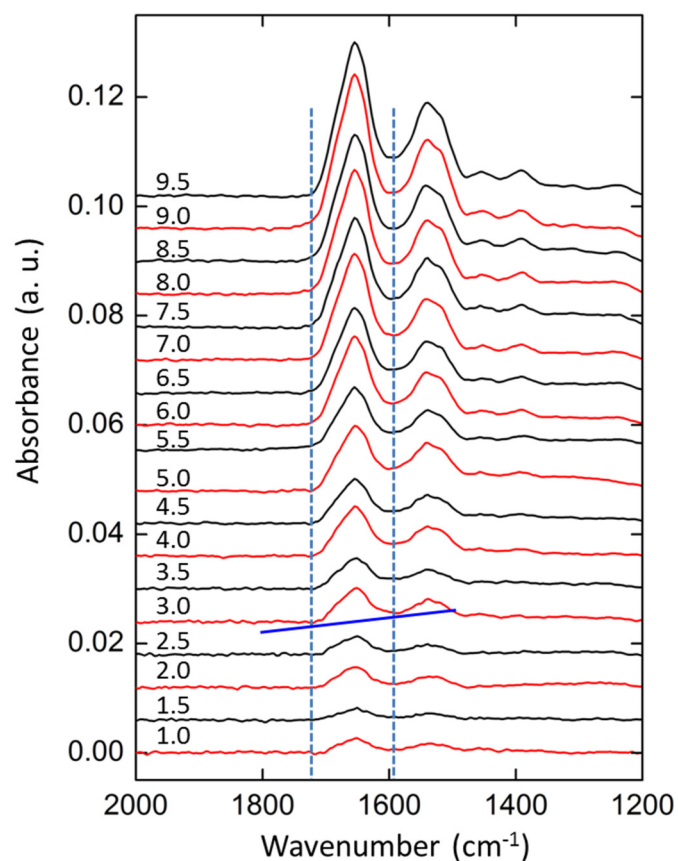


Figure S3.4. FTIR spectra of (bPEI/GOX)_n planar films deposited in 10 mM HEPES buffer (pH 7.0) on silicon wafers. The number of bilayers, *n*, is indicated left above the curve. The peak height at 1654 cm⁻¹ (amide I) was estimated by drawing a baseline (1760 cm⁻¹, 1590 cm⁻¹), which is proportional to the content of enzyme in LbL films. Independent samples were prepared and then used for FTIR measurements. The curves are shifted vertically by 0.006 for clarity.

These FTIR measurements are used to prove that the adsorbed GOX enzymes are not released by the following adsorption of bPEI layer. While (bPEI/GOX)_n/bPEI has a slightly decreased thickness compared to (bPEI/GOX)_n, demonstrating that a limited amount of bPEI is adsorbed on GOX layer.

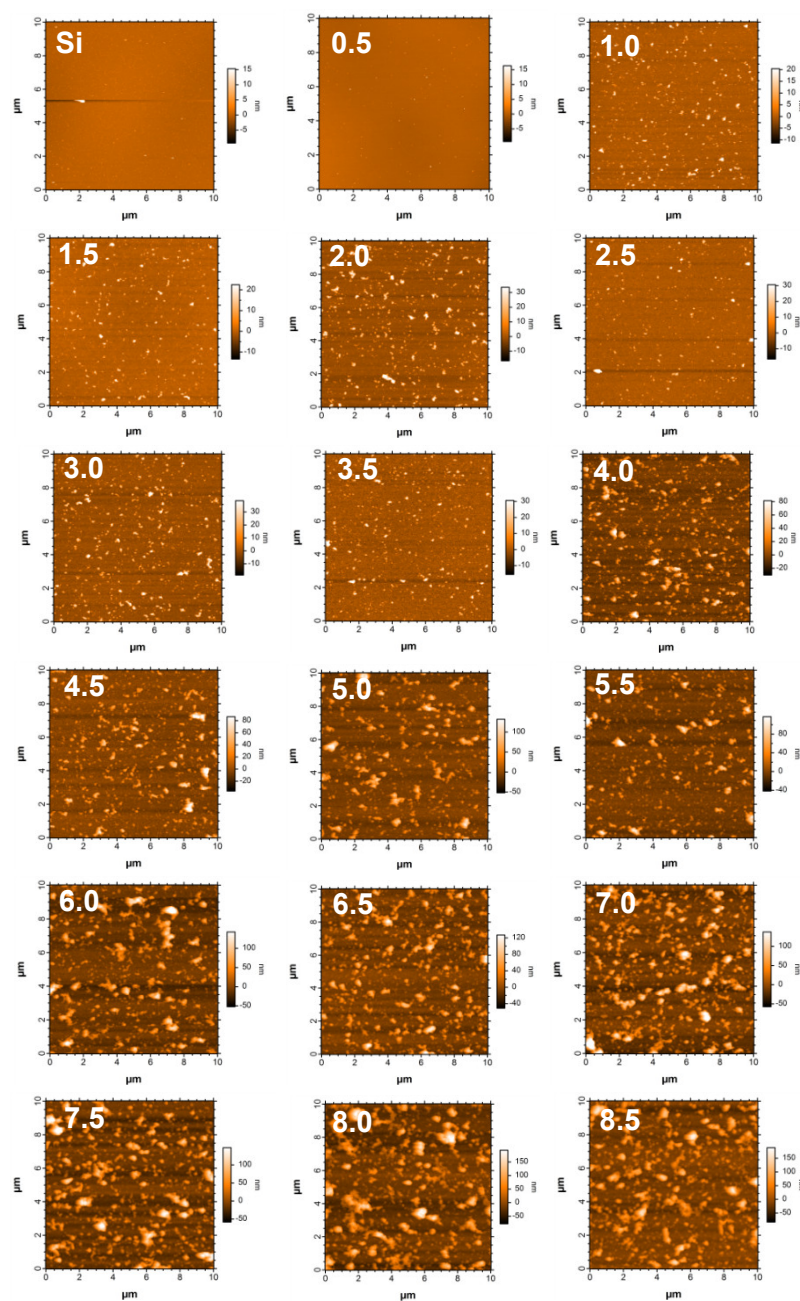


Figure S3.5. AFM height images ($10 \times 10 \mu\text{m}^2$) of (bPEI/GOX)_n flat films with different number of bilayers deposited in 10 mM HEPES buffer (pH 7.0) on silicon wafers. The number of bilayers (n) is indicated in each AFM image.

Supporting Information

These AFM images were collected on independent samples prepared at the same time to reveal the surface structure.

From Figure S3.5, we can derive the surface roughness values to quantitatively compare the change in surface morphology, in order to support the surface reorganization hypothesis.

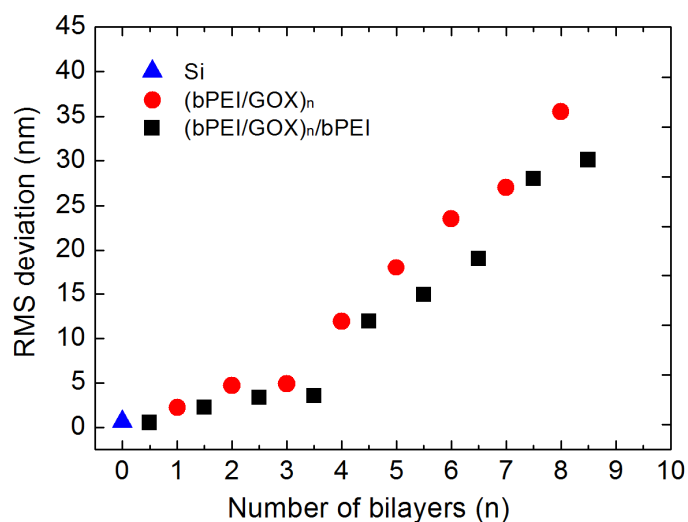


Figure S3.6. RMS surface roughness of (bPEI/GOX)_n flat films deposited in 10 mM HEPES buffer (pH 7.0) on silicon wafers at different number of bilayers. RMS values were calculated from corresponding images (10 x 10 μm²) shown in Figure S3.5. The surface becomes smoother when a limited amount of bPEI was adsorbed on GOX layer, suggesting that surface structure reorganization was probably induced by bPEI adsorption.

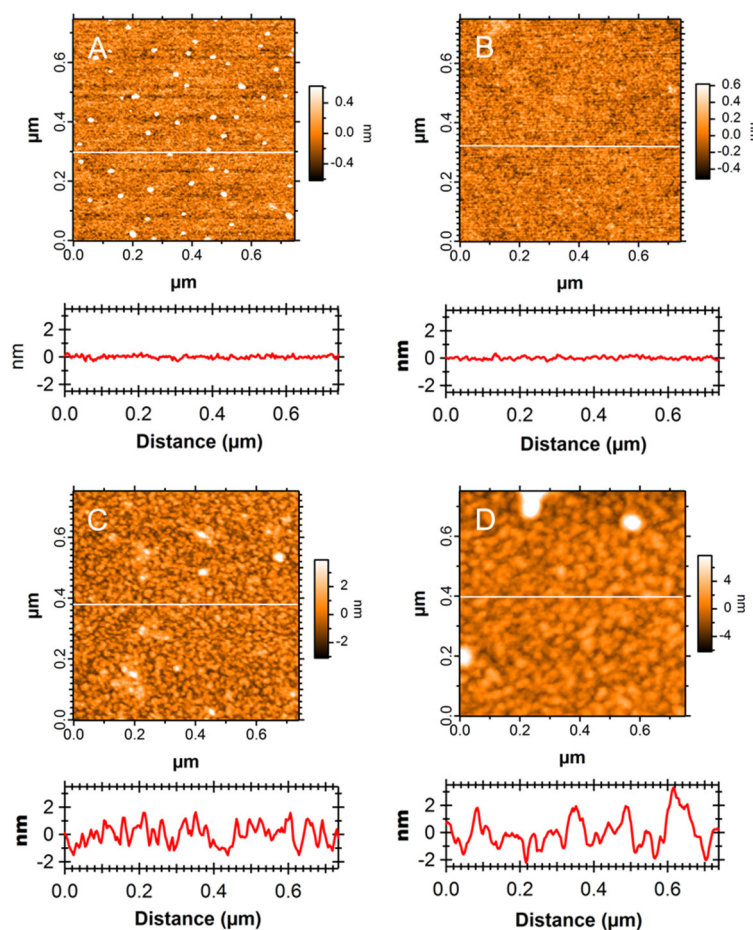


Figure S3.7. AFM height images ($0.75 \times 0.75 \mu\text{m}^2$) of bare silicon (A), Si/bPEI (B), Si/(bPEI/GOX) (C) and Si/(bPEI/GOX)₂ (D) films deposited in 10 mM HEPES buffer pH 7.0. Profiles were taken at the locations indicated in each image by solid lines. These images were taken in order to show the film growth evolution in the initial region of deposition in terms of surface morphology. The first bPEI layer on bare silicon leads to limited changes in surface morphology, while 1, and 2-bilayer films become rough due to the adsorption of globular GOX enzymes. The dots observed on the surfaces of 1, and 2-bilayer films probably result from the complexation of bPEI and GOX. Compared to other films, it seems that the surface of the silicon substrate is highly covered by 2-bilayer film.

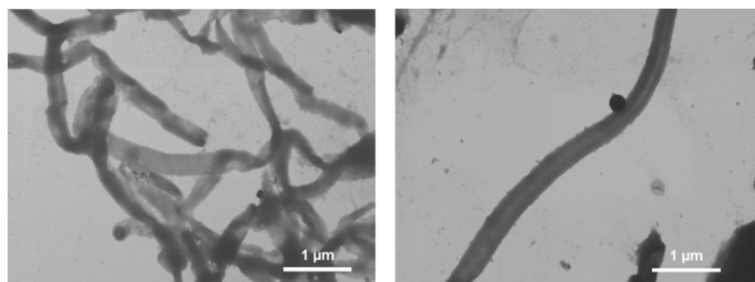


Figure S3.8. STEM images of (bPEI/GOX)₆ nanotubes prepared in 10 mM HEPES buffer (pH 7.0) showing their hollow core, even though they are collapsed after collection. Nanotubes were collected from aqueous dispersion of liberated nanotubes.

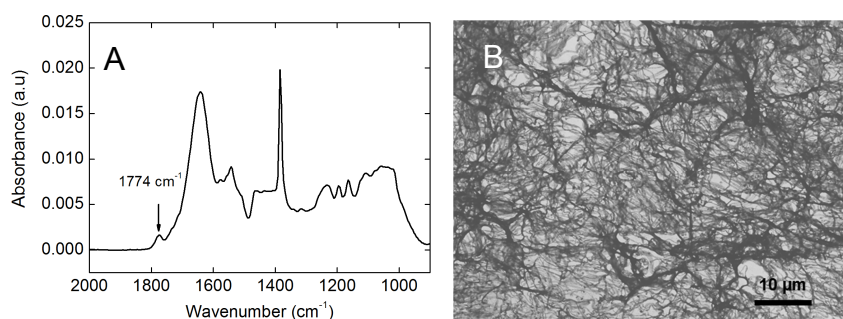


Figure S3.9. FTIR spectrum (A) and STEM image (B) of a thin but dense film of nanotubes prepared using the ice collection route (Scheme 3.2), then transferred to a TEM grid. The nanotubes were assembled in 10 mM HEPES buffer (pH 7.0).

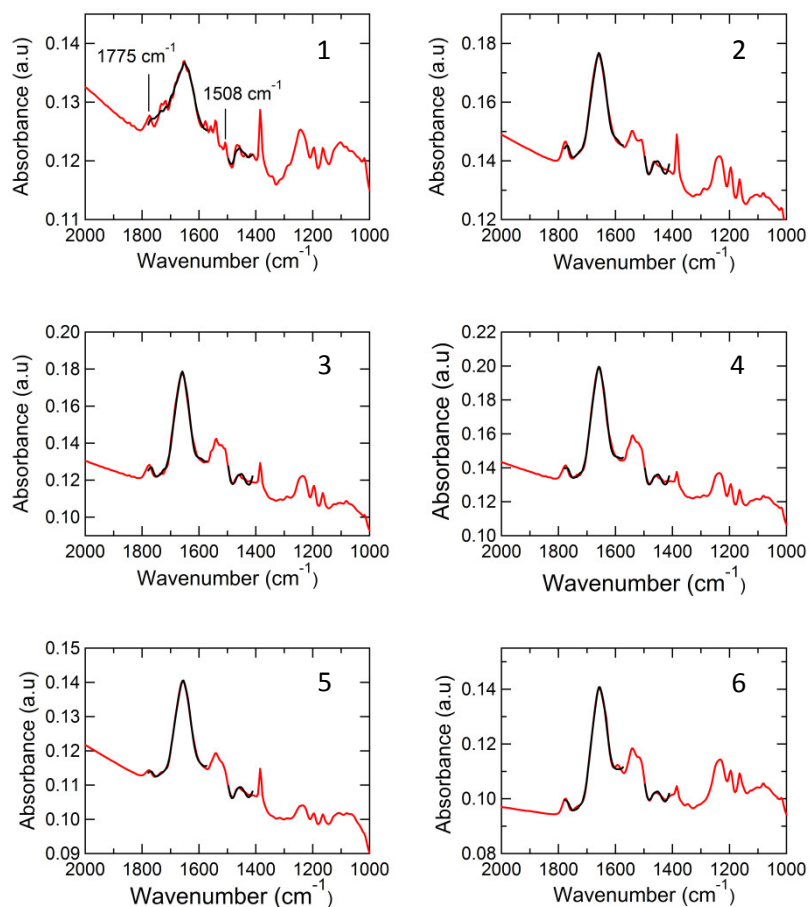


Figure S3.10. Fits (black lines) of the raw spectra of (bPEI/GOX) $_n$ nanotubes (red lines). The number of bilayers, n , is indicated in the graph. The nanotubes were assembled in 10 mM HEPES pH 7.0 buffer.

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Chapter 4

Collection and activity of liberated enzyme nanotubes

In this chapter, we aim at gaining insight in the activity kinetics of embedded enzyme of liberated nanotubes compared to those still confined inside templates. To achieve this goal, it was of primary importance to quantitatively collect as-synthesized LbL nanotubes from polycarbonate templates and to redisperse them in aqueous medium, prior to performing enzymatic reactions. Therefore, a soft extraction method of nanotubes from the template, namely, adjuvant-assisted filtration methodology was developed, and different water-soluble yet CH_2Cl_2 -insoluble adjuvants were evaluated with respect to collection efficiency and most importantly, structural stability of free nanotubes.

4.1 Introduction

Template-synthesis is a powerful tool in material engineering for construction of various functional nanostructures, by replicating either the exterior of the template such as colloidal particles or nanorods¹⁻⁵, or the well-confined void spaces within template⁶⁻¹⁰. Among these artificial nanostructures, well-defined nanowires or nanotubes shaped by nanoporous membranes have particularly fascinated researchers due to their novel properties^{11,12} and their potential applications in many different technological areas^{9,13-17}. By combining template-synthesis with different methods such as electrochemical deposition, sol-gel, solution/melt-wetting, chemical vapor deposition (CVD), chemical polymerization and layer-by-layer (LbL) assembly, a wide range of materials ranging from metal to semiconductors, organic polymers, polyelectrolytes and, biomacromolecules could be employed to build nanotubes or nanowires^{7,9,13-25}. In particular, LbL assembly, which involves consecutive adsorption of polyanions and polycations from solutions²⁶, is a suitable method for fabricating nanotubes including proteins thanks to its mild processing conditions. These synthesized protein nanotubes could be used as smart biological devices in appropriate medium for drug delivery^{27,28}, and as nanoreactors^{29,30}.

However, few reports focus on the morphology and properties of nanotubes composed of natural enzymes liberated from their templating membrane. Martin *et al.*³¹ first reported on the fabrication of glucose oxidase (GOX) nanotubes based on the AAO template-synthesis. Although the liberated nanotubes retained the enzymatic activity of immobilized GOX, most of them were broken during the template dissolution process due to the harsh conditions required for the removal of AAO (phosphoric acid solution). The soft extraction of polymer nanotubes from the template framework and subsequent redispersion of them in aqueous medium without deformation is indeed still a challenge for template-assisted synthesis³².

Recently, Komatsu *et al.*^{12,30,33} proposed an effective approach to collect protein LbL-nanotubes, released from a PC polymer membrane. After the nanotubes were liberated by dissolution in N,N-dimethylformamide (DMF), they were precipitated from the resultant polymer solution and washed with fresh DMF by centrifugation. The nanotubes were then rapidly freeze-dried to produce a lyophilized powder which could be dispersed in deionized water to produce a slightly turbid suspension. This process allowed the protein-

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incorporated nanotubes not only to retain their tubular structures but also to preserve their biological functions in an aqueous environment, for instance, binding affinity for ligands ¹². Although efficient, this procedure sometimes leads to the formation of aggregates of nanotubes, which cannot be redispersed in water. Rubner *et al.* extracted hybrid tubes of micrometer size by dissolving the PC template in dichloromethane, followed by a filtration step. The collected microtubes were then scraped off the filter surface and transferred to aqueous solution ³⁴.

Our group has been active in the LbL template-synthesis field for almost 10 years ³⁵ during which tubular nanostructures have been investigated for various potential biomedical applications, for instance gene delivery ³⁶ and temperature-responsive biocatalysis ³⁷. The key issue towards the practical application of these nanostructures in biomedicine is to efficiently recover them from templates and to efficiently redisperse them in an aqueous medium. Therefore, we dedicated a lot of effort to develop an efficient nanotubes collection method ^{11,38}.

In this chapter, we synthesized glucose oxidase (GOX) nanotubes under the optimized conditions (see Chapter 3) and then developed an efficient collection strategy to recover quantitatively sticky soft enzyme nanotubes without degradation. Consequently, for the first time, a quantitative comparison of the activity of membrane-embedded and released enzymatic nanotubes was possible. The developed method is quite universal allowing the quantitative collection of membrane templated one dimensional nano-objects of different sizes, composition, and rigidity ¹¹.

4.2 Experimental section

4.2.1 Materials

Glucose oxidase (GOX from *Aspergillus niger*, type x-s lyophilized powder), horseradish peroxidase (HRP, type VI, lyophilized powder), branched polyethyleneimine (bPEI, M_n ca. 60,000 g·mol⁻¹, M_w ca. 750,000 g·mol⁻¹, 50 wt. % in H₂O), *o*-dianisidinedihydrochloride, glucose, fructose and dextran (M_r ~70,000, *Leuconostoc* spp.) were purchased from Sigma-Aldrich. 2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulfonic acid (HEPES free acid), Potassium bromide (FT-IR grade, ≥ 99%) and dichloromethane (99.8%, for analysis) were purchased from Acros Organics. bPEI and GOX solutions were freshly prepared at a concentration of 1.0 mg/ml in 10 mM HEPES buffer at pH 7.0. Milli-Q water (Millipore) with 18.2 MΩ·cm resistivity was used in all experiments. Single-side polished silicon wafers (100) and track-etched polycarbonate (PC) membranes of 300 nm average pore diameter, 25 μm thickness and 1×10⁸ cm⁻² pore density, were used as templates, for LbL deposition, whereas poly(ethylene terephthalate) (PET) membranes of 200 nm average pore size, 23 μm thickness and 5×10⁸ cm⁻² pore density, were used as filters to collect nanotubes; both types of membranes were provided by the it4ip company, Louvain-la-Neuve, Belgium (<http://www.it4ip.be>).

4.2.2 Templated synthesis of bPEI/GOX nanotubes

The enzyme-containing nanotubes were fabricated following the procedure shown in Figure 4.1. Briefly, bPEI solution in 10 mM HEPES buffer (pH 7.0) first allowed the initial bPEI layer adsorption onto the channel surfaces in 30 min after 15 min sonication to aid diffusion of polycation into nanopores, then followed by two washing steps of 3 min each in fresh 10 mM HEPES buffer (pH 7.0) to remove loosely attached polycation chains. The bPEI-modified PC membrane was then placed in a GOX solution of 10 mM HEPES buffer (pH 7.0), following the same procedure as the one used for bPEI adsorption. The LbL crust adsorbed on the top and bottom surfaces was mechanically cleaned by a cotton swab during the washing step in buffer solution. All depositions were performed manually at ambient temperature. No drying treatment was carried out during the whole process until desirable number of bilayers was reached. Prior to dry step at the end of buildup, the nanotube-

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implanted membrane was washed in pure water for 1 min to remove the buffer salt, during which a final decrusting treatment was applied to eliminate the crust completely. The samples were stored at 4 °C in the dry state in a fridge and were used for activity measurements within 2 days.

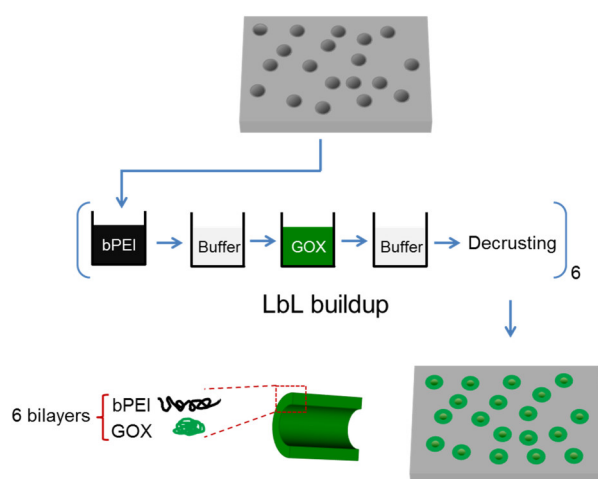


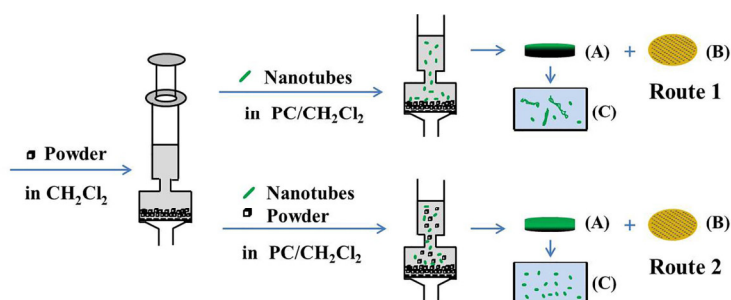
Figure 4.1. Templated-synthesis of enzyme nanotubes by LbL technology.

4.2.3 Collection of enzyme nanotubes

The nanotube-containing PC membrane was dissolved in CH_2Cl_2 to release the nanotubes, which were collected by filtration over a PET nanoporous membrane. Preliminary experiments showed that the nanotubes could not be released in water from the filter, once having been in contact with the virgin PET filter. Therefore, free nanotubes were recovered by a filtration method involving water-soluble powder as filtration adjuvant, as shown in Scheme 4.1. In principle, any water-soluble powder could be used to assist the collection of nanotubes if the adjuvant is compatible with nanotubes as well as the test conditions. NaCl and fructose have high solubility in water and are compatible with biological environment; dextran is a neutral polysaccharide made of glucose and commonly used in the biomedical field because of its good biocompatibility, nontoxicity, and degradation by dextranases³⁹. For all these reasons, these 3 adjuvant candidates were selected in this work. A finely-crushed powder (Vibrating mill, Perkin-Elmer & Co. GMBH) was first suspended in CH_2Cl_2 and filtered on a nanoporous PET membrane clamped in a stainless steel syringe holder (13 mm diameter,

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Millipore Corp.). This resulted in the formation of a thin powdery cake on the filter, which subsequently served as leaky barrier layer preventing the nanotubes from reaching the PET membrane. Then, the same powdered adjuvant was either added to the nanotube suspension in PC/CH₂Cl₂ or not, and the resulting suspension was filtered over the previous cake and rinsed three times with fresh CH₂Cl₂ for complete elimination of the dissolved PC (Scheme 4.1). At the end of the process, the resulting cake was easily detached from the PET filter; immersion in water dissolved the cake, leading to an aqueous suspension. We found that if the adjuvant was not mixed with nanotube suspension in PC/CH₂Cl₂, large pieces of undispersed chunks would be appearing in the nanotube aqueous suspension (Route 1, Scheme 4.1). However, in route 2, where the adjuvant was used to assist the filtration of nanotubes from the PC/CH₂Cl₂ suspension, a clear aqueous suspension of well-liberated nanotubes would be obtained.



Scheme 4.1. Collection of enzyme nanotubes through two different routes by adjuvant-assisted filtration. Both routes result in (A) a cake, (B) Au covered PET filter after filtration and removal of the cake, and (C) aqueous suspension obtained by dissolving the whole cake. In route 1, no adjuvant powder was added to nanotube/dissolved PC/CH₂Cl₂ suspension, resulting in nanotube stacking up irreversibly over the barrier cake, and as a consequence, undispersed chunks. In route 2, the adjuvant powder was mixed with nanotubes in a second collection step, leading to a clear dispersion of free nanotubes.

4.2.4 SEM and STEM characterization of enzyme nanotubes

The efficiency of the collection process of nanotubes and the morphology of the enzyme nanotubes were evaluated with a field emission scanning

electron microscope (JSM-7600F, JEOL Ltd.), equipped with a transmission detector, operated at 15 kV for the scanning mode (SEM) and 30 kV for the scanning transmission mode (STEM), respectively. To easily identify whether the nanotubes arrived at the filter through the barrier cake by SEM, the filter was covered by a 20 nm gold layer (Cressington Sputter Coater 208HR). For the observation of liberated nanotubes in aqueous solution, the samples for SEM were prepared either by filtration of an aqueous suspension of nanotubes through a 20 nm-Au/Cr coated PET membrane or by dipping an Au/Cr-coated PET membrane in the nanotube suspension (2 mL), and then washed by pure water. For STEM observations, a carbon-coated grid (200 mesh, Electron Microscopy Sciences) was immersed in the aqueous suspension of nanotubes and gently stirred for several minutes, then washed by pure water and air-dried at room temperature.

4.2.5 Atomic Force microscopy (AFM)

The thickness of dried (in air) and hydrated (in aqueous solution) LbL flat enzyme films was measured by a Nanoscope VIII Multimode AFM (equipped with a fluid cell, Bruker) with peak force tapping mode (PeakForce QNM). Scratch lines were made by prep blades to uncover the silicon substrate and measure the film height (thickness). The samples were scanned in air and then in aqueous medium over an appropriate area, which consists of film and exposed silicon substrate under peak force of 1-2 nN at a scan rate of 0.3-0.5 Hz with ScanAsyst-air and ScanAsyst-fluid probes (Bruker), respectively. Before AFM measurements in aqueous solutions using fluid cell, a small droplet of solution was placed on the samples that previously scanned in air and allowed for 40 min to reach dynamic equilibrium. Collected Images (20 x 20 μm^2) were used to compute the film thickness by statistical analysis after flattening the substrate area (film area excluded) using 1st order polynomials in a line-by-line type in X direction (Igor Pro program). The swelling degree (SD) of LbL films on silicon in aqueous solutions was calculated as follows:

$$SD = \frac{d_{\text{aqueous}} - d_{\text{air}}}{d_{\text{air}}}$$

Where d_{aqueous} and d_{air} are the thicknesses of films measured in air and in aqueous solution, respectively. The scanned areas in air and in fluid are exactly identical in order to avoid the influence of variation in film thickness.

4.2.6 FTIR measurements

Fourier transform infrared (FTIR) spectra were collected at 8 cm^{-1} resolution (128 passes) on a FTIR spectrometer (Nicolet Nexus 870, Thermo Scientific). For nanotubes extracted directly from PC template, the samples were prepared using the methods described in Chapter 3, experimental section 3.2.7. To probe nanotubes present in aqueous solution, the sample was prepared following the procedures described below. Briefly, the liberated nanotubes in aqueous solutions were firstly collected by filtration of the nanotube suspension through 200 nm-PC membranes followed by pure water rinsing and air-dry treatment, then finely-milled KBr powder (Vibrating mill, Perkin-Elmer & Co. GMBH) was placed on the top of the PC membrane in a pellet die. A strong pressure was required to form a transparent KBr pellet with PC membrane attachment at the bottom. Samples of nanotubes for FTIR were obtained after complete removal of entire PC membrane from the KBr disk by simply manual detachment (Figure S4.1). Herein, PC membrane was recommended rather than PET membrane, since the PC membrane is strong enough compared to the brittle PET membrane.

4.2.7 Enzyme activity

The activity of liberated nanotubes was evaluated as follows: The extraction of nanotubes from the PC membrane (disks of 10 mm diameter) was achieved as described in section 4.2.3, the only difference being that the obtained powder/nanotube cake was immediately dissolved for some time (depending on the nature of powder) on a shaker in a well (12 well culture plate) containing a mixture of 2.00 mL of a 0.3 mM o-dianisidinedihydrochloride solution in 50 mM sodium acetate buffer (pH 5.5) and 0.20 mL of a HRP solution, until the cake was dissolved completely. The solution was collected in a cuvette (light path, 1 cm) to measure the absorbance at 460 nm every 1 or 2 min immediately, after addition of 0.50 mL of a 3.0 mM glucose solution. The enzyme activity was defined as the initial rate of the coupled reaction, *i.e.*, the initial increase per min of the absorbance at 460 nm, $\Delta A_{460\text{ nm}}/\text{min}$.

4.3 Results and discussion

4.3.1 Collection of free nanotubes in water

To achieve our goal of measuring the activity of free nanotubes, these should be extracted from the PC template and dispersed in water, the essential environment where enzymatic reactions occur. This has to be performed with minimal losses, as quantitative comparisons require to work on a constant number of nanotubes. However, once in contact with the PET filter for recovery after PC dissolution, the nanotubes did not disperse again in water, irreversibly sticking to the PET membrane and together. A modified filtration method was thus developed to collect the nanotubes from the polymer solution, namely adjuvant-assisted filtration (Scheme 4.1). Sequential filtrations of a dispersion of finely ground adjuvant powder in CH_2Cl_2 , then of a suspension of this adjuvant powder added to the nanotubes/dissolved PC in CH_2Cl_2 , were carried out on a PET membrane of 200 nm pore size. The adjuvant powder, NaCl, fructose, or dextran, was selected so as to be soluble in water and insoluble in CH_2Cl_2 .

The first step of our collection procedure, the filtration of the adjuvant powder, results in the formation of a thin porous cake over the filter, acting as a barrier layer preventing the nanotubes from reaching the PET filter while still allowing filtration. Very few nanotubes can pass through this barrier layer and reach the PET filter regardless of the details of the following filtration of the suspension of nanotubes (Figure 4.2A-C), implying that this barrier of adjuvant powder is efficient against spurious adsorption of the nanotubes on the PET filter. However, one should be aware that this efficiency depends on the powder size, since an increased number of nanotubes were observed on the filter beneath the barrier cake when large crystal particles such as fructose were not milled into finer particles (Figure S4.2), consequently leading to a lower activity. In our collection procedure, fine powders of a size in the 10 μm range (insets of Figure 4.2A-C) were found to lead to optimal results.

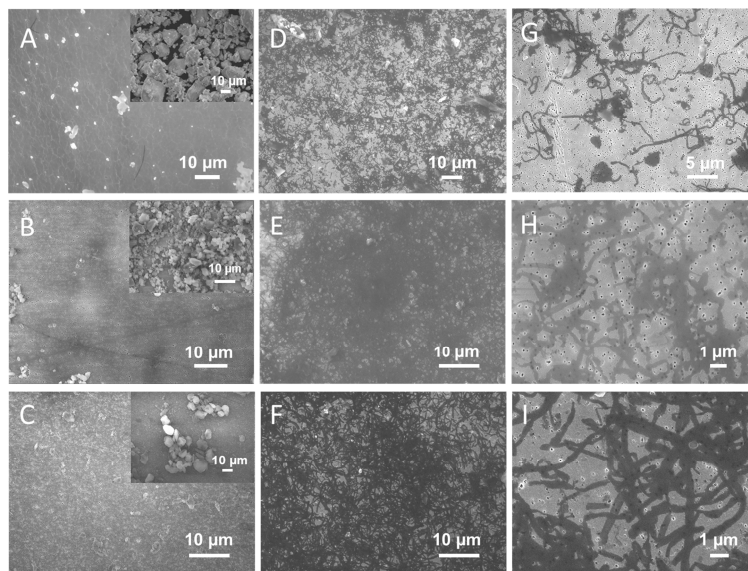


Figure 4.2. SEM images of the samples obtained by the adjuvant-assisted collection process. (A, D, G) with NaCl as adjuvant; (B, E, H) with fructose as adjuvant; (C, F, I) with dextran as adjuvant. Images (A-C) show the surface of the PET filter after removal of the cake, with almost no residual nanotube on the surface; insets give the morphology of the adjuvant powder. Images (D-I) are nanotubes recovered over an Au/Cr-covered PET membrane by filtration from an aqueous dispersion obtained by dissolving the corresponding adjuvant/nanotube cake in water. When NaCl was used as adjuvant, the nanotubes were collected over the adjuvant cake without adding NaCl adjuvant in the nanotube suspension in the second step of the collection procedure (Scheme 4.1, route 1); for other adjuvants, the procedure of route 2 in Scheme 4.1 was used.

The second step of the collection procedure, namely the filtration of the suspension of nanotubes mixed with the adjuvant powder, creates a mixed adjuvant/nanotube layer over the bottom adjuvant barrier, which can be rinsed safely by successive washing/filtration steps with pure CH_2Cl_2 to eliminate residual PC. Next, the cake is collected and dissolved in water, leading to a suspension of nanotubes in water. These tubes can then be collected by simple filtration over a PET membrane for imaging purposes and evaluation of the collection procedure (Figure 4.2 D-I).

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We found that the intimate mixing of nanotubes and adjuvant fine powder in the second step of the collection procedure is required to avoid the formation of aggregates of nanotubes. Indeed, the simple filtration of the nanotube suspension over the previously prepared adjuvant cake, without adding adjuvant in the nanotube suspension, led to the formation of clusters of nanotubes (Scheme 4.1, route 1). In this case, there was no adjuvant medium present among nanotubes, resulting in nanotube stacking up irreversibly over the barrier cake, regardless of the nature of adjuvants used for barrier cake. Figure 4.3 showed the FTIR spectra of enzyme nanotubes directly extracted from PC membrane and undissolved chunks collected from aqueous adjuvant solutions (NaCl, fructose and dextran, respectively). These chunks (Figure 4.3D-F) have similar FTIR spectral features than the free enzyme (Figure 4.3B) and the enzyme nanotubes (Figure 4.3C). In particular, the appearance of the characteristic peak of GOX enzyme at 1654 and 1541 cm^{-1} revealed the presence of enzyme nanotube component in these undissolved clumps. This was also confirmed by SEM and STEM. Figure 4.4 displays undispersible chunks collected from the fructose solution. In some thin positions of these clumps, nanotube coils were clearly observed in STEM image (Figure 4.4D). In many cases, a large clump of aggregated nanotubes was even visible to the naked eye in the aqueous solution. Consequently, the amount of free nanotubes collected from the aqueous suspension was limited, most of them being aggregated in this clump. As a result, the enzyme nanotubes suspension obtained without addition of fine adjuvants during the second filtration (route 1 of Scheme 4.1) exhibited weak activity in case where fructose and dextran were used as adjuvants (Figure 4.5). However, a comparable activity was observed with and without addition of fine NaCl powders in the nanotube suspension, owing to the release of free enzyme in solution from the clumps by the action of strong electrolyte NaCl (Figure 4.5). In contrast, Figure 4.2E and F show that a much larger amount of free nanotubes are collected (compared to the case of Figure 4.2D) when using the collection route 2 of Scheme 4.1. In addition, no trace of aggregated nanotubes was visible in the aqueous nanotube suspension with this collection strategy, resulting in high activity (Figure 4.5B). This demonstrates that almost all nanotubes are well trapped within the reservoir cake in route 2, and can be well dispersed in water during cake dissolution.

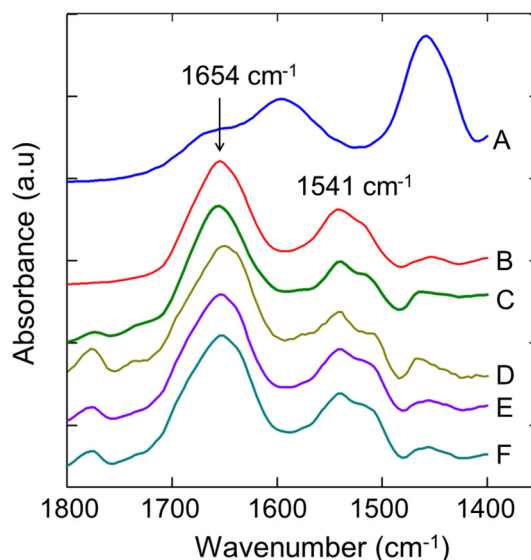


Figure 4.3. FTIR spectra of pure bPEI (A), GOX (B), (bPEI/GOX)₆ nanotubes (C), and undissolved clusters of nanotubes (6 bilayers) collected from aqueous NaCl (D), fructose (E) or dextran (F) solutions, respectively. The spectra are shifted vertically for clarity. For the collection of the clusters, the nanotube suspension in CH₂Cl₂ was simply filtered over the adjuvant cake barrier, without adjuvant added to the CH₂Cl₂ suspension of nanotubes in second filtration step (route 1, Scheme 4.1). After dissolution of the adjuvant cake in water, nanotube clusters were visible to the naked eye; they were picked up, rinsed in pure water and then dried for FTIR measurements.

The presence of adjuvants in nanotube suspension in CH₂Cl₂ in second filtration step is crucial to efficient recovery of free nanotubes and redispersion of them in aqueous solution. We also found that the size of adjuvant crystal is also a critical parameter regarding nanotubes collection efficiency and enzymatic activity. For instance, raw fructose is made of large crystals with several hundreds of micrometers (Figure S4.3A). Compared to the fine fructose powder (ca. 10 μm size, insert in Figure 4.2B), larger crystals (ca. 100 μm, Figure S4.3B) co-filtered in second filtration step lead to a lower activity (C compared to B in fructose section in Figure 4.5). This results from tiny nanotube aggregations during the collection procedure, as larger crystals increase the possibility of contact among nanotubes.

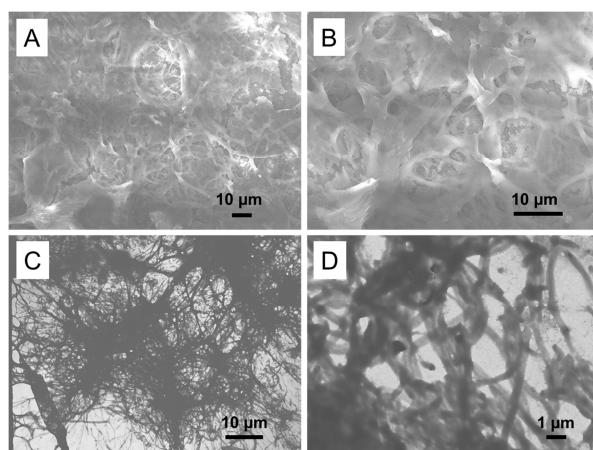


Figure 4.4. SEM (A,B) and STEM (C,D) images of clusters of nanotubes formed over the adjuvant barrier cake when the adjuvant powder is not added to the CH_2Cl_2 suspension of nanotubes in the second step of the collection procedure (route 1, Scheme 4.1). The adjuvant is finely-ground fructose.

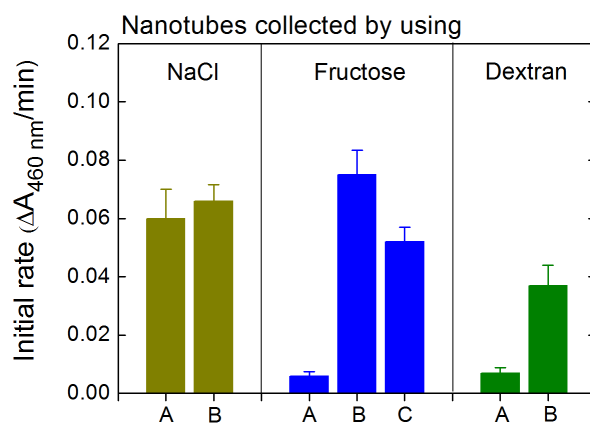


Figure 4.5. Activity of six-bilayer nanotube suspension harvested by using NaCl, fructose, and dextran as adjuvant, respectively, following two different collection routes in Scheme 4.1: (A) solution obtained by route 1 and (B,C) solutions harvested by route 2. The bigger fructose crystals (shown in Figure S4.3B) were used in second filtration step of route 2 instead of fine ones, leading to a lower activity (C). When fine NaCl was used as adjuvant, the comparable activity of nanotubes suspension (A) to (B) is ascribed to the enzymes released from nanotube caused by salt, as will be discussed later.

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Although efficient in collection route 2 of Scheme 4.1, the method apparently leads to broken and deformed nanotubes losing their tubular shape (Figure 4.2G-I and S4.4A-C), with only a limited number of fully intact nanotubes when NaCl is used (Figure 4.2G); however, nanotubes collected using fructose and dextran are less affected by the collection (Figure 4.2H,I). In contrast, the nanotubes mostly retain their tubular shape after dissolution of the PC templates in CH_2Cl_2 (Figures 3.16 and 3.17 in Chapter 3), indicating that deformation and fracture occur during at least one of the steps of our collection procedure. These steps involve (local) changes of ionic strength, generate concentration gradients and mechanical forces, and some of them could be detrimental to the integrity of the morphology of the nanotubes.

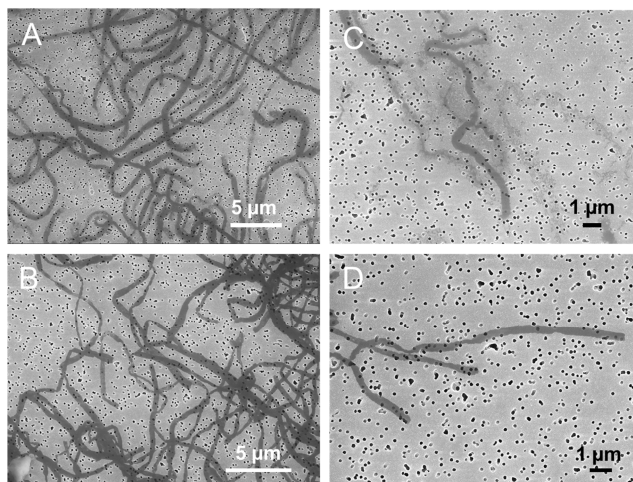


Figure 4.6. Images of nanotubes collected by adsorption on an Au/Cr-covered PET membrane from a dispersion in water obtained by the NaCl (A,C) and dextran (B,D)-aided collection method (route 2 of Scheme 4.1). The nanotube morphology is preserved by this gentler collection route.

We suspected that the collection step for imaging purposes of the nanotubes from their aqueous dispersion was the main cause of nanotube damage, because filtration required the application of a significant force. Therefore, in order to observe more directly the nanotubes floating in the aqueous solution, Au/Cr-coated PET membranes and TEM carbon-coated grids were simply immersed in the aqueous dispersion of free nanotubes. The nanotubes adsorbed on these surfaces, allowing us to observe many intact cylindrical

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nanostructures (Figure 4.6 and 4.7), different from the deformed ones obtained after filtration (Figure S4.4A,C). Despite the fact that a few fragments still exist with this gentler collection method, it is obvious that the morphology of the nanotubes after filtration from an aqueous suspension does not reflect the one of nanotubes in the aqueous suspension; nanotubes in water essentially preserve their integrity.

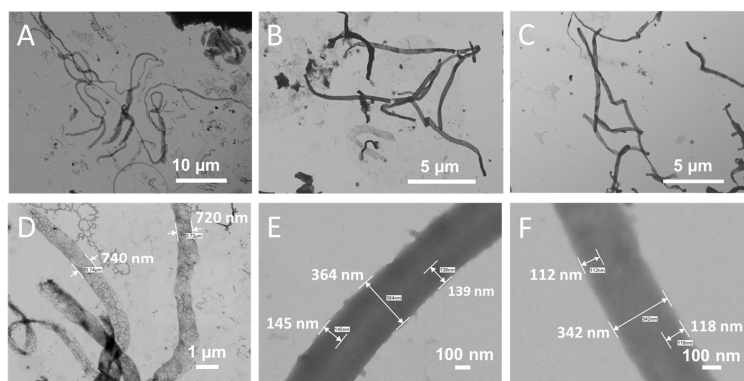


Figure 4.7. STEM images of nanotubes collected by adsorption on a TEM grid from a dispersion obtained by the adjuvant-aided collection method following route 2 of Scheme 4.1, with NaCl (A,D), fructose (B,E), and dextran (C,F) as adjuvants.

Our hybrid nanotubes are certainly soft and swollen in water, being therefore prone to bending or fracture, especially because of their large aspect ratio (ca. 80 in this work). From our images, the maximum length was estimated to be 25 μm , while the external diameter of nanotubes depends on the adjuvant used (Figure 4.7D-F); when using NaCl, it is ca. 700 nm, twice the value found when fructose and dextran were used (ca. 350 nm). Moreover, the nanotubes collected with NaCl as adjuvant are porous (Figure 4.7D), which we attribute to salt-induced degradation resulting from the screening of electrostatic interactions between the polyelectrolytes in the NaCl-rich aqueous solution obtained after dissolution of the adjuvant; this hypothesis is also supported by the observation of enzyme release during collection (results shown later). More robust nanotubes are obtained when fructose and dextran are exploited as adjuvants (Figure 4.7E,F). In these cases, no significant morphological differences were found between nanotubes collected from CH_2Cl_2 and from water, except for the higher flexibility of

water-collected nanotubes. As a conclusion, the extraction of nanotubes from polymer templates in a quantitative amount, followed by dispersion in water, was successfully achieved by the collection methodology of Scheme 4.1 (route 2), yielding a clear aqueous dispersion of intact nanotubes when fructose or dextran is used as filtration adjuvant. It is noteworthy that this approach can also be applied to a variety of nanotubes or nanorods, as will be reported elsewhere¹¹.

4.3.2 Activity of membrane-immobilized and liberated nanotubes

Since the relative composition in GOX is about constant in the nanotubes, independent of the number of bilayers, the total amount of enzyme contained in the nanotubes simply scales linearly with the deposited volume V of the film, which can be directly obtained from the measured pore diameters R according to $V = \pi L(R_m^2 - R^2)$, where R_m is the radius of the bare pore and L its length. Figure 4.8 shows that the pore diameter decreases regularly with the number of deposition cycles until 8 bilayers, then saturates; in parallel, the nanotube wall thickness regularly increases then saturates. The volume deposited in the pore is also displayed in Figure 4.8; within experimental precision, the adsorbed volume and therefore the amount of incorporated enzyme, increases linearly with the number of cycles until it saturates above 8 cycles.

The growth mechanism of polyelectrolytes in nanoporous templates was discussed extensively for the case of simple immersion in a previous article⁴⁰. Two regimes exist in the evolution of wall thickness with number of deposition cycles, with a transition between the regimes for pores filled by ca. 70% (in the dry state). Below this threshold, the growth occurs as in flat films, with however quantitative differences in the amount of polyelectrolyte deposited per cycle. In the second regime when the pores are filled by more than ca. 70% (in the dry state), the pores are actually completely filled in solution due to multilayer swelling. Therefore, freshly brought polyelectrolytes only slowly diffuse in the highly hydrated filled nanopores, resulting in the formation of a progressively denser gel, and in a much slower relative increase of the dried wall thickness of nanotubes. In our case, 70% filling in the dried state corresponds to the transition from 8 to 9 bilayers,

which explains why the growth of the LbL is slowed down at this stage. In this growth regime, polyelectrolyte diffusion into the nanopores filled with the swollen multilayer becomes the limiting factor.

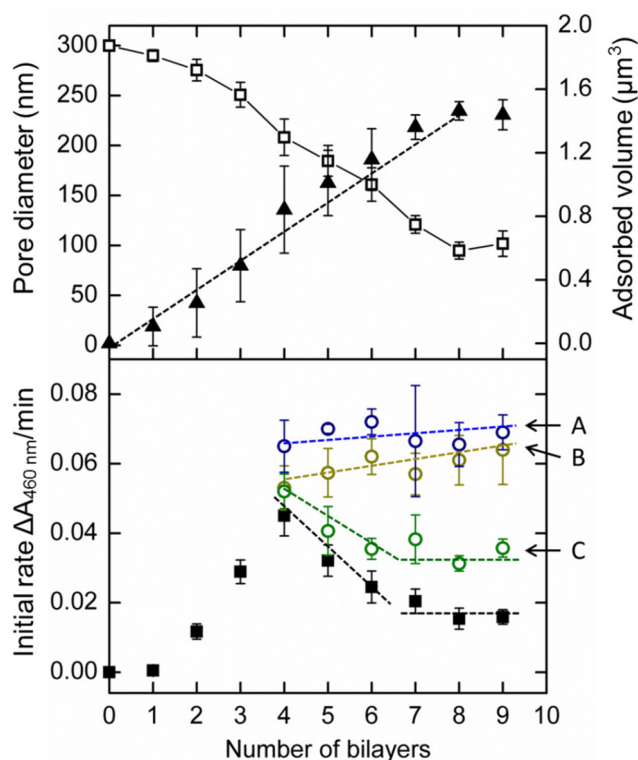


Figure 4.8. Pore diameter of LbL-filled membranes (open squares, left axis), adsorbed volume per pore (solid triangles, right axis), and activity of embedded (solid squares) and freed (open circles) nanotubes as a function of the number of bilayers. The free nanotubes were collected with fructose (blue, A), NaCl (yellow, B), and dextran (green, C) as adjuvant during the collection. GOX in 10 mM HEPES buffer (pH 7.0) was alternately assembled with bPEI in 10 mM HEPES buffer (pH 7.0), followed by rinsing with the same buffer. The nanotube suspension was obtained by the adjuvant-aided collection method following the route 2 of Scheme 4.1, except for samples collected by using NaCl, in which case no NaCl was added to nanotube suspension in PC/ CH_2Cl_2 in the second step of collection (route 1). The same amount of membrane sample was used for all nanotube collections and activity measurements. The dotted lines are drawn to guide the eye.

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Although the amount of enzyme in the nanotubes increases close to linearly up to 8 bilayers, the enzymatic activity of membrane-embedded nanotubes exhibits a maximum for 4 bilayers (Figure 4.8, full squares). Below this maximum, the activity logically increases with the number of bilayers due to the incorporation of larger amounts of enzyme; above this maximum, the activity decreases due to the restricted diffusion of the substrate into the increasingly narrower open channels of the tubes. Above 8 bilayers, when the pores are fully filled by the swollen multilayers, the activity remains constant, corresponding to the limited variation of enzyme content in this regime and almost constant diffusion hindrances.

For activity measurements of liberated nanotubes, the role of the adjuvant used during the collection process was investigated in details. The use of NaCl as adjuvant results in a strong release of the enzyme from the nanotubes, due to the partial disassembly of the tubes in the presence of the relatively high ionic strength resulting from the dissolution of the adjuvant (Figure 4.9). The activity of an aqueous suspension of nanotubes was measured just after the dissolution of the adjuvant cake (A in Figure 4.9); then, intact nanotubes were removed by filtration after 5 min of gentle stirring, and the activity of the nanotube-free solution was measured again (B in Figure 4.9). For the NaCl adjuvant, the activity of the nanotube-free solution was high and identical to the initial one, demonstrating that the enzymes are released from in the nanotubes: they are essentially present as free enzymes, or are complexed in very tiny fragments of tubes which are not filtered out. In contrast, when collected with fructose or dextran as adjuvants, the activity of filtered suspensions of nanotubes was much lower than prior to the removal of the nanotubes (Figure 4.9, compare columns A and B), even after 2h agitation of the nanotube suspension (columns E), demonstrating that these adjuvants essentially do not release the enzyme from the tubes. These conclusions were fully confirmed by monitoring the activity of nanotubes still embedded in the membranes, after exposure to aqueous solutions containing 22.5 mg/mL of added NaCl, fructose or dextran (Figure S4.5): while the addition of NaCl released a large amount of enzymes, no release was observed from the membranes exposed to fructose or dextran.

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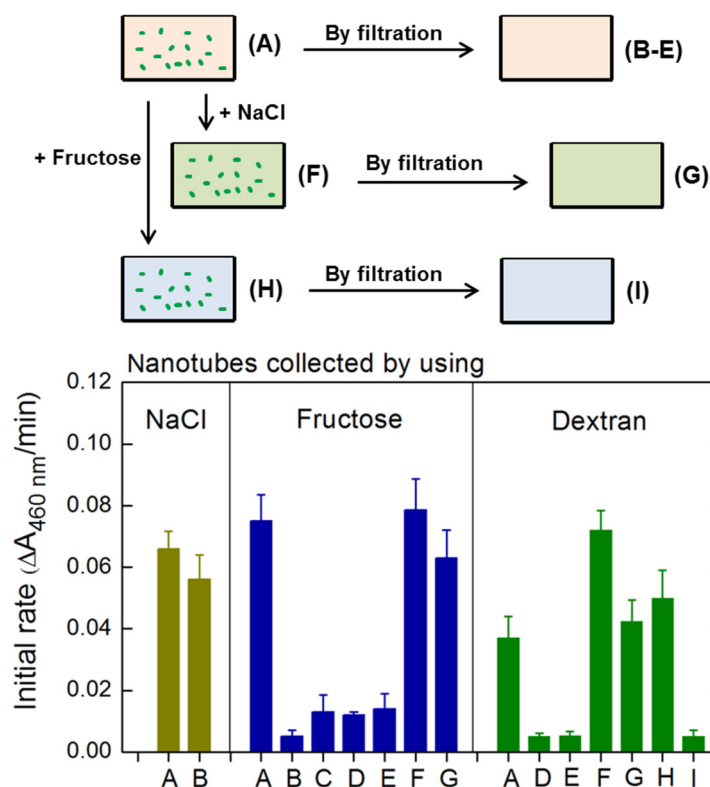


Figure 4.9. Activity of 6-bilayer nanotube suspensions (columns A) obtained by using NaCl, fructose, and dextran as adjuvant, respectively, following the route 2 of Scheme 4.1 (dissolution times were 5 min for NaCl and fructose, and 45 min for dextran); activity of solutions after removal by filtration of the nanotubes from the relevant suspensions shown in columns A, after agitation of the aqueous nanotube suspensions for 5 min (columns B), 20 min (columns C), 1 h (columns D) and 2 h (columns E). Columns F and G show the activities of the 6-bilayer nanotube aqueous suspensions, followed by (columns F) addition of 45 mg NaCl and 20 or 45 min gentle stirring for fructose- or dextran-based collected samples, respectively, and (columns G) removal of the nanotubes from this salted solution by filtration. Columns H and I follow the same protocols as columns F and G (dextran), respectively, except that 45 mg fructose was added instead of NaCl. The scheme shown on top is meant to describe the protocol used to evaluate the influence of adjuvant on the activity of liberated nanotubes in aqueous dispersion.

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Nevertheless, significant differences are observed between the dextran and fructose-based collection methods, with a significantly smaller activity of the nanotubes observed when dextran is used as adjuvant (Figure 4.9, columns A). To check that these differences are not due to variations in the number of collected nanotubes, we added NaCl to the aqueous nanotube suspensions after their collection, in order to partially destroy the tubes and release as much as possible the GOX enzyme; the activity of these suspensions was then found to be nearly identical for the two adjuvants (Figure 4.9, columns F), suggesting that the amount of enzyme is indeed similar for the nanotubes collected in the presence of fructose or dextran. In addition, we monitored the activity of the free enzyme towards glucose in the presence of fructose or dextran, and found no influence of the adjuvant (Figure S4.6); furthermore, no activity was exhibited by free GOX enzymes in the absence of glucose and in the presence of fructose or dextran (Figure S4.6). This confirms the highly specific catalytic activity of GOX towards its substance, D-glucose, and the absence of synergetic effects with fructose or dextran.

It has been reported that dextran can improve the stability of some enzymes towards the pH, temperature, for instance horseradish peroxidase⁴¹ and β -fructofuranosidase⁴², by probable adsorption onto these enzymes through various interactions. This adsorbed dextran might protect catalytically-active enzyme structure; however, it does not induce the limitation of access of substrate to active sites of enzymes, since we did not observe a decreased activity of free GOX enzymes in the presence of dextran.

The influence of adjuvant on the structure of liberated nanotube in aqueous dispersion may play an important role in the activity of nanotubes, since it may change the permeability of nanotubes' wall, which is related to the ease of substrate access to confined enzymes. However, the adjuvant is always present in aqueous dispersion of nanotubes; the control sample without adjuvant is unavailable. Hence, the catalytic activity towards glucose of planar (bPEI/GOX)_{6.5} films over silicon substrate in the presence and absence of adjuvant was tested, in order to check whether the diffusion of the adjuvant into the LbL films modifies or not the film permeability towards glucose (Figure S4.7). Similarly to the free GOX enzymes, the activity of the 6.5-bilayer planar films towards glucose was found to be constant at ca. 0.015 min⁻¹, independent of the added fructose concentration, and identical to the one measured in the absence of fructose. However, the presence of dextran reduced slightly the activity to ca. 0.013 min⁻¹. This small variation

suggests that dextran might slightly impede the diffusion of glucose in the films, maybe due to its limited adsorption on the film surface. At any rate, this barrier effect is minor, and cannot explain the large difference of activity between nanotubes collected in the presence of fructose or dextran (about a factor of 2 as shown in Figure 4.9). Figure 4.10 shows the FTIR spectra of pure dextran and dextran-collected nanotubes. It is difficult to identify whether there is dextran present on/within nanotubes because of the peak superposition in FTIR spectra. However, the amount of adsorbed dextran is certainly extremely limited.

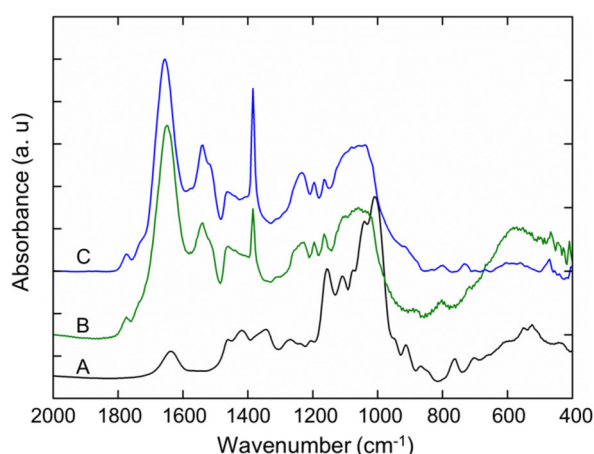


Figure 4.10. FTIR spectra of pure dextran (A, black line), dextran-collected nanotubes (6 bilayers) in aqueous suspension (B, green line), and 6-bilayer nanotubes extracted directly from PC template (C, blue line). The liberated nanotubes suspended in dextran aqueous solution was gathered by filtration for FTIR measurement following the approach described in section 4.2.6.

The main difference between fructose and dextran is their molar mass, and therefore the associated difference of osmotic pressure resulting from the difference of concentration of the adjuvant in the aqueous solution and in the nanotube. Fructose is a small molecule, while dextran is a neutral polymer with large molecular dimension (radius of gyration, *ca.* 8 nm for dextran 70)^{43,44}, existing as an expandable coil in the solution. As a result, much larger swelling forces are exerted on the nanotubes when they are in presence of fructose than in the dextran solution, which might very well explain the subsequent large permeability of the nanotubes towards glucose. An

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experiment was performed to support this hypothesis: the nanotubes were collected with either fructose or dextran as adjuvant, then NaCl was added to the suspensions to disrupt the nanotubes. The suspensions were then filtered to remove intact nanotubes, and the residual activity of the nanotube-free solutions were measured again (columns G in Figure 4.9). The residual activity was significantly larger for the fructose collection route compared to the dextran-based one, indicating that the nanotubes were much less resistant to NaCl in the presence of fructose than in the presence of dextran. Furthermore, the addition of fructose in the nanotube suspension obtained with dextran as adjuvant increases moderately the activity (column H in Figure 4.9), although this effect is weaker than when adding NaCl.

The dry and wet thicknesses of flat 6.5-bilayer films in different aqueous media were measured by AFM, in order to evaluate the swelling behavior under different conditions (Figure 4.11 and S4.7), summarized in Table 4.1. To eliminate the contribution of variation in film thickness from one sample to another to swelling degree, the thickness in dry and subsequent wet state was probed at exactly the same position for each aqueous media, as shown in Figure S4.8 and Figure 4.11. Identical surface features of films were observed in the dry and wet state with only difference in the size of dots present on the film surface. In the wet state, these dots appear larger than in the dry state, showing that the film is well swollen by the various aqueous media. The average dry thickness of flat 6.5-bilayer film was determined to be ca. 20 ± 3 nm. The appearance of thick film of 25 nm (Figure S4.8C and Table 4.1, acetate buffer) demonstrated the rationality to take the variation in flat film thickness into account. By measuring the wet thickness of the same film under different aqueous media (Figure 4.11 and S4.8), the swelling degree was found to be significantly different, 44%, 63%, 118% and 79% in water, acetate buffer, fructose and dextran solution, respectively (Table 4.1). These data show that compared to pure water, solutes present in solution induce further swelling of the LbL film. Therefore, the sole contribution of fructose or dextran solute to film swelling was calculated by subtracting the swelling degree in acetate buffer from that in relevant adjuvant solution of acetate buffer.

The 'deformation' images collected in peak force quantitative nanomechanical mapping (PeakForce-QNM) model allow us to estimate the tip penetration in soft enzyme films (Figure S4.9), since this deformation is approximately equal to the tip indentation. The tip penetration in dry and

hydrated films was ca. 0.2 and 7 nm, respectively. Larger penetration in hydrated films leads to a slightly underestimated swelling degree. However, these values are relatively small; in particular, the penetration in hydrated film was similar, regardless of media, thus causing a negligible influence on the comparison of the swelling degree of films in different media.

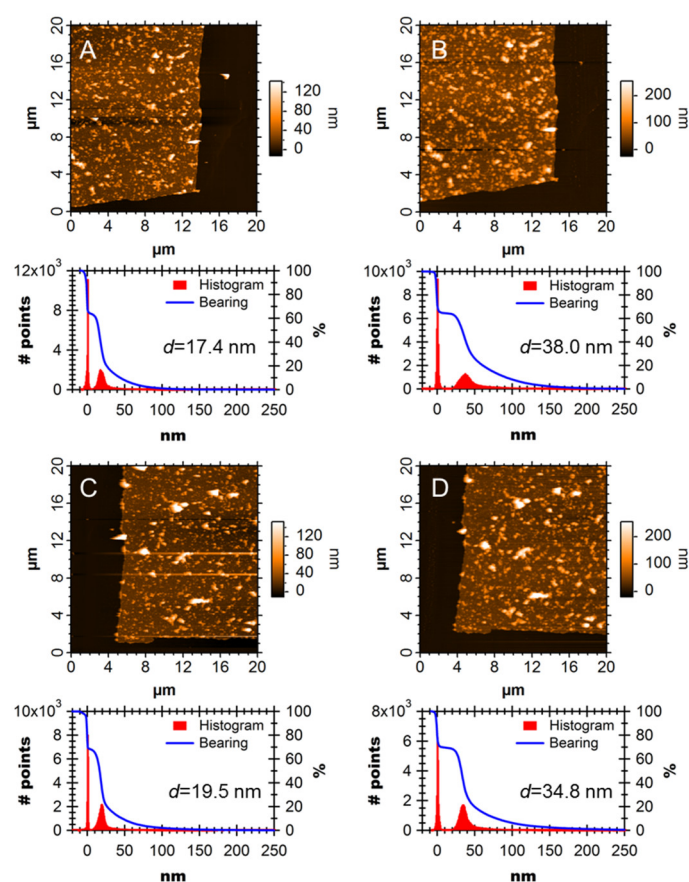


Figure 4.11. Dry (A, C) and wet thickness in fructose (B) and dextran solution (D) of 6.5-bilayer film prepared in 10 mM HEPES pH 7.0 measured by AFM. The concentration of fructose and dextran for fluid cell is 22.5 mg/mL in 50 mM sodium acetate buffer pH 5.5, identical to the solution for activity measurements of fructose/dextran-collected nanotubes. The thickness, d was obtained by statistics analysis (beneath each AFM image), indicated in histogram graphs.

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Table 4.1. Swelling degree of 6.5-bilayer planar films in different media measured by AFM*.

Medium	Dry thickness ^c (nm), d_{air}	Wet thickness ^d (nm), $d_{aqueous}$	Swelling degree (%)
Pure water	19.5 ^{c1}	28 ^{d1}	44
Acetate buffer ^a	25 ^{c1}	41 ^{d1}	64
Fructose in buffer ^b	17.5	38	117
Dextran in buffer ^b	19.5	35	79

* Only one sample was used for each aqueous medium. The dry and wet thickness obtained on exactly the same film area was used to calculate the swelling degree for each aqueous medium, leading to reliable results.

^a 50 mM sodium acetate, pH 5.5.

^b 22.5 mg/ml fructose or dextran in 50 mM sodium acetate, pH 5.5.

^c The thicknesses measured on as-prepared films by AFM in air before samples were imaged in fluid. ^{c1} Figure S4.8.

^d The thicknesses of the identical samples in aqueous conditions. ^{d1} Figure S4.8.

Swelling induced by fructose and dextran solutes were found to be approximate 53 % and 15%, respectively, suggesting that fructose exerts a larger osmotic force that increases the swelling of films further than dextran. However, further swelling by fructose solutes does not increase the activity of the flat film significantly further and neither does dextran (Figure S4.7).

One should be aware that compared to the macroscopic ‘open’ film systems, nanotubes have a specifically nanoconfined structure. Thus the wall swelling might play a more important role in the ease of glucose access to enzymes confined in nanocompartments.

These observations support the notion that fructose swells more strongly the nanotubes than dextran and contributes to their disruption. Therefore, we also associate the larger activity of the nanotubes in the presence of fructose to their higher degree of swelling and therefore easier accessibility of

Collection and Activity of Liberated Enzyme Nanotubes

glucose to the enzymes. In contrast, the accessibility of glucose to the enzyme in dextran-collected nanotubes is hindered to some extent by their lower degree of swelling. Therefore, in the sequel, we concentrate on the dextran-based route, as it is more innocuous to the structure of the nanotubes.

As was evidenced, the amount of enzyme incorporated in the nanotubes increases linearly with LbL growth, until saturation is reached for 8 bilayers. This is not the case for the activity, shown in Figure 4.8 for the membrane-embedded nanotubes, the dextran-collected free nanotubes, fructose-collected free nanotubes, and the NaCl-collected free (and largely destroyed) nanotubes. No significant difference of activity is seen for these three systems for 4 bilayers; below this number, free intact tubes could not be collected due to their too weak mechanical properties. However, the activity of membrane-embedded nanotubes simply scales linearly below 4 bilayers, and it is most likely also the case of the free nanotubes reported before³¹. Above 4 bilayers, the nanotubes collected with NaCl and fructose as adjuvant exhibit a much larger activity than membrane-embedded nanotubes, due to the massive release of the enzyme for NaCl-collected nanotubes and the higher swelling of fructose-collected nanotubes in this collection condition.

In contrast, liberated nanotubes collected in the presence of dextran exhibit an activity which follows a similar trend as nanotubes embedded in the membrane, although their activity is significantly higher. Contrarily to nanotubes embedded in the templates, the free dextran/fructose-collected nanotubes have no limitation of accessibility around their outer surfaces, thereby enabling the diffusion of glucose and products in and out of nanotubes not only from their ends and inner surface but also through their hydrated external walls as reported elsewhere³¹. It is significant that the observed difference of activity of membrane-embedded and dextran-collected free nanotubes is about a factor of 2 (in the plateau region), which corresponds roughly to the increase of the accessible surface for free nanotubes compared to embedded ones.

4.4 Conclusions

Robust glucose oxidase-loaded nanotubes were synthesized by template-assisted layer-by-layer assembly. A quantitative collection methodology was developed based on adjuvant-assisted filtration, with either NaCl, fructose or dextran as adjuvant. While NaCl induces a release of enzymes from liberated nanotubes, clear aqueous suspensions of nanotubes of intact tubular morphology could be obtained with fructose or dextran. Nevertheless, the larger osmotic pressure of fructose compared to dextran leads to less stable and more swollen nanotubes; as a consequence, the use of dextran is recommended, leading to the efficient and quantitative extraction of nanotubes from the membrane templates. The enzymatic activity of the nanotubes either embedded in the membrane or released in suspension was measured. The activity of embedded nanotubes exhibits a maximum for 4 bilayers due to the interplay between the amount of enzyme embedded in the tubes and the accessibility of glucose to the enzyme; dextran-collected freed nanotubes display a similar trend, but have a higher activity than embedded nanotubes thanks to the increased diffusion of the enzyme substrate through external lateral surfaces. As for fructose-collected free nanotubes, they exhibit an even higher activity most probably due to very swollen walls of the nanotubes. The larger swelling degree of enzyme films in fructose solution compared to dextran solution was observed, confirming the possible contribution of fructose adjuvant to increased activity of liberated nanotubes. These nanotubular enzymatic structures thus appear as promising swarming agents for the transformation of glucose in a solution; the generalization of this approach to other enzymes would broaden even further perspectives of application.

Supporting information

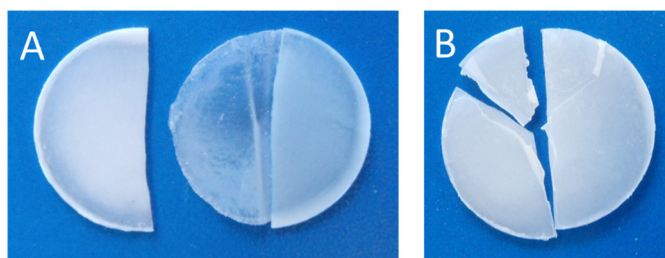


Figure S4.1. Preparation of FTIR samples of free nanotubes collected from aqueous suspension by using nanoporous PC (A) or PET (B) membrane as a filter. The PC membrane is strong enough to be entirely detached from the KBr pellet manually, while the brittle PET membrane makes it difficult to be removed completely. The residual PET would contaminate the FTIR samples; therefore, the PC membrane is recommended.

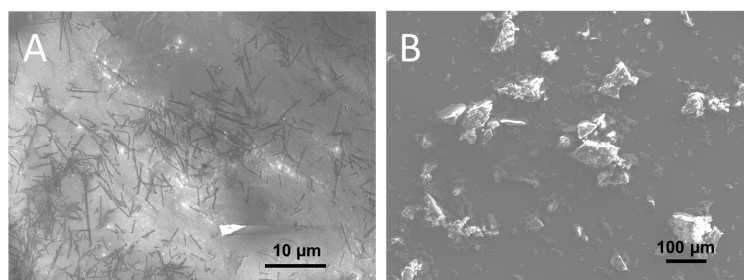


Figure S4.2. Microscopy images of 6-bilayer nanotubes (A) on the filter after removal of the adjuvant cake when fructose of large powder size (B) is used in the fabrication of the barrier cake layer. The microporous structure of barrier layer resulting from the large size of irregular fructose powder allows nanotubes passing through the fructose layer during the filtration, leading to lower collection efficiency.

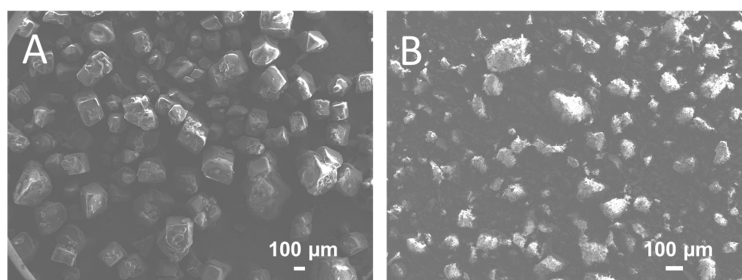


Figure S4.3. SEM images of raw (A) and slightly milled (B) fructose crystals (Vibrating mill, Perkin-Elmer & Co. GMBH). The fructose crystals and a ball are added into a small stainless steel tubes with detachable caps; then this assembly is placed in vibrating mill machine to crush fructose crystals for different times.

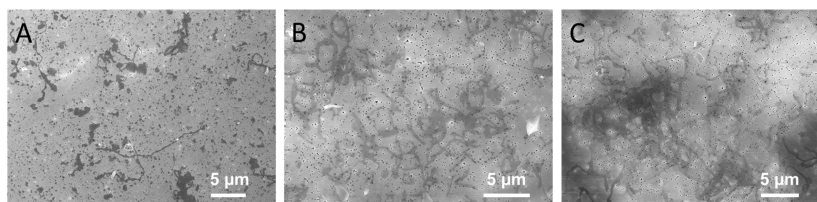


Figure S4.4. SEM images of 6-bilayer nanotubes collected by filtration over an Au-coated PET membrane from an aqueous suspension of nanotubes obtained by the NaCl (A), fructose (B), and dextran (C)-assisted collection strategy following route 2 of Scheme 4.1.

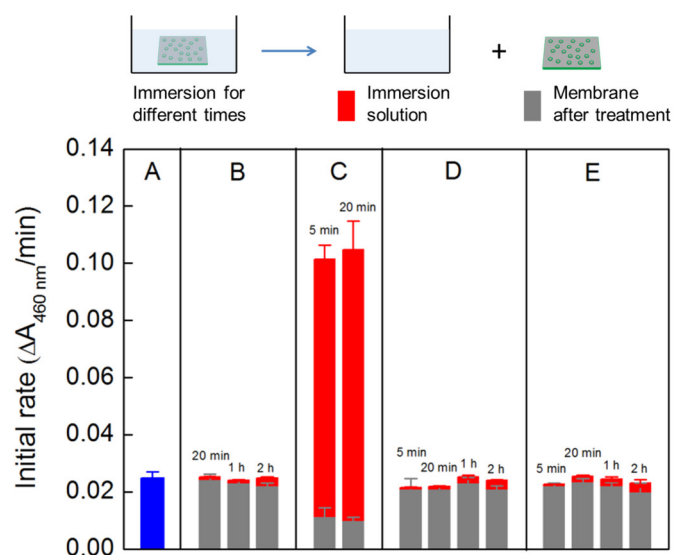


Figure S4.5. Investigation of the effect of adjuvants on the stability of 6-bilayer nanotubes embedded in the templates, by measuring the activity of the initial nanotube-immobilized membrane (A) and membranes after immersion of the membrane in (B) 50 mM sodium acetate buffer, pH 5.5, (C) 45 mg NaCl in 2 mL of 50 mM sodium acetate buffer, pH 5.5, (D) 45 mg dextran in 2 mL of 50 mM sodium acetate buffer, pH 5.5, and (E) 45 mg fructose in 2 mL of 50 mM sodium acetate buffer, pH 5.5, for times varying from 5 min to 2h. The activity measurements of all membranes were performed in 50 mM sodium acetate buffer, pH 5.5 in a period of 20 min; this 20 min is different from the immersion time described above. For initial nanotube-immobilized membrane (A), it has contact with 50 mM sodium acetate buffer, pH 5.5, only when the measurement of activity is conducted (in blue). For membranes (B-E), these membranes were first immersed in 50 mM sodium acetate buffer, pH 5.5 for different times, and then were taken out from the buffer followed by wash in pure water and dry. Afterwards, the activity of these immersion-treated membranes was tested in 50 mM sodium acetate buffer, pH 5.5 in another period of 20 min (in gray). The activities of the immersion buffer solutions after removal of the membranes were tested as well, and are shown in red.

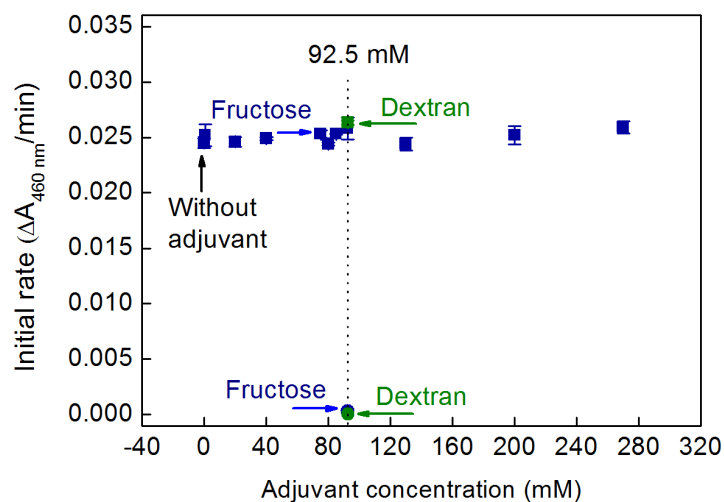


Figure S4.6. Effect of the presence of the adjuvants (fructose, full blue squares; or dextran, full green squares) on the activity towards glucose of free GOX in solution. The full circles indicate the activity towards either dextran (green) or fructose (blue), in the absence of glucose. Activities were tested by following a protocol identical to the one used for nanotube-embedded membranes, with a GOX and glucose concentration in the cuvette of 0.925 $\mu\text{g}/\text{ml}$ and 0.56 mM, respectively (except for the full circles, for which no glucose but 0.5 ml of H_2O was added in the cuvette). The vertical dotted line at 92.5 mM adjuvant corresponds to the final concentration of adjuvant in the cuvette, for our other experiments measuring the activity of suspensions of freed nanotubes. For dextran, the molarity was computed from the molar mass of the repeat unit (glucose, 180 g/mol).

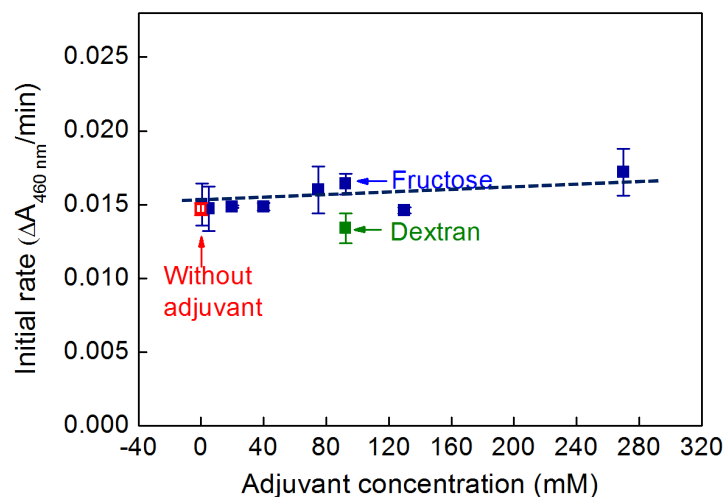


Figure S4.7. Effect of the presence of an adjuvant (fructose, full blue squares; dextran, full green squares) on the activity of flat (bPEI/GOX)_{6.5} LbL films prepared in 10 mM HEPES buffer, pH 7.0, throughout. The red open square is the activity of this flat film towards glucose in the absence of added adjuvant. The dry thickness of the 6.5-bilayer films was approximately 35 nm, as measured by ellipsometry. The dotted line is drawn to guide the eye.

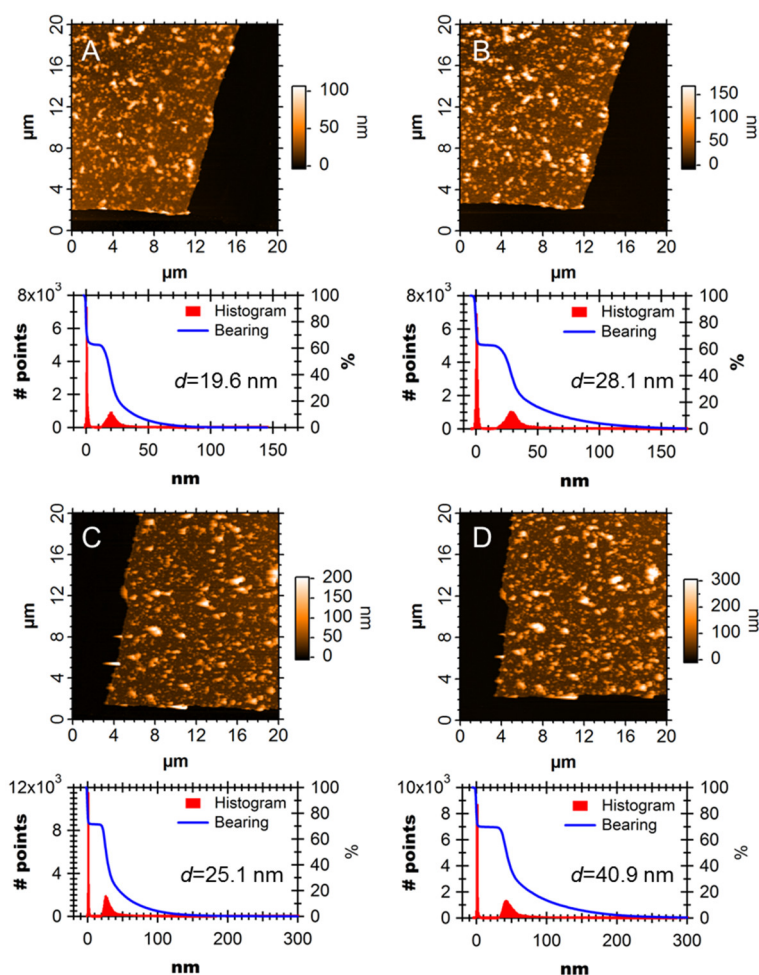


Figure S4.8. Dry (A, C) and wet thickness in pure water (B) and 50 mM acetate buffer (pH 5.5) (D) of (bPEI/GOX)_{6.5} film prepared in 10 mM HEPES pH 7.0 measured by AFM. The thickness, d was obtained by statistics analysis (beneath each AFM image), indicated in histogram graphs.

During the collection of topography images on hydrated film in different media, we also obtained the 'deformation' information about the films. This allows us to get some information on the tip penetration in hydrated film, which might affect the wet thickness measured by AFM.

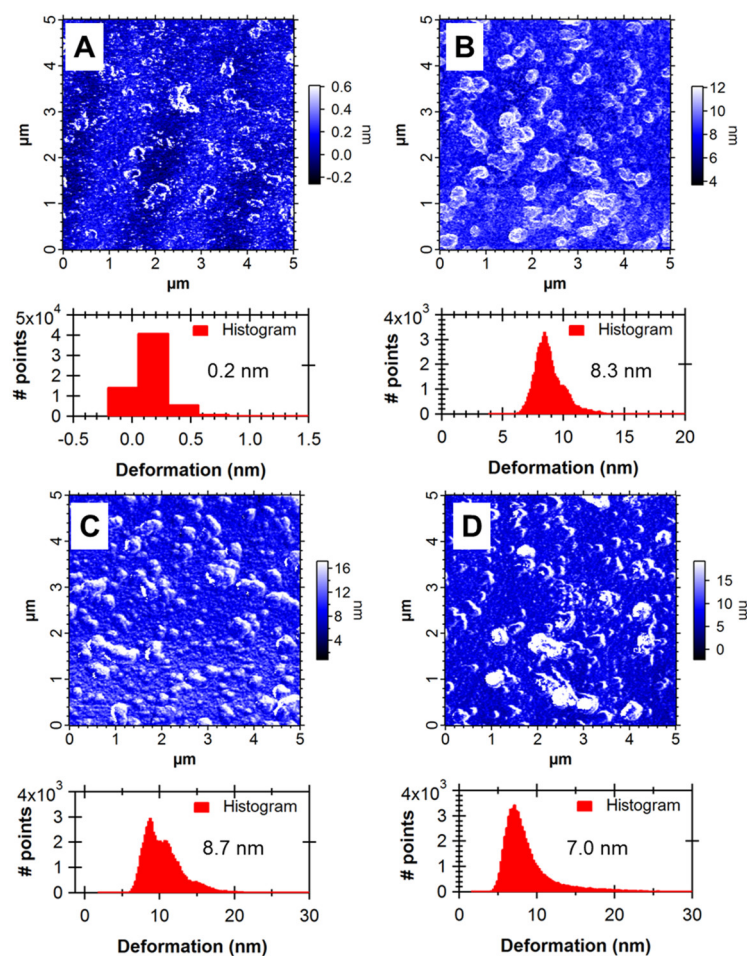


Figure S4.9. 'Deformation' images (AFM) collected in PeakForce-QNM model on dry 6.5-bilayer film in air (A), and hydrated 6.5-bilayer films in (B) 50 mM acetate buffer, pH 5.5, (C) 50 mM acetate buffer, pH 5.5 containing 22.5 mg/mL fructose, and (D) 50 mM acetate buffer, pH 5.5 containing 22.5 mg/mL dextran. These films were prepared in 10 mM HEPES buffer, pH 7.0 at the same time, and an independent film was used in each condition. The average deformation value (indicated in histogram graphs) was obtained by the statistics analysis (beneath each image).

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Chapter 5

Mats of layer-by-layer assembled nanotubes

In this chapter, we demonstrate the filtration-based fabrication of 'paper' mats made of nanotubes prepared by layer-by-layer (LbL) assembly in the nanopores of sacrificial polycarbonate (PC) membranes. Adjuvants are also exploited to assist the construction of the mats during filtration, aiming at producing self-standing mats. The structure of the resulting mats, which is investigated by SEM and AFM, depends not only on the collection protocol but also on the nature and properties of the nanotubes. The activity of enzyme nanotube mats prepared over a nanoporous PET filter is evaluated, demonstrating these systems to be promising platforms for bio-purification.

5.1 Introduction

Networks of fibrillar proteins such as elastin, collagen, and keratin are often present in natural tissues and organisms, acting as structural and functional scaffolds to define tissues, guide a biochemical process, or provide support to cells¹⁻⁷. These sophisticated nanofibrous architectures created by nature are providing researchers with an inspiring guide for the design of artificial scaffolds made of synthetic functional nanomaterials. In addition to natural nanofibrillar components (fibrillar proteins), artificial nanofibers or nanotubes made of a variety of natural and synthetic polymers or nonfibrillar proteins can also be generated by appropriate technologies⁸⁻¹⁰, among which electrospinning received most efforts. Electrospun nanofibers or nanotubes incorporating functional components have a breadth of potential applications in fields as diverse as tissue engineering^{8,11-15}, environment¹⁶⁻¹⁸ and energy¹⁹⁻²³. However, electrospinning requires applying voltage between a spinneret and a grounded target that is placed at a fixed distance from the tip of the spinneret. Moreover, limiting factors are also the requirements for an adequate solvent able to lead to bead-free nanofibers and for crosslinking to improve the stability of electrospun fibers, and the high shear forces which may disturb the secondary structure of proteins during the electrospinning process^{8,24-28}. For instance, it has been demonstrated that smooth and uniformly distributed nanofibers can be obtained efficiently only when the electrospinning solution is less than 50% in enzyme content²⁶. In addition, significant changes were reported in the secondary structure of collagen during its electrospinning²⁷. The relatively harsh processing conditions thus make electrospinning not suitable for the encapsulation of substantial amounts of biomacromolecules into nanofibers, especially enzymes prone to denaturation, which may lose bioactivity in electrospun protein fibers²⁸.

An alternative method of biofunctional nanofiber fabrication is template-synthesis combined with layer-by-layer (LbL) assembly, leading to nanofibers or nanotubes²⁹⁻³¹ replicating the nanochannels of a polymer matrix. LbL assembly is an extremely versatile technology involving alternated adsorption of complementary species through electrostatic, hydrogen bonding, or hydrophobic interactions³²⁻³⁴. The most compelling feature of the LbL approach is its aqueous adsorption conditions compatible with a variety of biomacromolecules, allowing for gentle incorporation of these biomacromolecules in nanofibers or nanotubes; thereby, ensembles of

LbL-nanofibrils or nanotubes consisting of biofunctional components could preserve their biological activity³⁵⁻⁴². Moreover, enzyme components were found to dominate LbL enzyme nanotubes with in favorable cases a content higher than 80%^{42,43}. Templated LbL technology thus provides an elegant way for incorporating substantial amounts of proteins in nanotubes or nanowires.

Well-defined nanotubes/wires can be achieved by LbL assembly in nanoporous membranes used as sacrificial templates. A tridimensional network of nanotubes is harvested when a polymer matrix with an intersecting nanochannel network is used as template. When template membranes with vertically aligned nanochannels are used, well-separated LbL nanotubes are obtained, and can be used as building blocks for the construction of nanotube networks after release. Networks consisting of vertically aligned LbL-nanotubes were used recently for ionic separation, in which LbL-nanotube modified membranes were sequentially deposited on a porous support with intermediate dissolution of sacrificial templates to produce a framework of well-packed vertically-aligned nanotubes⁴⁴. Recently, clusters of LbL nanotubes were also serendipitously observed during the collection of enzyme nanotubes by our group⁴². Thereby, we surmised that mats of randomly positioned LbL-nanotubes can be obtained by a type of paper-making process, consisting of the simple filtration and sedimentation of LbL-nanotubes onto a sacrificial support.

Herein, we use vacuum-based filtration to prepare LbL-nanotube 'paper' mats, whose detailed structure is investigated by SEM and AFM. In one protocol, sacrificial adjuvants are exploited to assist the fabrication of a self-standing mat by filtration; in another one, the nanofiber mat is directly collected on a filtration membrane. LbL-nanotubes with different structure and composition were prepared to investigate the impact of the procedures used during the filtration process as well as the nature of the nanotubes on the resulting mat surface morphology and internal structure with the aim of elaborating a porous structure in which LbL-nanotubes retain their integrity. Finally, the activity of enzyme nanotube mats fabricated on support filters is examined for the fabrication of biocatalytic artificial 'papers' for purification.

5.2 Experimental section

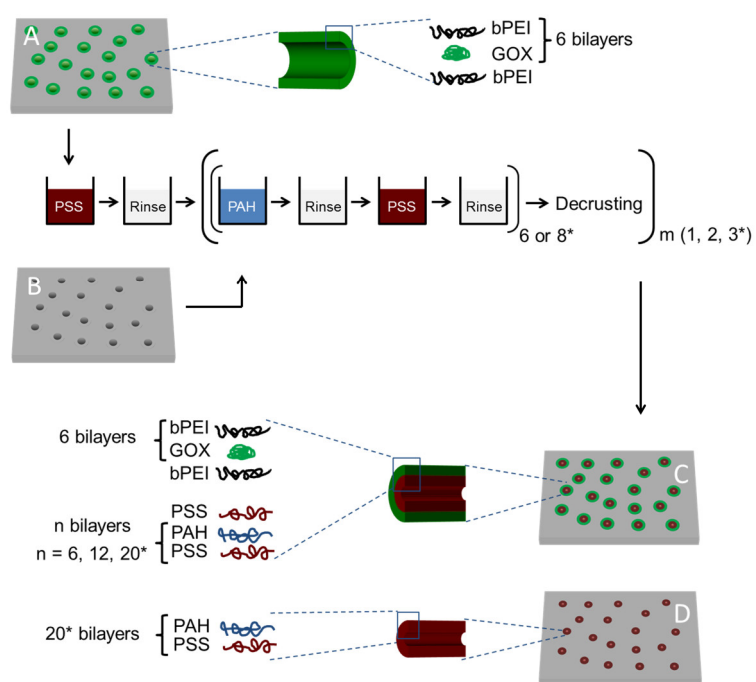
5.2.1 Materials

Glucose oxidase (GOX from *Aspergillus niger*, type x-s lyophilized powder), horseradish peroxidase (HRP, type VI, lyophilized powder), branched polyethyleneimine (bPEI, M_n ca. 60,000, M_w ca. 750,000 g·mol⁻¹, 50 wt. % in H₂O), poly(sodium 4-styrenesulfonate) (PSS, M_w ca. 70,000 g·mol⁻¹), sodium chloride, *o*-dianisidinedihydrochloride, glucose, fructose and dextran (M_r ~70,000, *Leuconostoc* spp.) were purchased from Sigma-Aldrich. 2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulfonic acid (HEPES free acid), potassium bromide (FT-IR grade, ≥ 99%), calcium chloride and dichloromethane (99.8%, for analysis) were provided by Acros Organics. Poly(allylamine hydrochloride) (PAH, M_w , 120,000-200,000 g·mol⁻¹) was obtained from Alpha Aesar. All polyelectrolyte solutions were freshly prepared at a concentration of 1.0 mg/ml in either 10 mM HEPES buffer at pH 7.0, or water at pH 7.0 with 0.5 M NaCl and 5 mM CaCl₂ as supporting electrolytes. Milli-Q water (Millipore) with 18.2 MΩ·cm resistivity was used in all experiments. Track-etched polycarbonate (PC) membranes of 300 nm and 200 nm average pore diameter, 25 μm thickness and 1×10⁸ cm⁻² pore density, were used as templates for LbL deposition. Poly(ethylene terephthalate) (PET) membranes of 200 nm average pore size, 23 μm thickness and 5×10⁸ cm⁻² pore density, were used as filters (support) to construct nanotube mats. Both types of membranes were provided by it4ip, Louvain-la-Neuve, Belgium (<http://www.it4ip.be>).

5.2.2 Templated synthesis of nanotubes

The (bPEI/GOX)₆ nanotubes were constructed in nanoporous PC membranes following procedures identical to the ones described in section 4.2.2, and will thus not be described here. However, in some cases, a reinforcing core of (PAH/PSS)_n was built inside (bPEI/GOX)₆ nanotubes to obtain a core-shell structure (Scheme 5.1). Since PAH adsorption induces the release of previously adsorbed GOX^{45,46}, bPEI-terminated (bPEI/GOX)₆/bPEI nanotubes were constructed prior to PAH/PSS core buildup (Scheme 5.1A). For (bPEI/GOX)₆/bPEI nanotubes, a decrusting treatment of the faces of the membranes was performed in buffer after the final bPEI deposition; in this case, no rinsing step in pure water was applied

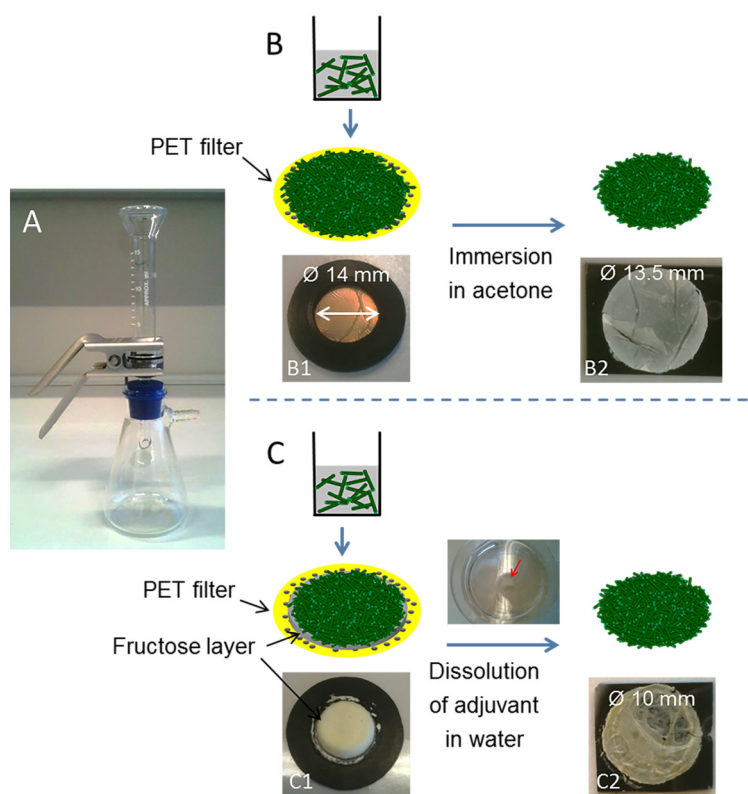
at the end of the entire bPEI/GOX shell buildup process. The (bPEI/GOX)₆/bPEI modified membranes were stored in the dry state in a fridge (4 °C) overnight, before starting the sequential construction of the PSS/(PAH/PSS)_n core in 10 mM HEPES at pH 7.0 through the whole process. To investigate the effect of the rigidity of core-shell nanotubes on mat structures, 6, 12 and 20 bilayers (n) of PAH/PSS core were built, defined hereafter as 6/6, 6/12, 6/20 (shell/core) nanotubes, respectively. During the preparation of these samples, a decrusting treatment was carried out only after each 6 bilayers of PAH/PSS deposition. Between two sequential depositions of 6 bilayers, the samples were always stored in the dry state in a fridge (4 °C) overnight. For the 20 bilayered PAH/PSS core sample (n=20), the last 8 bilayers were done at once before decrusting. For these three systems, prior to being dried at the end of the entire buildup (6, 12, 20 bilayers), the nanotube-containing membranes were washed with pure water for 1 min to remove buffer salt, and a final decrusting treatment was applied to completely eliminate the crust. For (PAH/PSS)₂₀ nanotubes in 200 nm nanochannels (Scheme 5.1B), the buildup process followed the same protocol as used in the construction of (PAH/PSS)₂₀ cores, except that water with the addition of 0.5 M NaCl and 5 mM CaCl₂ (pH 7.0) was used for PAH and PSS adsorptions, followed by pure water rinsing. The enzyme nanotube-filled membrane samples were stored at 4 °C in the dry state in a fridge before subsequent mat construction and activity measurement.



Scheme 5.1. Schematic buildup processes and structures of nanotubes. Enzyme nanotube-modified (A) or virgin (B) PC membranes were sequentially placed for 30 min in PSS and PAH polyelectrolyte solutions (10 mM HEPES buffer at pH 7.0, or pure water with 0.5 M NaCl and 5 mM CaCl_2 at pH 7.0), followed by two intermediate rinses of 3 min each in 10 mM HEPES buffer at pH 7.0 or just pure water, leading to the formation of core-shell (C) or (PAH/PSS) (D) nanotubes, respectively. *For (PAH/PSS)₂₀, after two cycles ($m=2$), the remaining 8 bilayers were deposited at once before final decrusting treatment.

5.2.3 Construction of nanotube mats

The nanotube mats were prepared by vacuum-filtration over a filter (effective diameter, 14 mm) without and with a finely-milled adjuvant powder being first deposited to form a porous barrier layer (Scheme 5.2). Briefly, an appropriate amount of nanotube-containing PC membranes was first dissolved in CH_2Cl_2 with the aid of ultrasonication for several seconds to enable the dispersion of nanotubes. Then, this resultant suspension of nanotubes was filtered through a solvent-resistant Au-coated PET membrane resting over a grille support, followed by abundant CH_2Cl_2 rinse to remove dissolved PC, leaving nanotubes stacked over the PET filter to form a supported mat of nanotubes. The Au coating is used to reduce the interactions between the resulting mat and PET filter, facilitating the detachment of self-standing mats. For the adjuvant-aided filtration, an adjuvant layer was first prepared over the PET membrane, followed by filtration of the nanotube suspension over the adjuvant layer and subsequent rinsing with pure CH_2Cl_2 , leading to the formation of a mat of nanotubes resting over the adjuvant cake. After drying to remove CH_2Cl_2 trapped in the cake, the entire solid cake covered by the nanotube mat could be easily detached from the PET filter and was then placed in water where the adjuvant dissolved, resulting in a supernatant mat floating in water. This mat was washed abundantly by immersion in pure water to remove the dissolved adjuvant and then collected over a silicon wafer. It should be pointed out that the formation of a flat and homogeneous adjuvant cake prior to the filtration of the nanotube suspension is crucial to obtain an intact mat of good-quality, since the mat replicates the large-scale topography of the adjuvant cake. We found that prior to adding the nanotube suspension to the funnel, free CH_2Cl_2 solvent has to be fully evacuated to flatten the adjuvant cake (however, the trapped CH_2Cl_2 in the adjuvant has to be left to avoid cake contraction). Only when starting from a flat and homogeneous cake did we subsequently obtain an intact and homogeneous nanotube mat (Figure S5.1A-I). We observed that the presence of free CH_2Cl_2 above the cake invariably results in a very rough and sometimes broken mat of nanotubes (Figure S5.1a-f). All mats were dried in air prior to analysis.



Scheme 5.2. Illustration of the fabrication of nanotube mats by vacuum-filtration (A) without (B) and with (C) a sacrificial adjuvant layer. In route B, the mat is either left over the supporting PET membrane (B1), or can be released from the PET filter (if coated by Au) upon immersion in acetone, yielding a free mat (B2, collected on a silicon wafer). As for route C, a free-standing mat (marked by a red arrow in water) can be directly obtained thanks to a screening adjuvant cake layer. It is crucial to fabricate a flat adjuvant cake prior to sequential filtration of the nanotube suspension (Figure S5.1).

5.2.4 SEM and STEM characterization

Nanotubes and their mats were observed with a field emission scanning electron microscope (JSM-7600F, JEOL Ltd.), equipped with a transmission detector, operated at 5 kV for the scanning mode (SEM) and 30 kV for scanning transmission (STEM), respectively. For SEM observation, the mat was prepared on Au-coated PET (Scheme 5.2B1) when in the absence of adjuvants. Then the samples were covered by 1.5-2.0 nm of gold (Cressington Sputter Coater 208HR) to reduce surface charge effects, during which we found that the mat detached from the Au-coated PET spontaneously due to the vacuum (Figure S5.2A). A small piece of free mat was then taken and placed on conductive tape with either its top or bottom surface up (Figure S5.2B). In some cases for which the mat could not be released from the filter when applying the vacuum, a conductive tap was used to peel off the mat from the filter for the observation of its bottom face. As for mats prepared by the adjuvant-aid approach, the mats were collected over a silicon wafer with either their top or bottom surface up in order to eliminate the influence of the substrate on the topography. Prior to SEM measurements, all samples were sputtered with 1.5-2.0 nm of gold in order to improve the contrast, including cross-sections and bottom surfaces. For STEM observation of nanotubes, a carbon-coated copper grid (200 mesh, Electron Microscopy Sciences) was used to collect the nanotubes from a PC/CH₂Cl₂ suspension, followed by washing in pure CH₂Cl₂ and drying in air at room temperature.

5.2.5 Atomic force microscopy (AFM)

The nanotube mats were imaged with a Nanoscope VIII Multimode AFM (equipped with a fluid cell, Bruker) in peak force tapping mode (PeakForce QNM) at a scan rate of 0.387 Hz with ScanAsyst-air and ScanAsyst-fluid probes (Bruker), respectively. Prior to AFM measurements in aqueous solutions in the fluid cell, a small droplet of solution was placed on the samples for 40 min to reach equilibrium. Collected images were flattened by subtracting 1st order polynomials in a line-by-line fashion in the X direction (home-made program).

5.2.6 FTIR measurements

Fourier transform infrared (FTIR) spectra were collected at 8 cm^{-1} resolution (128 passes) in transmittance mode on a FTIR spectrometer (Nicolet Nexus 870, Thermo Scientific), equipped with a FT-IR purge gas generator (Whatman). A standard KBr disk was prepared for all mat samples.

5.2.7 Enzyme activity

The activity of the enzyme nanotube mats was evaluated by the same coupled enzymatic reactions as described in section 3.2.8. Briefly, disks of 6 mm diameter were cut out from the mat resting over PET membranes, and then glued to the bottom of a well (12-well cell culture plate). 2.00 mL of a 0.3 mM *o*-dianisidine dihydrochloride solution in 50 mM sodium acetate buffer (pH 5.5), and 0.20 mL of a horseradish peroxidase (HRP) solution (containing approximately 60 units/mL) were mixed in the well with the mat, and air-equilibrated. 0.50 mL of a 3.0 mM glucose solution was then added to the well and the increase in absorbance at 460 nm (maximum absorption) of the solution was recorded at room temperature every two minutes immediately after mixing, for 12 min during which continuous shaking was applied (KS-15 shaker, Edmund Bühler GmbH), and for another 12 min during which the solution was left in a cuvette (the solution was taken and placed in a cuvette in order to be separated from the mat which stays in the well). The enzyme activity is here defined as the initial rate of the coupled reaction, *i.e.*, the initial increase per min of the absorbance at 460 nm, $\Delta A_{460\text{ nm}}/\text{min}$.

5.3 Results and discussion

5.3.1 Formation of mats of enzyme-rich nanotubes

A simple and efficient approach to prepare a LbL-nanotube mat from a suspension is filtration activated by a vacuum, as presented in Scheme 5.2A. All technical details are in the experimental section and supporting information. Herein, apart from the conventional procedure to fabricate nanotube mats by filtration of a nanotube suspension over a nanoporous membrane filter (Scheme 5.2B, PET filter), an adjuvant-assisted filtration methodology (Scheme 5.2C) was also developed to prepare self-standing nanotube mats, in which an adjuvant cake was deposited as a leaky buffer layer resting over a PET filter prior to filtration of the nanotube suspension. Nanotubes were filtered and stacked up over the adjuvant cake rather than over the PET filter during filtration, thus avoiding direct contact with the PET filter, therefore eliminating adhesion to the filter. After rinsing with fresh CH_2Cl_2 to remove traces of polycarbonate, the as-formed adjuvant cake covered by the nanotube mat was transferred into pure water and dissolved (sacrificial layer). As shown in Scheme 5.2C, after the dissolution of the adjuvant in pure water, followed by abundant water rinse, a self-standing nanotube mat can be obtained and thus collected over a silicon wafer. In another filtration route, denoted here as adjuvant-free filtration (Scheme 5.2B), nanotube mats are directly deposited on the top of a PET filter; when the PET filter is coated by a thin Au layer, we found that the mat can be released from the filter by simple immersion in acetone, resulting in a self-standing mat as well. The same approach was recently reported to release carbon nanotube mats from polyvinylidene fluoride filters⁴⁷. Hence, these two methods were exploited here to fabricate nanotube mats whose structures are studied and compared by SEM and AFM.

Enzyme-containing (bPEI/GOX)₆ nanotubes were used as building blocks to construct an enzymatic 'paper-like' mat for biocatalysis. The morphologies of the top surface of the mats prepared by the two routes described above are displayed in Figure 5.1. Nanotubes approximately 200 nm in diameter were observed on both surfaces by AFM, smaller than the initial pore size of the templates (300 nm) due to the deformation and shrinkage of the soft enzyme nanotubes during the dispersion and/or filtration process. Nevertheless, moderate differences in surface morphology between adjuvant-free and

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fructose-aided collections were observed. The surface of the mat prepared by the fructose-free collection route displays a significant number of holes, or 'craters' (arrows in Figure 5.1A,C), compared to the mat prepared in the presence of adjuvants. This phenomenon results from the inhomogeneous piling up of the nanotubes on the PET filter depending on the random location of the pores of the membrane, as supported by the fact that larger 'craters' are visible when a lesser amount of enzyme nanotubes is used for the construction of the mats (Figures S5.3 and S5.4). Therefore, a large amount of nanotubes is required to construct mats wherein the influence of the bottom filter is fully screened. In the sequel, mats having a thickness on the order of micrometers are therefore prepared.

A cross-sectional observation by SEM was then performed not only to measure the thickness of the mats but also to probe their internal structure. Figure 5.2 shows that the mats are very homogeneous over a thickness of *ca.* 10 μm (A) and 4 μm (B). In the magnified images, dense and 'bulk'-like internal structures rather than layered nanotubes are observed, regardless of the methods used in mat construction, demonstrating that the enzyme nanotubes collapse (possibly during filtration and/or drying in the SEM) and lose their tubular structures, merging with neighboring ones owing to their soft nature. Interestingly, many nanodots of *ca.* 20 nm size are observed on the fracture surfaces, probably resulting from the complexation of bPEI and GOX.

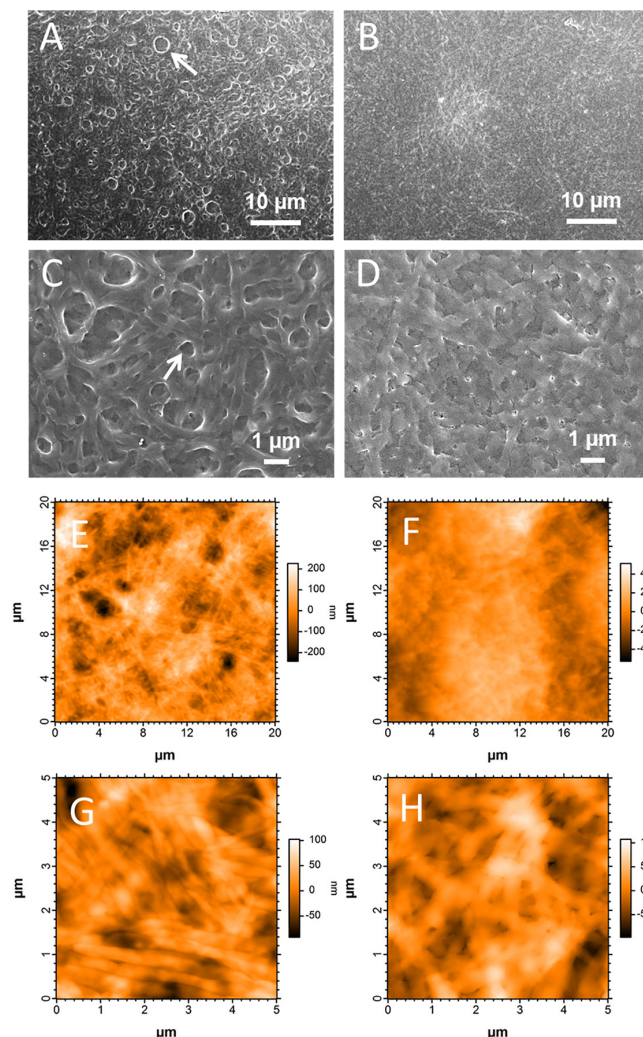


Figure 5.1. SEM images (A-D) and AFM images (E-H) of the top surface of (bPEI/GOX)₆ enzyme nanotube mats constructed by fructose-free (A,C,E,G) and fructose-aided (B,D,F,H) filtration, respectively. The arrows in A and C point to crater-like features appearing in the mats made by the adjuvant-free route. (E,F 20x20 μm²; G,H 5x5 μm²)

At this point, one should be aware that in adjuvant-aided fabrication of mats, the formed adjuvant layer is very crucial to determine the homogeneity, morphology as well as the internal structure of mats, depending on the

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adjuvant nature (dextran or fructose, Figures S5.5 and 5.2) and on the details of the filtration procedures (Figure S5.1). Careful optimization of the filtration procedure, leading to a flat compact fructose cake resulted in the formation of a continuous homogenous enzyme nanotube mat (Figure S5.1, Scheme 5.2C2). Therefore, this optimized protocol will be used in the sequel.

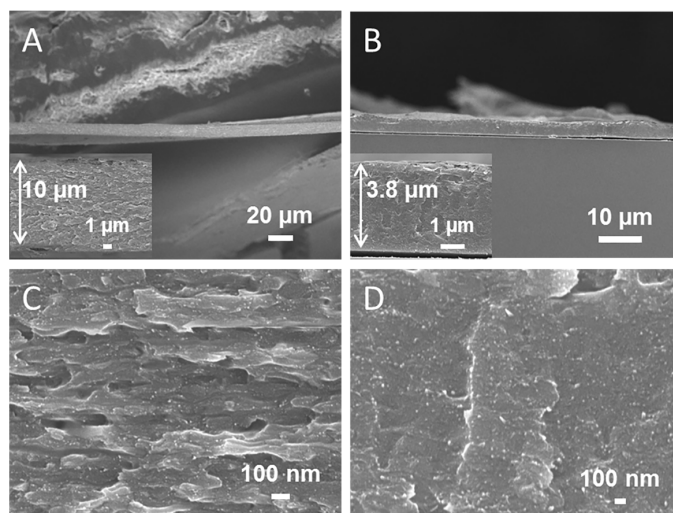


Figure 5.2. Cross-sectional SEM images of (bPEI/GOX)₆ enzyme nanotube mats fabricated by fructose-free (A,C) and fructose-assisted (B,D) vacuum-filtration, respectively. The insets show the thickness of each mat. A larger amount of nanotubes was used in the fructose-free route, yielding a thicker mat in this case.

Owing to the soft nature of (bPEI/GOX)₆ nanotubes, mats made of (bPEI/GOX)₆ nanotubes invariably exhibited a nonfibrous internal morphology. The porous structure of the scaffold plays a critical role in determining the biological properties of the porous nanomaterials, since porosity is very important in many biological processes⁴⁸. In order to obtain a fibrillar scaffold of nanotubes, a robust (PAH/PSS) core was introduced to strengthen the nanotubes. Therefore, (bPEI/GOX)₆ enzyme nanotubes with 6, 12, and 20 bilayers of PAH/PSS core were prepared, and their structures were investigated by SEM and STEM. As reported before⁴², the soft (bPEI/GOX)₆/bPEI nanotubes burst along their length (Figure S5.6B) due to the capillary forces exerted on the soft nanotubes upon drying during

observation in the microscope. Adding a PAH/PSS core of 6 bilayers was found to improve their structural stability to a limited extent (Figure S5.6D), while intact and nice nanotubes were observed when the number of bilayers of the PAH/PSS core reached 12 or 20 (Figure S5.6E-H). Moreover, in magnified STEM images (insert in Figure S5.6H), the hollow structure of the 6/20 nanotube is clearly revealed, with a wall thickness of *ca.* 100 nm, implying that *ca.* 30 nm of PAH/PSS core was grown inside the enzyme shell (subtracting the 70 nm of enzyme shell ⁴² from the whole thickness). These observations confirm that LbL assembly of PAH and PSS occurs inside the enzyme shells and indeed enhances their structural stability. The successful incorporation of PAH/PSS into enzyme nanotubes was further evidenced by FTIR. Figure 5.3 shows that the characteristic peaks at 1127, 1039 and 671 cm^{-1} absent in the spectrum of (bPEI/GOX)₆ nanotubes appear in the spectra of core-shell enzyme nanotubes, corresponding to the SO_3^- stretching vibration (1129, 1040 cm^{-1}) and aromatic =C-H deformation vibration (674 cm^{-1}) from the PSS component (Figure S5.7), respectively. However, no characteristic peak of the PAH component shows up in the spectra of core-shell nanotube mats, indicating that PSS dominates the PAH/PSS core; this is also confirmed by absence of characteristic peaks of PAH in the spectrum of pure PAH/PSS nanotubes (Figure S5.7C,D). Moreover, the strong amide I and amide II bands of the enzyme at *ca.* 1650 and 1541 cm^{-1} were retained in the spectra of core-shell mats, confirming the presence of enzymes and indicating that the introduction of the PAH/PSS core does not significantly release the enzyme from the shell of the nanotubes. A weak but apparent peak at 1775 cm^{-1} was also observed in the spectra of all mats, corresponding to the strong O=C-O stretching vibration of residual traces of PC trapped in the mats.

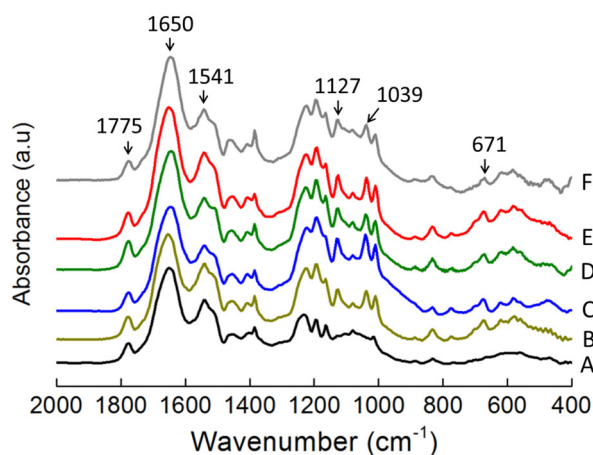


Figure 5.3. FTIR spectra of mats made of (bPEI/GOX)₆ (A), and of core/shell nanotubes comprising a PAH/PSS core of 6 (B), 12 (C,E), and 20 (D,F) bilayers. The mats (A-D) were constructed by using fructose as adjuvant, whereas no adjuvant was used for the fabrication of mats (E,F). The spectra are shifted vertically for clarity.

Mats made of 6/6, 6/12 and 6/20 (shell/core) enzyme nanotubes were then constructed by the adjuvant-free and fructose-aided routes, respectively, as described in Scheme 5.2. For these core-shell nanotubes, the morphology of the mats depends not only on the rigidity of the building nanotubes, hence on the number of bilayers in the PAH/PSS core, but also on the protocol used for mat construction.

Nanotubes could be identified on the top surface of all mats (Figures 5.4 and 5.5); compared to the (bPEI/GOX)₆ nanotube mats, much more intact (although still flattened) core-shell nanotubes are visible on the surfaces of all mats made of core-shell nanotubes, regardless of the fabrication route. For adjuvant-free mats, an evolution of the surface morphology was observed as a function of the number of bilayers in the core (Figure 5.4). A clear nanofibrous microstructure consisting of coiled nanotubes is present starting from 12 bilayers in the core (Figure 5.4B,C), totally different from (bPEI/GOX)₆ and 6/6 mats in which the fibrous structure is blurred by tube smearing. These results agree with the improved intrinsic stability of core-shell nanotubes revealed by SEM and STEM, as discussed above (Figure S5.6). As for the mats obtained by the fructose-aided collection route, they

appear to be smoother with a more smeared nanofibrous texture in all cases (Figure 5.5), akin to that of adjuvant-free (bPEI/GOX)₆ mat. Nevertheless, the core-shell nanotubes exhibit improved structural rigidity and are less deformed even with only 6 bilayers of PAH/PSS core.

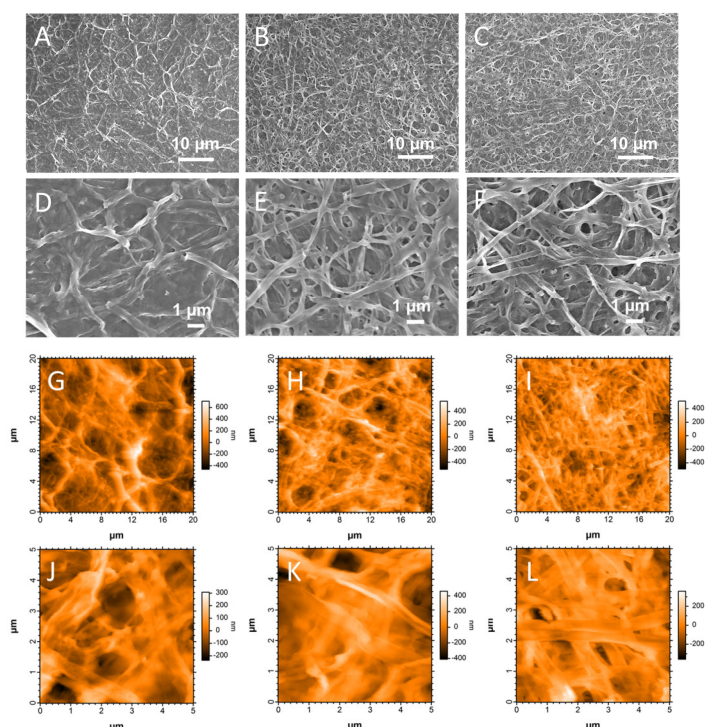


Figure 5.4. SEM images (A-F) and AFM images (G-L) collected on the top surface of core-shell enzyme nanotube mats fabricated by the adjuvant-free filtration route (6/6, A,D,G,J; 6/12, B,E,H,K; 6/20, C,F,I,L). (G-I 20x20 μm^2 ; J-L 5x5 μm^2)

In order to understand the smoother surface microstructure of mats collected by the adjuvant-aided route, one should be aware that such mats are placed in pure water during recovery (Scheme 5.2), before being dried in air (Scheme 5.2C2). Therefore, it is possible that the smoother surface arises from one step of this process. To check this possibility, a mat made by the adjuvant-free collection route was immersed in water and acetate buffer and then imaged by AFM. No change of surface structure and nanotube morphology was observed, except for the hydration of the nanotubes

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resulting in cylindrical rather than flattened shapes (Figure S5.8). The subsequent drying of these mats did also not modify the surface morphology of the mats (Figure S5.9), even after immersion in a strong acetate buffer, indicating that mats prepared by the adjuvant-free route are stable and resistant to drying and immersion in aqueous solutions. Likewise, no change of surface morphology of mats collected by the fructose-aided route was induced by contact with water and subsequent drying in air. Indeed, SEM images of the as-prepared mat resting over the fructose cake, before cake dissolution, revealed a similarly smooth surface (Figure S5.10).

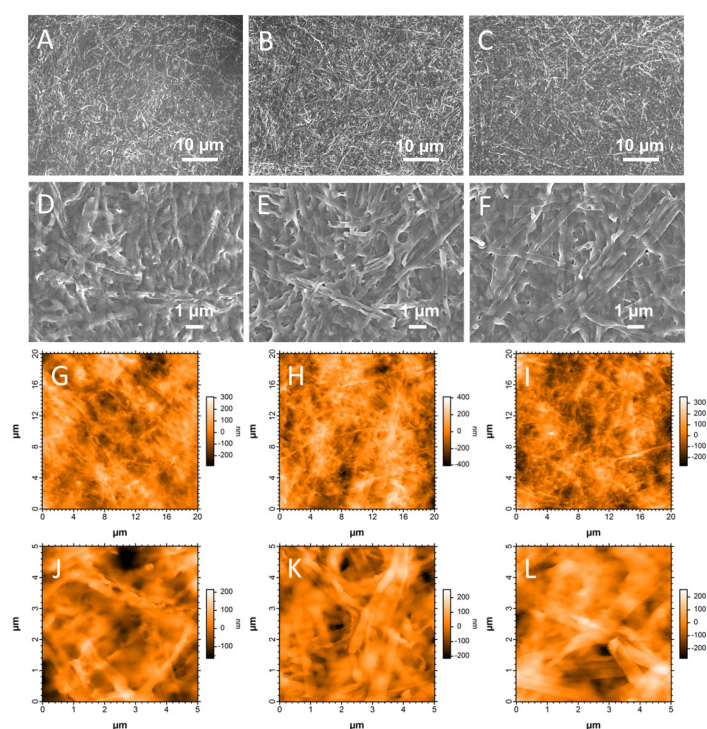


Figure 5.5. SEM images (A-F) and AFM images (G-L) recorded on the top surface of core-shell enzyme nanotube mats fabricated by the fructose-assisted filtration route (6/6, A,D,G,J; 6/12, B,E,H,K; 6/20, C,F,I,L). (G-I 20x20 μm^2 ; J-L 5x5 μm^2)

Hence, it is probable that the differences seen on the outer surfaces of the mats prepared by the two collection routes originate from differences in the very deposition of the tubes, arising from different local hydrodynamic forces

resulting from the different permeability of the leaky fructose cake and of the bare PET membrane.

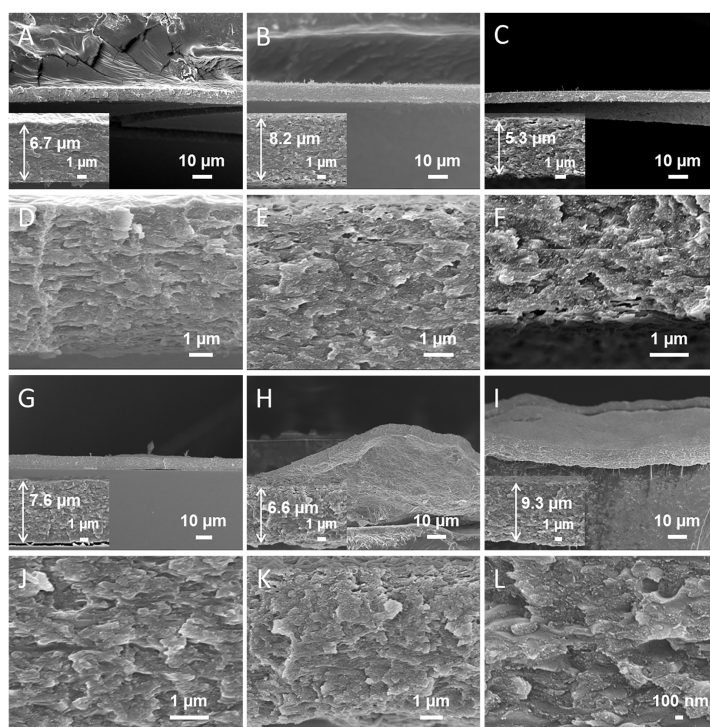


Figure 5.6. Cross-sectional SEM images of core-shell enzyme nanotube mats fabricated by adjuvant-free (A-F) and adjuvant-aided (G-L) filtration routes over nanoporous PET membranes. The insets give the thickness of each mat. (6/6, A,D,G,J; 6/12, B,E,H,K; 6/20, C,F,I,L)

The cross-sectional structures of these core-shell nanotube mats were also investigated by SEM. Mats prepared by simple filtration were always very flat and homogeneous (Figure 5.6A-C), owing to the planar support (PET filter). On the contrary, mats collected by the fructose-aided route and transferred to silicon wafers, experienced deformation and partial detachment during drying, resulting in wavy 'papers'. This was in particular found for the 6/12 and 6/20 nanotube mats (Scheme 5.2C2 and Figure 5.6H,I); this problem was not observed for the simple (bPEI/GOX)₆ mat (Figure 5.2B), probably because the lower stiffness of the nanotubes prevented buckling to occur during drying. Inserts in Figure 5.6 show that all mats have comparable

thickness when prepared from the similar amount of nanotubes. Different from the simple (bPEI/GOX)₆ nanotube mats, small pores were observed in the cross-sections; however, the expected nanofibrillar structure was never really apparent, probably due to the sectioning process (Figure 5.6).

5.3.2 Formation of mats of PAH/PSS nanotubes

As evidenced above, mats made of nanotubes with enzymes in their shells exhibit limited fibrillar internal structures, even when having rigid PAH/PSS cores. We ascribe this observation to the stickiness of the soft (bPEI/GOX)₆ shell, which facilitates the smearing and merging of neighboring nanotubes to form a more compact layer. This can be demonstrated by fabricating mats made of pure PAH/PSS nanotubes.

The PAH/PSS nanotubes were prepared in PC templates in 10 mM HEPES buffer at 7.0, as in the previous assembly conditions. Similar to the mats made of core-shell enzyme nanotubes, PAH/PSS nanotubes were visible on the top surfaces of all mats (Figure 5.7 and S5.11), independent of the construction route. Only moderate differences in morphology were observed between the mats prepared by the two routes. As before, mats collected by the fructose-aided route detached from the silicon wafers spontaneously upon drying, resulting in wavy but homogenous mats (Figure S5.12A,B). However, the internal structure disclosed by SEM was fully different for the pure PAH/PSS nanotube mats, with a clearly layered fibrillar structure made of individual flattened PAH/PSS nanotubes observed, regardless of the fabrication route (Figure 5.8 and S5.12), confirming their integrity.

However, the separation of the nanotubes is still imperfect. This is because, in our assembly conditions of 10 mM HEPES buffer at pH 7.0, the low pH and low ionic strength lead to a slow nanotube growth⁴⁹. This is evidenced by the STEM image of single (PAH/PSS)₂₀ nanotubes (Figure S5.13B): 20 bilayers of PAH/PSS provide nanotubes of only ca. 35 nm wall thickness, slightly larger than the value estimated on cross-sections of mats (ca. 25 nm). Obviously, these nanotubes are still not strong enough to fully retain their tubular integrity when they stack up in a mat; this limited mechanical sturdiness is also supported by the observed flattening of the tubes, as revealed by their apparent diameter of ca. 600 nm (Figure S5.13B), while they were prepared in membranes of 300 nm pore diameter.

Mats of LbL Assembled Nanotubes

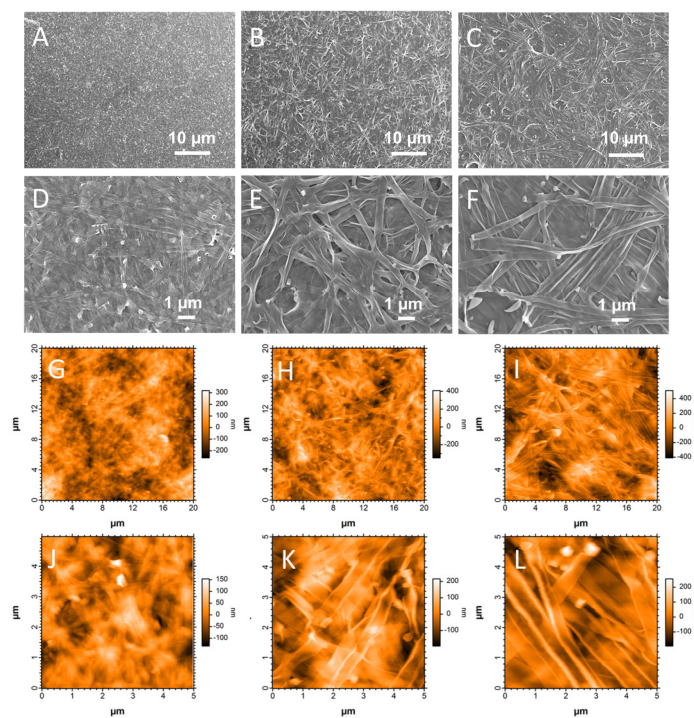


Figure 5.7. SEM (A-F) and AFM images (G-L) collected on the top surface of PAH/PSS nanotube mats fabricated by adjuvant-free filtration over a nanoporous PET membrane. (PAH/PSS)₆, A,D,G,J; (PAH/PSS)₁₂, B,E,H,K; (PAH/PSS)₂₀, C,F,I,L. (G-I 20x20 μm²; J-L 5x5 μm²)

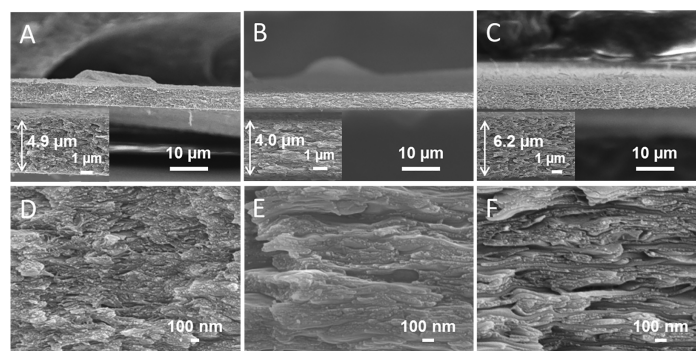


Figure 5.8. Cross-sectional SEM images of PAH/PSS nanotube mats fabricated by adjuvant-free filtration. (PAH/PSS)₆, A,D; (PAH/PSS)₁₂, B,E; (PAH/PSS)₂₀, C,F. These nanotubes were grown in 10 mM HEPES buffer (pH 7.0). The insets give the thickness of each mat.

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In order to reinforce the nanotubes and retain their hollow structure in the mats, we have increased the degree of filling of the nanopores during their construction. In order to achieve this goal without suffering from a longer deposition time, salt was added to the polyelectrolyte solutions, leading to a more coiled conformation of the polyelectrolyte chains, and therefore to the deposition of thicker LbL multilayers inside the nanopores^{9,50}. It has been reported that the size distribution of PSS coils in NaCl is broader than in CaCl_2 aqueous solution due to the absence of ion-bridging effects⁵¹. In addition, divalent Ca^{2+} ions in solution cause ionic bridging between some monomer units of PSS, resulting in a much stronger chain contraction than NaCl ⁵¹, which minimizes the radius of gyration of the polyelectrolyte chains and therefore should facilitate diffusion into the nanopores. However, it was reported that, if the concentration of the divalent salt exceeds 5 mM, the diffusion of the polyelectrolytes in the pores is reduced considerably, probably due to interchain aggregate formation^{44,51}. Therefore, 0.5 M NaCl and 5 mM CaCl_2 were employed as supporting electrolytes during the nanotube buildup process; a complete filling of pores of PC templates was previously demonstrated for the PAH/PAA pair⁴⁴ with these assembly conditions. Furthermore, a PC membrane with pores 200 rather than 300 nm in diameter was used.

As expected, the resulting nanotubes have a more rigid morphology with ca. 30 μm length (Figure S5.13C) and 200 nm outer diameter (Figure S5.13D), identical to the size of the nanochannels. The wall thickness of these robust nanotubes was found to be ca. 60 nm, accounting for this improved stability. Mats made of these smaller yet more robust nanotubes were constructed via the two collection routes. Nanotubes of ca. 200 nm diameter were observed on the surface of the mats (Figure 5.9B,D and S5.14B,D), confirming their integrity. Moreover, in contrast to mats based on soft enzyme nanotubes or on (PAH/PSS) nanotubes with a thinner wall, no difference was noted in the surface morphology of the mats prepared by the two routes (Figure 5.9 and S5.14), confirming that these robust nanotubes resist better the hydrodynamic forces due to solvent flow during filtration, independently of the nature of the collecting filter.

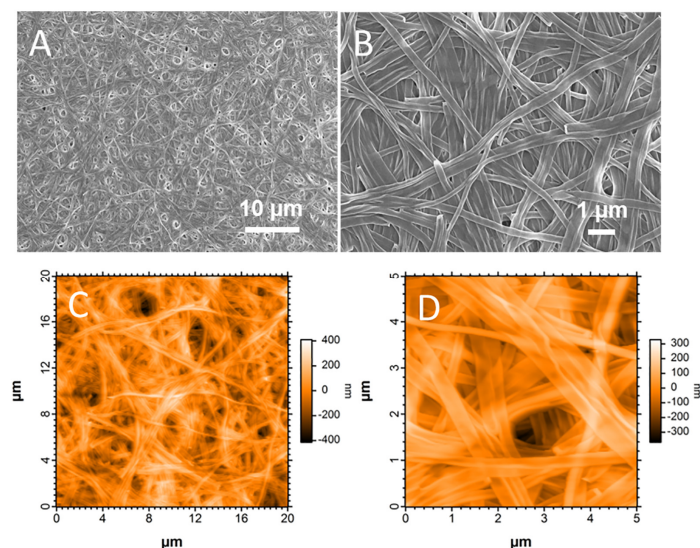


Figure 5.9. SEM (A,B) and AFM images (C,D) collected on the top surface of $(\text{PAH/PSS})_{20}$ nanotube mats prepared on a PET filter without adjuvant. $(\text{PAH/PSS})_{20}$ nanotubes were grown in the presence of 0.5 M NaCl and 5 mM CaCl_2 in the polyelectrolyte solutions. (C $20 \times 20 \mu\text{m}^2$; D $5 \times 5 \mu\text{m}^2$)

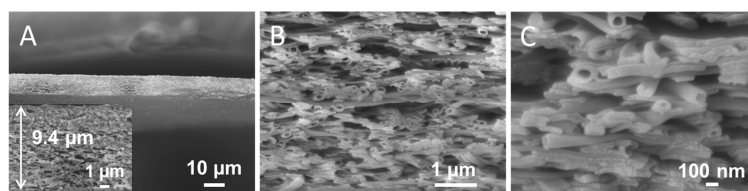


Figure 5.10. Cross-sectional SEM images of mats of $(\text{PAH/PSS})_{20}$ nanotubes collected over a PET filter in the absence of fructose adjuvant. The nanotubes were assembled from polyelectrolyte solutions (pH 7.0) with 0.5 M NaCl and 5 mM CaCl_2 added, in nanoporous PC membranes of 200 nm pore diameter. The insert in A shows the thickness of this mat. Image B displays the porous structure. In the magnified image C, the tubular structures are visible.

Logically, cross-sections of mats based on these robust nanotubes display a porous structure made of layered nanotubes (Figure 5.10 and S5.15). The hollow cylindrical structure of the nanotubes is preserved, obviously owing to

their enhanced sturdiness. The outer diameter and wall thickness of the nanotubes, estimated from Figure 5.10C, are *ca.* 150 nm and 50 nm, respectively, consistent with values obtained previously on the STEM image of single nanotube (Figure S5.13D). Hence, an interlocked framework of nanotubes was achieved by using these thicker and stronger nanotubes; inversely, we conclude that the lack of internal texture of the enzyme-based nanotubes is due to their softness.

5.3.3 Symmetry of the nanotube mats: imaging the bottom surface

The chemical and physical properties of the top and bottom surfaces of a mat may differ substantially. For mats fabricated by filtration over a conventional filter, the bottom surface is formed by direct contact with a filter, while the other, top surface, is generated by suspension in a liquid followed by drying in air. It has been reported that membrane filters in vacuum filtration could chemically affect the stability of as-prepared graphene oxide (GO) mats, owing to the ionic species extracted from the filter membrane⁵². Recently, Wang⁵³ showed that a physical imprint of the filter membrane was left on the bottom surface of reduced GO mats, resulting in an asymmetry of morphology and wettability of the mats. This is thus a common issue in mats prepared by conventional filtration.

Herein, as expected, a physical imprint of the PET filter was observed on nanotube mats prepared by the adjuvant-free route; however, its extent varies depending on the type of used nanotube. For very soft (bPEI/GOX)₆ and 6/6 nanotubes, the nanotubes are forced to enter the mouths of the filter nanopores, leading to the formation of protrusions on the bottom surface of the mat (Figure 5.11B,D), with a dimension comparable to the size of the nanopores of the underlying PET membrane (around 200 nm). These protrusions disappear when the nanotubes become stronger and are consequently denied entry into the filter pores: for instance, mats made of nanotubes with 12 (Figure 5.11E,F) or 20 bilayers (Figure 5.11G,H) of PAH/PSS core do not exhibit these protrusions. However, the tubes in contact with the filter are still strongly flattened, giving rise to a quite smooth bottom surface replicating the flat portion of the PET membrane, with a micro-porosity (Figure 5.11F,H). AFM measurements provide similar results

(Figure S5.16), even though the bottom surface of the 6/12 mat appears slightly rougher in AFM.

Similarly smoothed bottom surfaces were also generated for the mats made of (PAH/PSS)₆, (PAH/PSS)₁₂, and (PAH/PSS)₂₀ nanotubes built in HEPES buffer, as well as for smaller (PAH/PSS)₂₀ nanotubes built from polyelectrolyte solutions with added NaCl and CaCl₂. Individual nanotubes can be seen in all cases (Figure S5.17 and S5.18), especially for the mat made of the more sturdy 200 nm-(PAH/PSS)₂₀ nanotubes (Figure S5.19); however, the nanotubes are invariably flattened when contacting the surface of the PET filter. Hence, all mats prepared by direct filtration over PET membranes are asymmetric in their surface microstructure, independent of the sturdiness of the nanotubes.

In contrast, the fructose cake collection route prevents the mat to being in contact with a hard filter surface. We expected that the open yet flat fructose cake would reduce the impact of the support on the microstructure of the bottom surface of the mats. Hence, we fabricated mats made of different type of nanotubes, using finely-crushed fructose as adjuvant, and cut them in half in water before collection over silicon wafers, with the two parts displaying up the top or bottom surface of the mat (Figure S5.20). After drying, the samples were analyzed by SEM and AFM. As expected, the morphology of the bottom surfaces of the mats collected over the fructose cake (Figure 5.12 and Figure S5.21) was similar to that of their top surface (Figure 5.5 and Figure S5.11 and S5.14), and totally different from that of the flattened bottom surface of mats collected directly over a PET filter (Figure 5.11C-H and Figure S5.17B,C,E,F, S5.18 and S5.19), regardless of nanotubes used, indicating that the influence of the PET filter was fully eliminated by the presence of a soft fructose buffer layer in-between. Even though slight differences could sometimes be observed, especially in AFM images, these were of an extremely limited extent with the optimized protocol of fructose cake fabrication described before (Figure S5.1A-F).

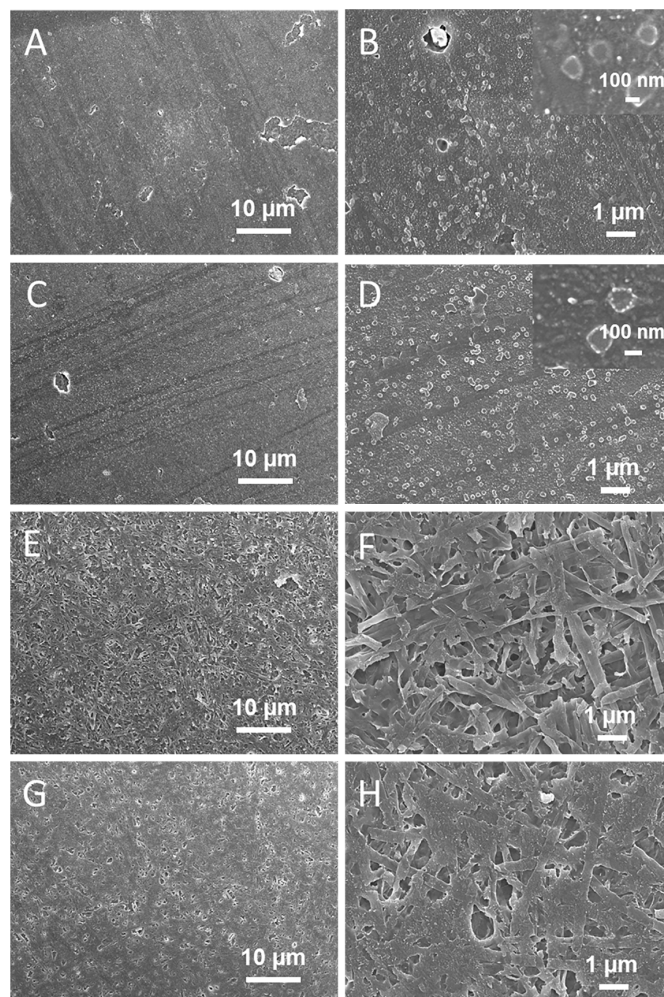


Figure 5.11. SEM images collected on bottom surfaces of enzyme mats made of (bPEI/GOX)₆ (A,B), 6/6 (C,D), 6/12 (E,F), and 6/20 (G,H) nanotubes. The mats were prepared by using adjuvant-free filtration. Insets in B and D show the crater-like imprint of nanopores of the PET filter on the bottom surfaces.

The structure of a rough mat prepared on a rough fructose cake prepared by the non-optimal procedure described in Figure S5.1a-c was also investigated by SEM. The top surface morphology was found to be identical to the one of mats of the same type of nanotubes prepared under the optimized filtration

protocol (compare Figure S5.21C,F and S5.22A,D). However, the bottom surface was extremely porous, displaying an open network structure of robust nanotubes (Figure S5.22B,E), presumably due to the much higher roughness of the fructose cake. The observation of a cross-section of this rough mat revealed that the entire mat is a 3D framework of nanotubes of very high porosity, with as a result a much higher thickness of 137 μm (Figure S5.22C,F). This porous structure is most probably arising from the rough surface of the fructose cake, and possible inclusion of fructose particles in the mat during sedimentation. This creates voids in the mats when the fructose powder is dissolved in water, leading to this type of very porous network structure. These experiments demonstrate the crucial influence of the filter/cake structure on the final morphology of the mat of nanotubes.

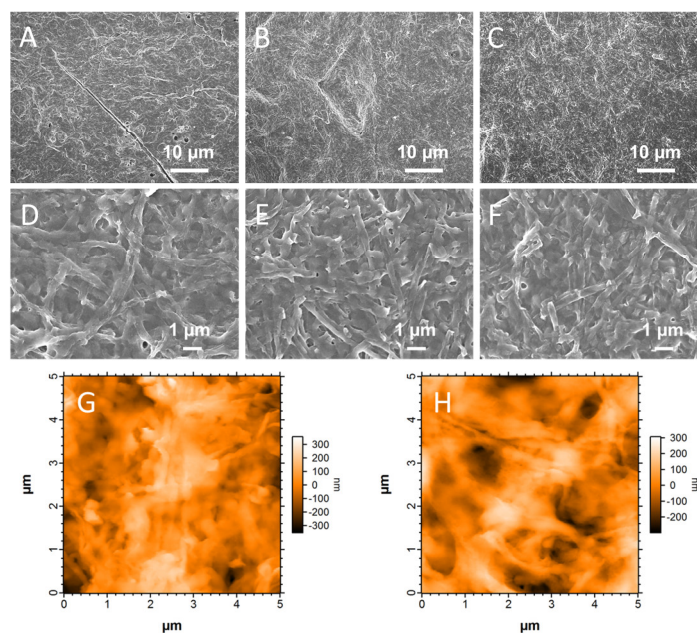


Figure 5.12. SEM (A-F) and AFM (G,H) images collected on the bottom surfaces of mats made of 6/6 (A,D,G), 6/12 (B,E) and 6/20 (C,F,H) nanotubes built from HEPES buffer. Mats were prepared by the fructose-aided filtration route. (G,H 5x5 μm^2)

5.3.4 Enzymatic activity of the nanotube mats

From the previous observations, mats of different morphology can be obtained depending on the details of the preparation protocol; presumably, these mats should also display different properties. Here, enzyme nanotubes are used to construct mats, to catalyze an enzymatic reaction which could be of interest for, e.g., bio-purification. We tested the activity of enzyme-nanotube mats prepared over nanoporous PET filters. These mats exhibited enzymatic activity (Figure S5.23), attributed to the immobilized enzymatic component. The activity of core-shell enzyme nanotube mats is *ca.* 0.0075 min^{-1} , slightly lower than that of the pure (bPEI/GOX)₆ nanotube mat (0.01 min^{-1}), but was found independent of the number of bilayers of the PAH/PSS core (Figure 5.13). This slightly reduced activity probably results from the slightly more limited accessibility of glucose to enzymes in the mats, due to the PAH/PSS cores of the nanotubes. The stability of the immobilized enzyme in nanotube mats was also evaluated by detecting possible released enzymes in solution. After measuring the activity of the mats over a period of 12 min, the solutions were taken out of the well and placed in a cuvette, and then the activity of these solutions in a cuvette without mats was monitored for further 12 min. Figure S5.23 shows that minute amounts of enzymes were released from the (bPEI/GOX)₆ nanotube mat, whereas no release could be detected for the core-shell nanotube mats, further confirming the enhanced stability of core-shell nanotubes.

The activity of nanotube mats could be normalized by the number of nanotubes, allowing us to quantitatively compare the activity of nanotube mats with nanotubes either embedded in templates or liberated in aqueous dispersion. The normalized activity of enzyme-loaded mats containing *ca.* 4.5×10^8 enzyme nanotubes was *ca.* 0.002 min^{-1} for 1×10^8 nanotubes. From Chapter 4, the estimated activity was *ca.* 0.03 and 0.05 min^{-1} for 1×10^8 nanotubes embedded in templates and liberated by dextran, respectively. In this respect, the nanotubes in the mat exhibit quite low activity, probably because of the nonporous structure of mats which limits the diffusion of substrates. However, the activity of mats over filters might be significantly improved by increasing the access of the substrate via the filtration of the solution through the enzyme mats. These enzyme-loaded nanotube mats deposited over nanoporous PET supports thus offer a promising filtration-based platform for bio-purification.

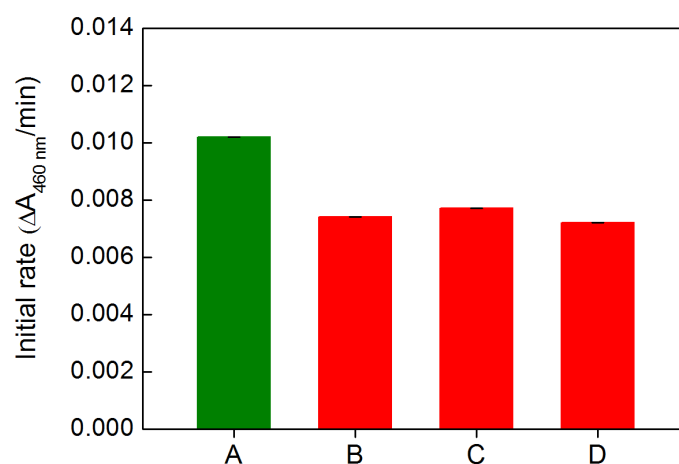


Figure 5.13. Enzymatic activity of mats consisting of (bPEI/GOX)₆ (A), 6/6 (B), 6/12 (C) and 6/20 (D) (shell/core) nanotubes, respectively. For comparison, the same amount of nanotubes was used for the formation of all mats. For each nanotube system, two mats of 6 mm diameter were cut out from a large mat and used for the activity measurements. The error bar is based on these two independent samples.

5.4 Conclusions

In this chapter, an alternative promising methodology was developed to create functional nanofiber/nanotube network frameworks by combination of templated layer-by-layer technology and simple filtration processes, leading to the formation of functional LbL-based 'papers'. Filtration was performed either directly on a nanoporous PET filter, or onto a leaky cake of finely-crushed fructose powder, in which case an optimized protocol of cake formation was proposed. Depending on the selected filtration route, the mats exhibit different top surface morphologies for soft enzyme nanotubes or core-shell nanotubes; however, an identical surface morphology is found for strong PAH/PSS nanotubes.

The internal structure of the mats made of these nanotubes also depends on the nature as well as on the rigidity of the nanotube building blocks. The sticky nature of pure (bPEI/GOX)₆ enzyme nanotubes facilitates the smearing and merging of neighboring nanotubes to form compact mats upon sedimentation of the nanotubes; this effect is decreased when adding a rigid PAH/PSS core, with as a consequence mats of such tubes displaying a limited porous structure. For the rigid pure PAH/PSS nanotubes, a fibrillar layered structure composed of partially flattened nanotubes is formed, and the hollow nanotube structure is preserved inside the mats. This illustrates that nanotubes with thick walls are strong enough to survive the construction process, leading to porous nanofiber networks with a two-level porosity: at the scale of the nanotube cores, and at the inter-tube scale.

The bottom surfaces of all mats are strongly affected by the nature of the bottom filter. When in direct contact with a hard PET filter, the bottom surface is smoothened by the flat regions of the PET filter; in addition, very soft nanotubes penetrate the pores of the filter, resulting in the imprint of the filter morphology on the bottom surface of the mat. This issue can be avoided by using a properly-prepared fructose cake between the nanotube mat and the collecting PET filter, leading to a mat symmetric with respect to surface microstructure. However, if the cake is improperly prepared, a very porous and rough mat results, which might be of interest for some applications.

The introduction of a rigid PAH/PSS core in enzyme nanotubes improves the nanotube stability without compromising the activity of the immobilized

enzymes, showing potential application in bio-purification. Our methodology thus provides a new and general way to create porous nanofiber structures, allowing for the incorporation of various functional components into nanofibers, leading to smart nanofibrillar 'papers' which offer attracting possibilities for applications in, *e.g.*, tissue engineering, biosensing and purification.

Supporting Information

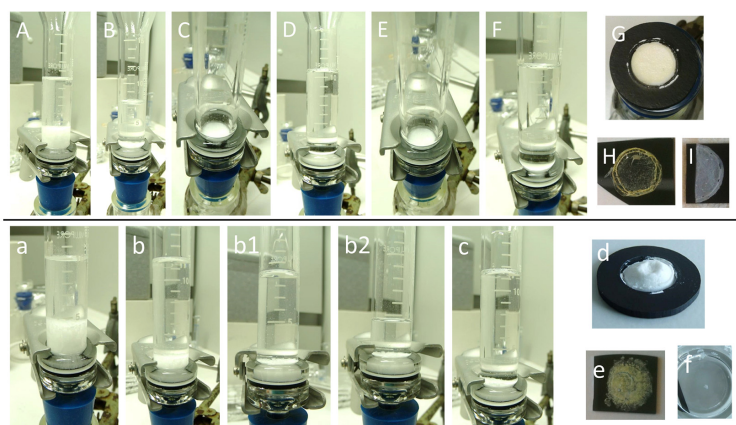


Figure S5.1. Illustration of the fructose-assisted filtration process with two different procedures for the formation of the fructose cake and of the mats. (A-F), a flat and homogenous fructose cake was formed and shaped by evacuation of free CH_2Cl_2 present over the loose sedimentary fructose cake (A-C). It is worthy to mention that this sediment cake (A, just after fructose powder suspension was added to funnel) becomes less loose upon the filtration (B), until the free CH_2Cl_2 level just reaches the top surface of the cake (C); at this moment, the trapped CH_2Cl_2 in the flatten cake is conserved in order to avoid shrinking of the cake. This optimized condition aims at stabilizing the cake and is crucial to obtain a flat cake for the subsequent formation of a nice nanotube mat. Then the nanotube suspension of CH_2Cl_2 is carefully added to the funnel by letting it flow along the interior wall in order to avoid the disruption of the adjuvant cake (D), followed by filtration until the nanotube suspension is sedimented (E, an identical protocol was applied as in C). After rinsing with fresh CH_2Cl_2 (F, an identical protocol was used as for D) and evacuation of CH_2Cl_2 completely in the funnel (including CH_2Cl_2 trapped in the fructose cake), a flat and homogeneous cake with a nanotube mat resting over it is harvested (G). Another key parameter is to leave the cake (coated with the mat) in air to evaporate CH_2Cl_2 fully before the cake is taken off from filter, since the cake with trapped CH_2Cl_2 is soft and easily broken. It is very important to obtain an intact disk of self-standing mat (as in H and I, made of $(\text{bPEI}/\text{GOX})_6$ and $(\text{PAH}/\text{PSS})_{12}$ nanotubes

prepared in 10 mM HEPES buffer at pH 7.0, respectively). For sample I, the entire disk was obtained, and was cut in half for further analysis.

In another protocol used in fructose-aided filtration (a-c), no filtration was applied on the fructose powder suspension in funnel (a), before careful addition of nanotube suspension to the funnel. As the flow of $\text{CH}_2\text{Cl}_2/\text{PC}$ was passing through the filter the loose sediment cake became compacted (b, b1), until only a small amount of nanotube suspension was left above the cake in the funnel (b2). Then, fresh CH_2Cl_2 was carefully added into the funnel to wash the mat sufficiently, resulting in the formation of a rough and irregular cake (d), as well as of rough and broken mats (e, f made of $(\text{bPEI}/\text{GOX})_6$ and $(\text{PAH}/\text{PSS})_{12}$ nanotubes prepared in 10 mM HEPES buffer (pH 7.0) and salty assembly condition (0.5 M NaCl + 5 mM CaCl_2 , pH 7.0), respectively).

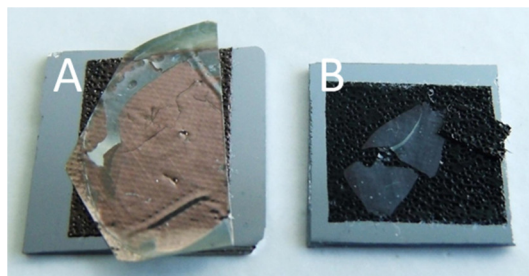


Figure S5.2. Photographs of a $(\text{PAH}/\text{PSS})_{12}$ mat atop an Au-coated PET filter, after 1.5 nm gold metallization (A) and a small piece of free-standing mat taken from A, then placed bottom up on conductive tape (B). Most part of the metallized mat in (A) was taken away for analysis, and only a small part is left in picture A, therefore exposing the bare Au-coated surface of the PET membrane. $(\text{PAH}/\text{PSS})_{12}$ nanotubes were grown in 10 mM HEPES buffer at pH 7.0.

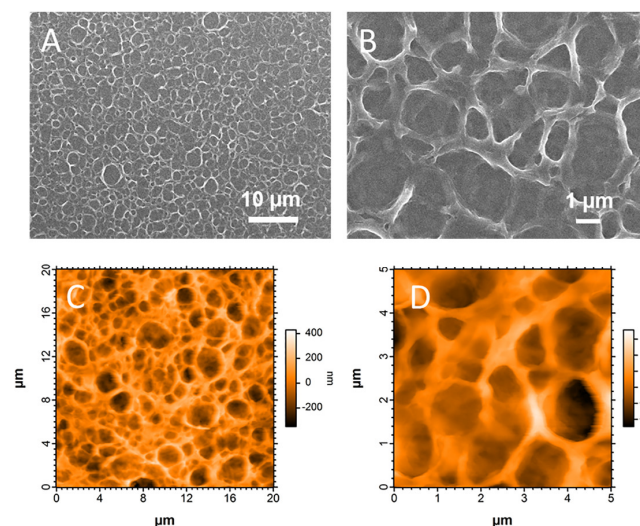


Figure S5.3. Surface morphology (SEM, A,B; AFM, C,D) of an enzyme mat constructed with a small amount of (bPEI/GOX)₆ enzyme nanotubes, by adjuvant-free filtration over nanoporous PET membranes. The small amount of enzyme nanotubes gives rise to a thin mat displaying large craters resulting from the influence of the porous bottom PET membrane. (C 20x20 μm^2 ; D 5x5 μm^2)

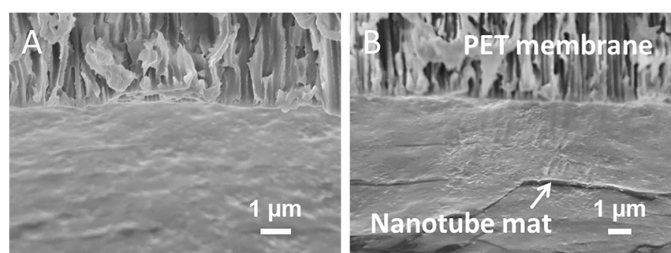


Figure S5.4. Cross-sectional SEM images of the mat constructed using a small amount of (bPEI/GOX)₆ enzyme nanotube, by the adjuvant-free filtration over nanoporous PET membranes. From the cracks of the mat in image B, the thickness was approximately estimated to be several hundreds of nanometers.

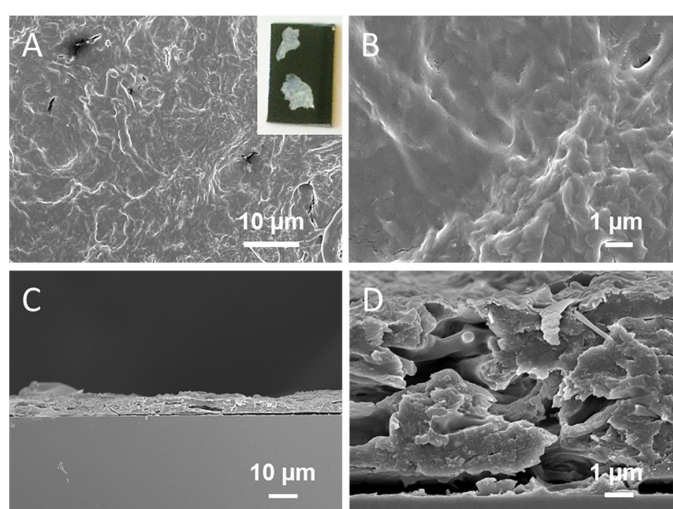


Figure S5.5. Top (A,B) and cross-sectional (C,D) views of (bPEI/GOX)₆ enzyme nanotube mats constructed by using dextran instead of fructose as adjuvant. Voids of micrometer size are present within the as-prepared mat, which is not the case when fructose is used as adjuvant (Figure 5.2 of the companion article).

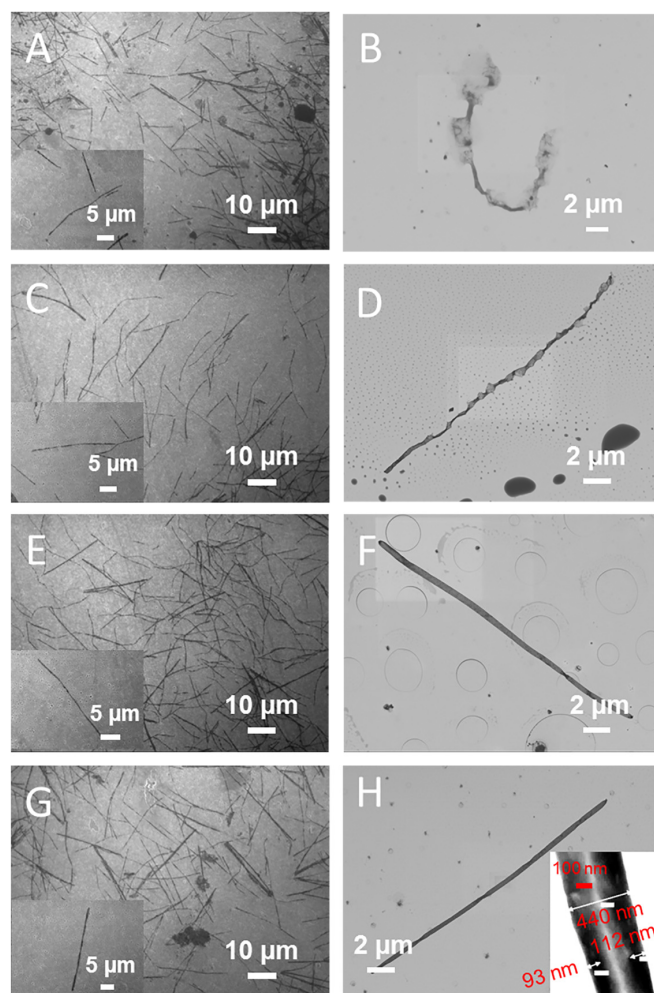


Figure S5.6. SEM (A,C,E,G) and STEM (B,D,F,H) images of (bPEI/GOX)₆/bPEI (A,B) and (bPEI/GOX)₆/bPEI/PAH/(PAH/PSS)_n nanotubes with n=6 (C,D), 12 (E,F), and 20 (G,H). For SEM sample preparation, an appropriate amount of nanotubes in PC/CH₂Cl₂ suspension was filtered through Au-coated PET membranes. The magnified image (insert in H) reveals the tubular structure with ca. 100 nm wall thickness. PC membranes with 300 nm pore size were used; the tubes flatten during drying.

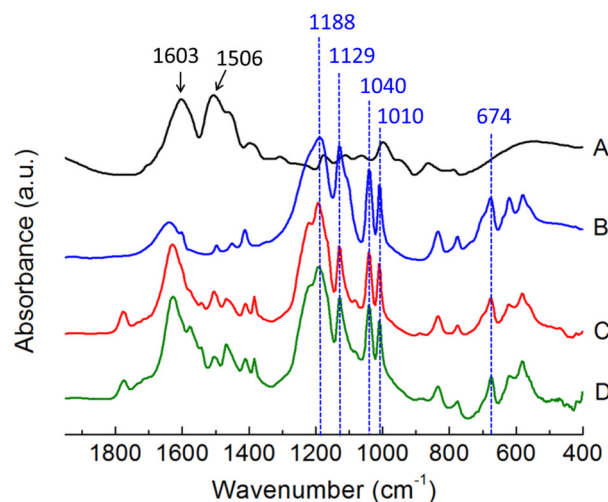


Figure S5.7. FTIR spectra of pure PAH (A), PSS (B), and mats of (PAH/PSS)₁₂ (C) and (PAH/PSS)₂₀ (D) nanotubes prepared by fructose-aided filtration. (PAH/PSS)₁₂ and (PAH/PSS)₂₀ nanotubes were built in 10 mM HEPES buffer at pH 7.0 in a 300 nm pore size PC membrane.

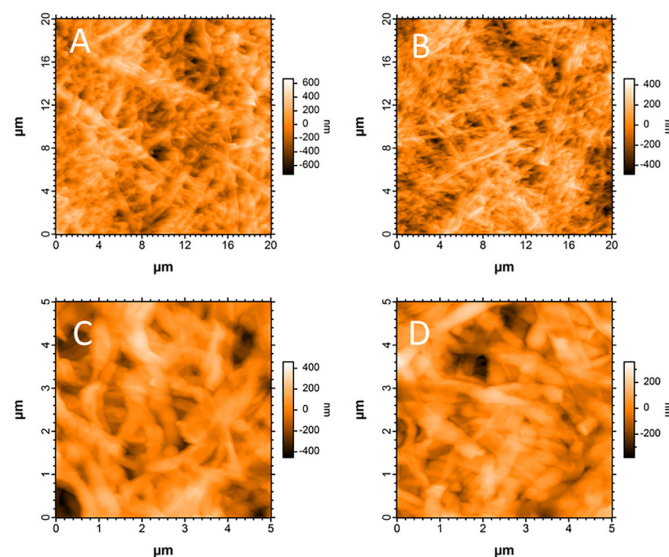


Figure S5.8. AFM images (in fluid) of the mat made of 6/20 nanotubes in water (A,C) and acetate buffer at pH 5.1 (B,D), respectively. The mat was prepared by adjuvant-free filtration.

Supporting Information

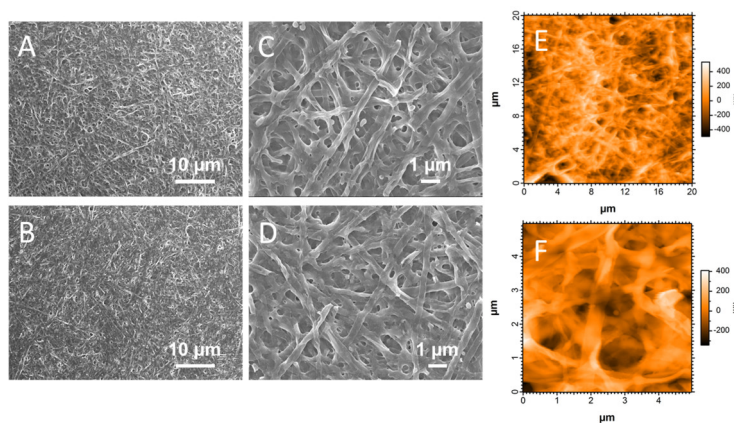


Figure S5.9. SEM (A-D) and AFM images (in air, E,F) of the air-dried mat of 6/20 enzyme nanotubes after more than 2h immersion treatment in either water (A,C,E,F) or acetate buffer (pH 5.1) (B,D). The mat was constructed by adjuvant-free filtration.

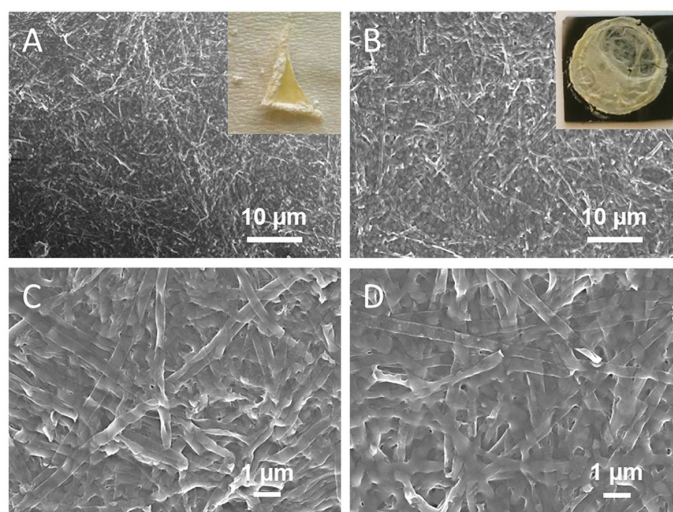


Figure S5.10. Topography of 6/20 enzyme nanotube mats at different stages of sample preparation. Mat prepared on a fructose cake and imaged directly (A,C; in this case the mat was detached manually from the fructose cake for SEM observation); and mat collected from water and then air-dried (B,D).

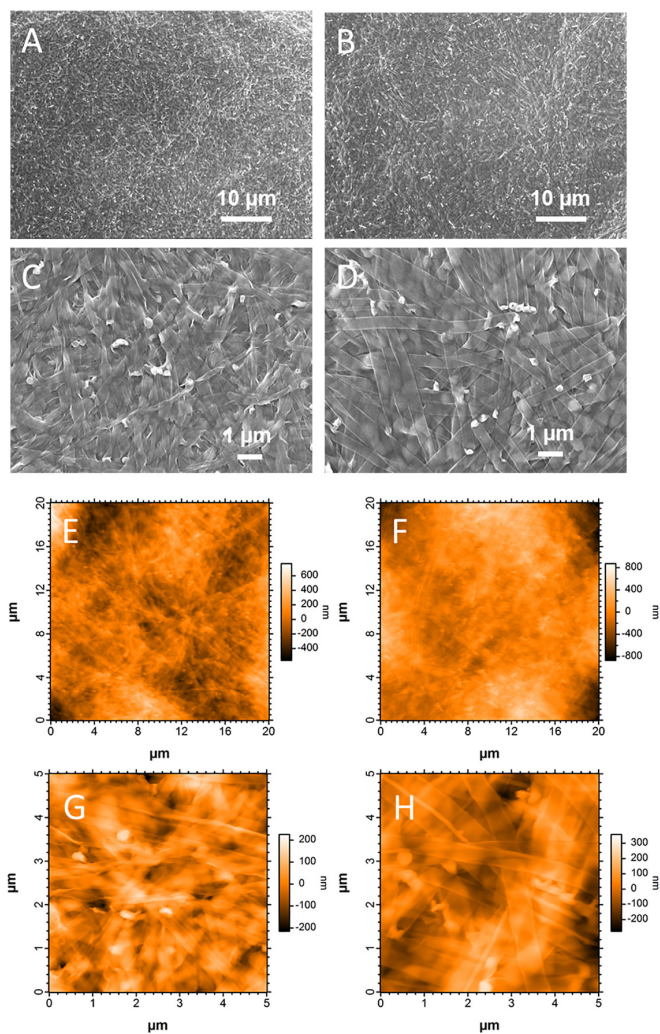


Figure S5.11. SEM (A-D) and AFM images (E-H) collected on the top surface of PAH/PSS nanotube mats fabricated by fructose-assisted filtration. (PAH/PSS)₁₂, A,C,E,G; (PAH/PSS)₂₀, B,D,F,H. Nanotubes were grown in 10 mM HEPES buffer at pH 7.0. (E,F 20x20 μm²; G,H 5x5 μm²)

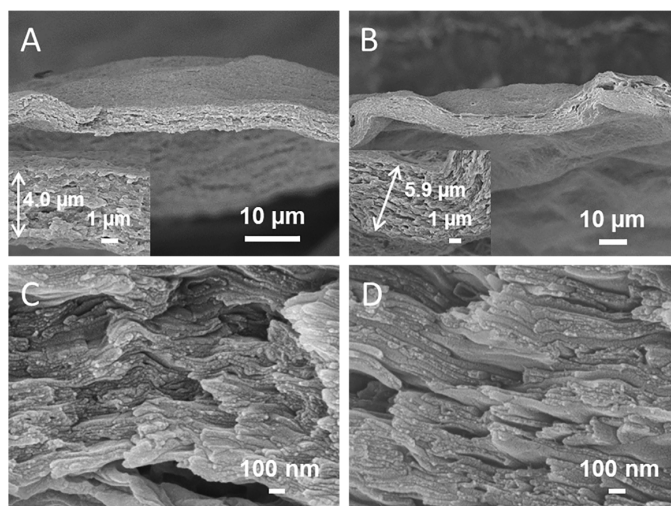


Figure S5.12. Cross-sectional SEM images of PAH/PSS nanotube mats fabricated by fructose-assisted filtration. (PAH/PSS)₁₂, A,C; (PAH/PSS)₂₀, B,D. The nanotubes were built in 10 mM HEPES buffer at pH 7.0. The thickness of each mat was indicated in inset images.

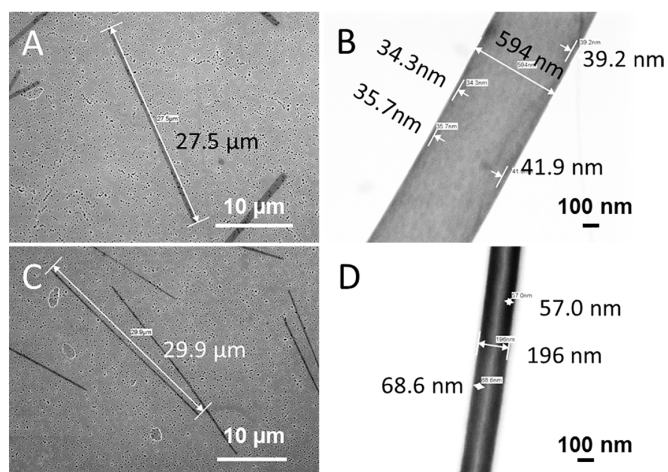


Figure S5.13. SEM (A,C) and STEM (B,D) images of (PAH/PSS)₂₀ nanotubes prepared in 10 mM HEPES at pH 7.0 throughout (A,B) and in 0.5 M NaCl and 5 mM CaCl₂ at pH 7.0 (C,D). PC membranes with a pore size of 300 nm (A,B) and 200 nm (C,D) were used as templates, respectively.

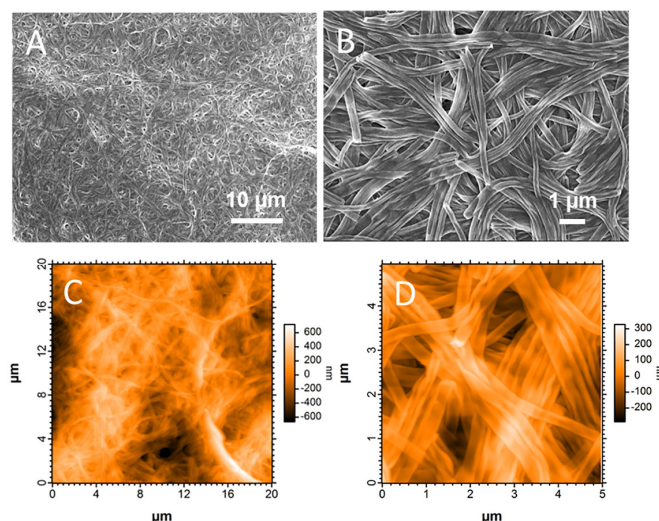


Figure S5.14. SEM (A,B) and AFM images (C,D) collected on the top surface of (PAH/PSS)₂₀ nanotube mats fabricated by fructose-assisted filtration. (PAH/PSS)₂₀ nanotubes were grown in the presence of 0.5 M NaCl and 5 mM CaCl₂ in polyelectrolyte solutions at pH 7.0.

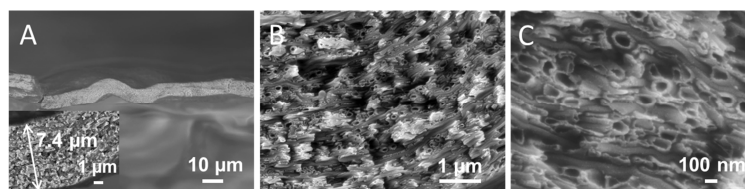


Figure S5.15. Cross-sectional SEM images of mats of (PAH/PSS)₂₀ nanotubes, prepared in the presence of fructose adjuvant. The nanotubes were assembled from polyelectrolyte solutions (pH 7.0) with 0.5 M NaCl and 5 mM CaCl₂ added, in nanoporous PC membranes of 200 nm pore diameter. The insert in A shows the thickness of this mat. Image B displays the porous structure. In the magnified image C, the tubular structures are revealed.

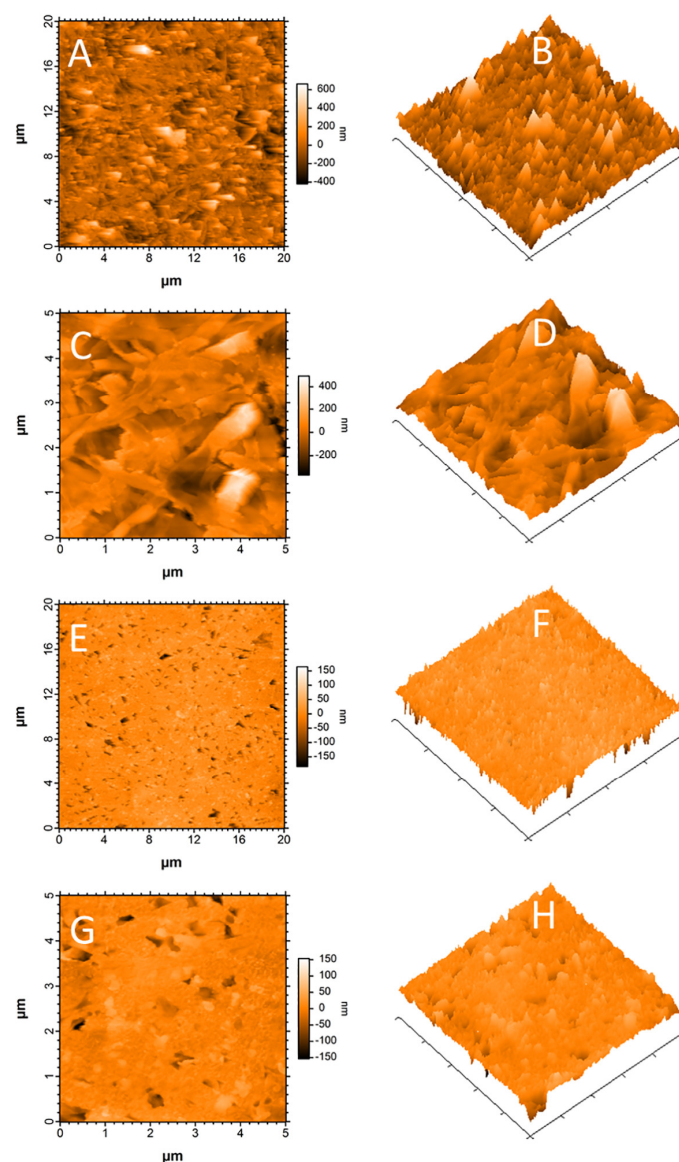


Figure S5.16. AFM images collected on bottom surfaces of 6/12 (A-D) and 6/20 (E-H) nanotube mats prepared by adjuvant-free filtration. B,D,F,H are corresponding 3D displays with the same scale.

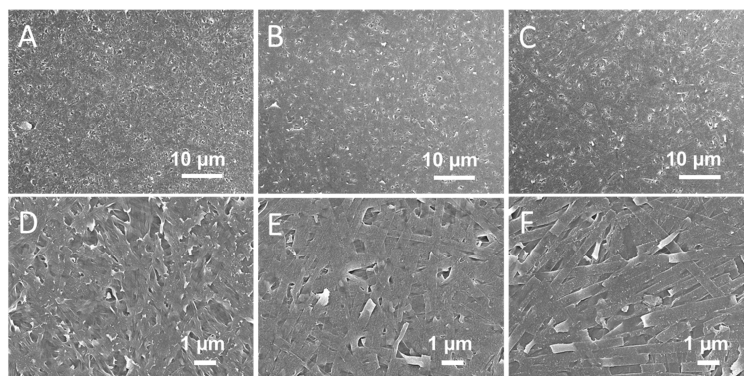


Figure S5.17. SEM images recorded on the bottom surfaces of mats consisting of (PAH/PSS)₆ (A,D), (PAH/PSS)₁₂ (B,E), and (PAH/PSS)₂₀ (C,F) nanotubes, fabricated from 10 mM HEPES buffer at pH 7.0. The mats were prepared by the adjuvant-free filtration route over Au-coated nanoporous PET membranes.

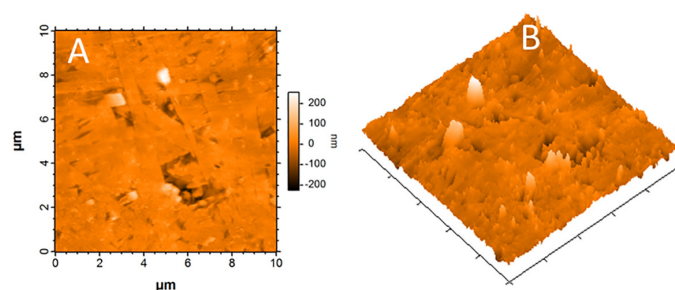


Figure S5.18. AFM images recorded on the bottom surfaces of a (PAH/PSS)₂₀ nanotube mat prepared by the adjuvant-free filtration route. The nanotubes were prepared from 10 mM HEPES buffer at pH 7.0. B is the corresponding 3D display with the same lateral scale as A (10x10 μm²).

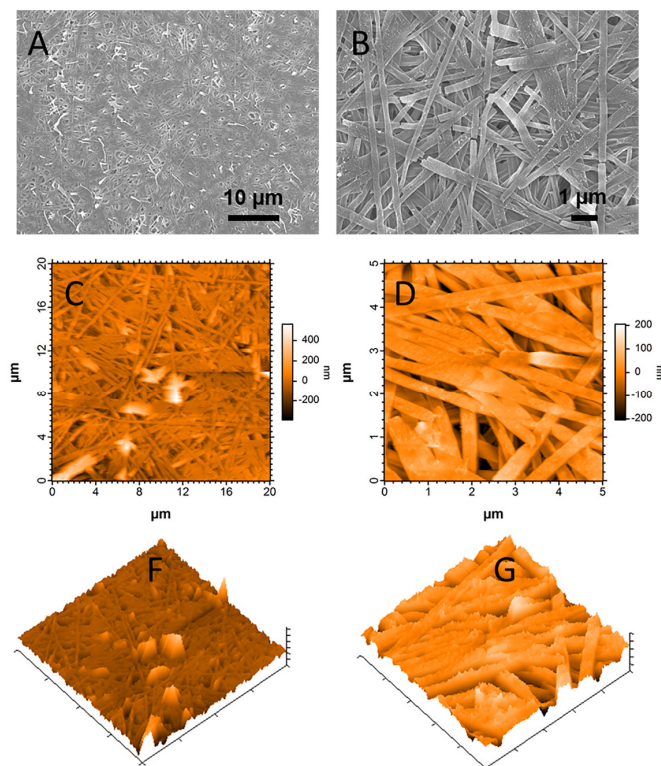


Figure S5.19. SEM (A,B) and AFM (C-F) images of the bottom surface of mats made of (PAH/PSS)₂₀ nanotubes prepared from the (NaCl and CaCl₂) salted assembly conditions with the adjuvant-free filtration route, within pores of 200 nm diameter. F and G are 3D displays corresponding to C and D, respectively. (C,F 20x20 μm²; D,G 10x10 μm²)

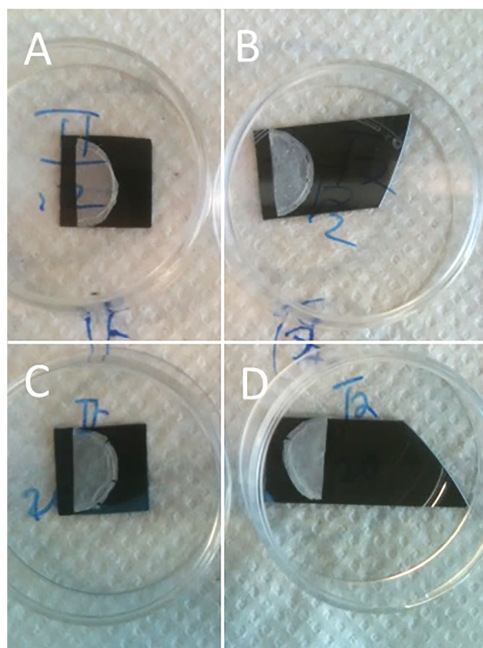


Figure S5.20. Photographs of $(\text{PAH/PSS})_{12}$ (A,B) and $(\text{PAH/PSS})_{20}$ (C,D) nanotube mats collected on silicon wafers with their top (A,C) and bottom (B,D) surface up. The nanotubes were built from 10 mM HEPES buffer at pH 7.0. These PAH/PSS mats were constructed by the fructose-aided collection route.

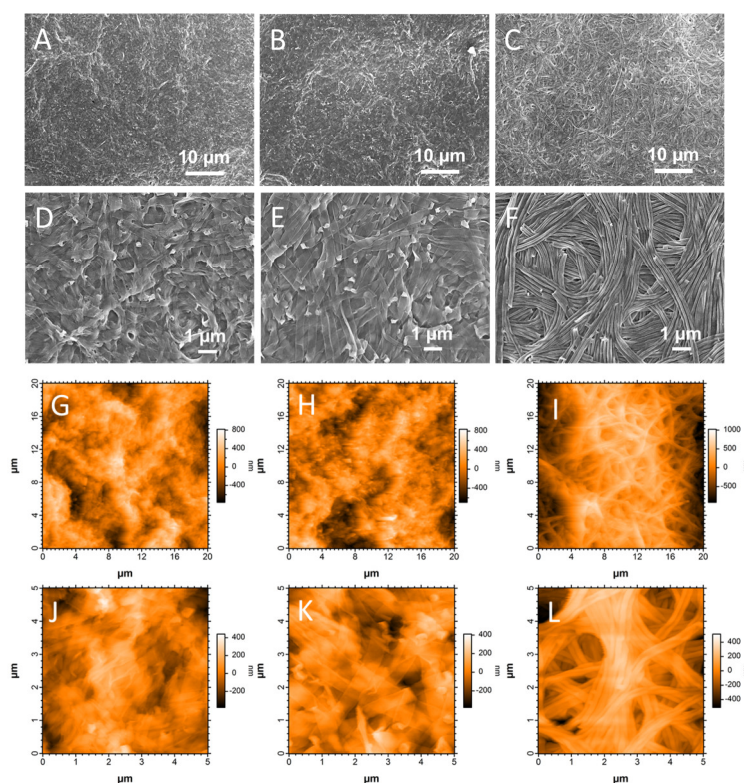


Figure S5.21. SEM (A-F) and AFM (G-L) images collected on the bottom surfaces of mats made of (PAH/PSS)₁₂ (A,D,G,J) and (PAH/PSS)₂₀ (B,E,H,K) nanotubes built from HEPES buffer, and of (PAH/PSS)₂₀ (C,F,I,L) nanotubes assembled from salted polyelectrolyte solutions. Mats were prepared by the fructose-aided filtration route. After the formation of the mat over the fructose layer, a floating mat could be obtained by dissolution of the sacrificial fructose cake (Scheme 5.2), followed by rinse in abundant water. The mat was then collected over a silicon wafer with its bottom surface up.

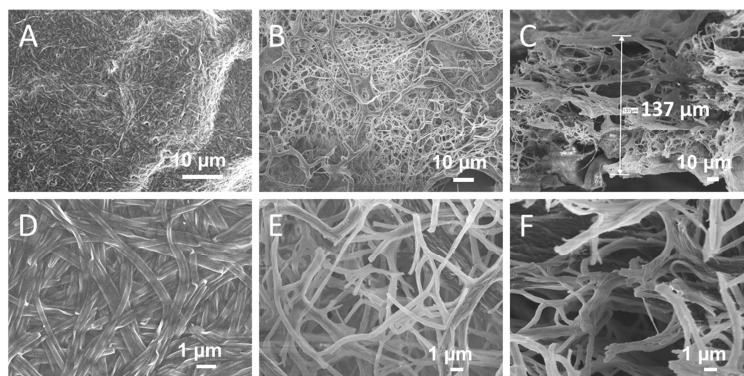


Figure S5.22. Top (A,D), bottom (B,E) and side (C,F) SEM views of mats made of 200 nm-(PAH/PASS)₂₀ nanotubes built in 0.5 M NaCl and 5 mM CaCl₂ (pH 7.0) assembly condition. This mat was constructed by fructose-aided filtration using the no-optimal procedure described in Figure S5.1a-c.

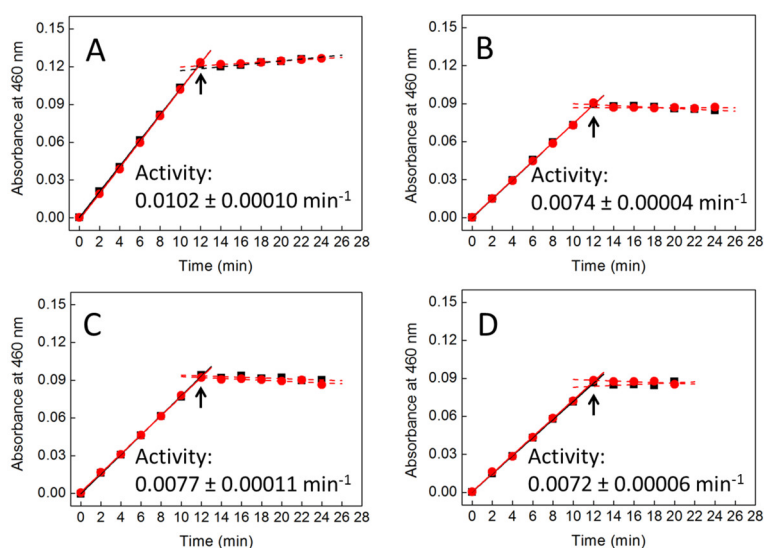


Figure S5.23. Increment of absorbance at 460 nm against reaction time, for enzymatic nanotube mats prepared by the adjuvant-free route. The mats were taken out from the solution after 12 min (indicated by arrows). (A) (bPEI/GOX)₆ nanotube mat, (B) 6/6 nanotube mat, (C) 6/12 nanotube mat, (D) 6/20 nanotube mats. The activity was calculated from linear fits (solid lines), also shown in each graph.

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Chapter 6

Conclusions and perspectives

In the present work, we went through the construction, collection, and the activity evaluation of tubular LbL-nanostructures containing bioactive components, as well as mats made of these bioactive nanotubes. These systems mimic natural structures and functions, and are one step towards a better understanding of nature's intelligence, thus providing guides for the design of novel nanomaterials and active assemblies for various applications.

In the first stage, the growth of GOX enzymes LbL-assembled with polycations (CHI, PEI) was investigated in depth either on flat substrate (silicon wafers) or within nanoporous templates (PC membranes) under various deposition conditions differing in terms of pH, nature and concentration of buffer, and ionic strength.

The odd assembly behavior of proteins with polycations results from the high complexity of the molecular envelope of proteins. A strong release of previously adsorbed GOX enzyme was caused by the subsequent deposition of chitosan in most test conditions, leading to a very inefficient accumulation of components and thus a quite slow growth on flat surfaces. The regular growth of GOX/CHI was observed only when pure water (pH 6.5) was used for the adsorption, however, buffer is recommended for the absorption of enzymes to maintain the bioactivity of enzymes in the long-term LbL buildup process. Hence, this GOX/CHI pair is not suitable for the construction of robust enzyme nanostructure. In contrast, the GOX enzymes were regularly accumulated in a layer by layer fashion in most cases (buffer used) when pairing with branched PEI (bPEI). The highly branched structure of bPEI is believed to stick to the enzymes effectively, since negligible

release of adsorbed GOX was caused by the following deposition of bPEI in some cases. However, a significant loss of GOX was still observed in high ionic strength conditions, indicating that lower ionic strength facilitates the buildup of enzyme-containing films. With these knowledge in hand, the LbL assembly of GOX and bPEI was then performed in the nanoporous PC membranes in view of the fabrication of stable enzyme nanotubes. By examining the activity of nanotube-containing PC membranes and the structure of the resulting nanotubes, 10 mM HEPES buffer (pH 7.0) was found among all test conditions to give nice tubular structures with a high activity; thus this condition was chosen for template-synthesis of robust enzyme nanotubes. The composition of enzyme nanotubes prepared in these optimized conditions was also analyzed by FTIR, revealing that the enzyme dominates the tubes by weight, with a content of ca. 80% regardless of the number of bilayers, and that the enzyme retains its protein secondary structure in the nanotubes after complexation with bPEI.

Once stable enzyme nanotubes were achieved, the second part focused on the extraction of these tubular structures from templates and on transferring them into aqueous medium, which is a key issue for using these promising functional nanotubes for biological applications. Nanotubes can be easily released from the hard PC template upon dissolution in CH_2Cl_2 . However, the difficulty is in the separation of liberated polymeric nanotubes from the polymer solution by centrifugation. Therefore, we developed an alternative approach to collect tubular structures and then efficiently disperse them again in aqueous solution. In this filtration-based collection strategy, water-soluble yet CH_2Cl_2 -insoluble adjuvants were exploited to serve as both leaky barrier layer and reservoir. The formation of a thin adjuvant layer over the filter (nanoporous PET membranes) prevents the contact between nanotubes and filter, since the interactions between liberated nanomaterials and filter are too strong to allow for dispersion of collected nanotubes in water. Moreover, the addition of adjuvants in the $\text{PC}/\text{CH}_2\text{Cl}_2$ suspension of liberated nanotubes leads to a mixed adjuvant/nanotube layer, resulting in a good distribution of nanotubes in the adjuvant matrix, thereby eliminating aggregates of nanotubes. No trace of nanotubes on the PET filter and of aggregated nanotubes in clear aqueous suspension was observed with this collection strategy, demonstrating that almost all nanotubes are trapped within the reservoir cake, and can be well dispersed in water during the cake dissolution. However, the dissolved adjuvant in nanotube suspension has

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side effects on the nanotube structures and consequently on the activity of aqueous suspensions of free nanotubes, depending on the nature of the adjuvant used. While NaCl induces a release of enzymes from liberated nanotubes by screening electrostatic interactions, clear aqueous suspensions of nanotubes of intact tubular morphology could be obtained with fructose and dextran. Nevertheless, the high osmotic pressure created by fructose compared to dextran contributes to loosening the nanotubular structure, leading to less stable and more swollen nanotubes; hence, dextran is recommended, since it is more innocuous to the structure of the nanotubes, benefiting from its neutral and polymeric nature.

The activity of nanotubes embedded in templates was found to reach a maximum for four bilayers, followed by a moderate decrease and then a plateau due to the interplay between the amount of enzyme embedded in the tubes and the accessibility of glucose to the enzyme. Dextran-collected freed nanotubes display a similar trend, but have a higher activity than embedded nanotubes thanks to the increased diffusion of enzyme substrate through the external lateral surfaces. As for fructose-collected nanotubes, they exhibit an even higher activity probably as a result of their more swollen walls. This part of our work succeeded thus to provide an elegant route to fabricate and collect functional tubular structures, paving the way to use them further as bioactive nanoreactors for biomedical applications.

Finally, enzyme-containing LbL-nanotubes were exploited to construct a biocatalytic 'paper' mat for possible bio-purification applications. In this regard, the nanotubes were collected directly from the PC/CH₂Cl₂ suspension after their release, by filtration either on a porous PET filter, or onto an adjuvant layer over a PET filter. The collected nanotubes tend to stick together, thereby providing cohesion to the mat, owing to the soft and blended nature of their outer surface. Moreover, nanotubes interlace with each other during sedimentation, improving the stability of the resulting mats as well, offering the possibility of entirely releasing the mats from the filters (hard PET membrane or adjuvant layer) either by immersion of the PET filter in acetone, or by dissolution of the sacrificial adjuvant layer in water. When the adjuvant is used to assist in mat preparation, an optimized protocol of adjuvant layer formation was proposed to produce an intact and nice mat. Then the structure of mats made of LbL-nanotubes was investigated in details by SEM and AFM. The top surface morphologies of mats were found

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to be different for soft enzyme nanotubes or core-shell nanotubes, depending on the selected filtration route; however, an identical surface morphology is observed for strong PAH/PSS nanotubes. Interestingly, the mats exhibit diverse internal structures, depending on the nature as well as on the rigidity of the nanotube building elements, from compact mats for sticky and soft enzyme nanotubes, limited porous structure when a rigid (PAH/PSS) core was introduced to enzyme nanotubes, to fibrillar layered structure for rigid pure PAH/PSS nanotubes. Thicker nanotubes are strong enough to conserve their tubular structure integrity, leading to a porous nanofiber scaffold with a two-level porosity: at the scale of the nanotube cores, and at the inter-tube scale. Moreover, the presence of the adjuvant layer eliminates the physical imprint of the PET filter observed on the bottom surface of mats when directly filtered over PET membranes, leading to a mat symmetric with respect to the surface microstructure. The preservation of the biological activity of enzymes in nanotubes and the improved stability of enzyme nanotubes when a rigid core is used, make such bioactive mats promising platforms for applications in bio-purification.

This thesis was mainly dedicated to the development of smart functional nanoassemblies with interesting biocatalytic applications as well as to the understanding of associated mechanisms. Our work on the assembly of proteins in nanochannels and on the collection of templated-synthesized bioactive nanotubes provides a robust route for the templated-construction of versatile multifunctional nanovectors incorporating biomacromolecules. Nevertheless, many challenges still remain on the way to the goal of utilizing such enzyme nanoreactors as bioactive organelles *in vivo* to perform a real task. Many investigations will still be required, for instance, on the compatibility, stability, as well as the cellular uptake behavior of these free tubular nanostructures.

The mat fabrication methodology discussed in Chapter 5 is just the first step to understand this construction process of mats and to demonstrate the capability of this new technology for the fabrication of functional nanofibrillar frameworks by using functional LbL-nanotubes. Following this work, there is a very wide range of studies that could be undertaken in the future. For instance, enzyme nanofibrous 'paper' incorporating carbon nanotubes would be an ideal nanomaterial as porous electrodes for enzymatic biofuel cell applications; sequential sedimentation of different functional LbL-nanotubes would result in multilayered mats, leading to, e.g., enzymatic cascade

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systems by using catalase nanotubes and glucose oxidase nanotubes. Another possibility is to fabricate hybrid multifunctional nanotube scaffolds for *in vitro* tissue engineering. Towards this aim, biocompatible materials and growth factors could be used and the cell growth behavior could be investigated with respect to, e.g., adhesion, proliferation, or differentiation. Afterwards, we could go further to incorporate cells into these biocompatible scaffolds to create hybrid tissue analogues, mimicking the extracellular matrix (ECM) environment and three-dimensional organization of biological tissue. This technology is thus very promising to produce various artificial tissues of interest.

The diversity of this nanofibrous mat fabrication strategy results from the way that nanotubes/nanofibers are produced, which allows utilization of a vast range of functional elements. By rational incorporation of quantum dots in nanofibers, hybrid mats could be obtained and used as light-harvesting nanomaterials. Layered nanotubes containing well-distributed quantum dots in the tube wall would result in a nice vertically-ordered configuration of embedded dots in an entire nano/microscaled paper, offering some hope for the proof-of-concept construction of a quantum dot solar cell of high conversion efficiency. In addition, the dimension of nanostructures (pore diameter and length) can be easily tuned by using appropriate pore size templates. These parameters can also be investigated with respect to the structure and properties of resulting 'paper' mats. For instance, polyelectrolyte nanofibrous papers can probably be used as nanofilters for selective ion and solute separation and the size and structural features may play an important role.

Beyond the mat comprising randomly positioned nanotubes/nanofibers, it is possible to horizontally align the nanotubes/nanofibers containing magnetic particles by magnetic field during the filtration-based preparation, giving rise to an anisotropic nanofibrous scaffold for possible fluidic applications. In addition, continuously changing the direction of the magnetic field in plane would result in a helical structure within the mat, which can then direct the deposition of an organized angle-ply, multi-lamellar extracellular matrix.

At the end of this thesis, templated LbL assembly is thus now a robust route to fabricate various functional structures, and there is still a large space at the bottom to be exploited by combination with crossover technologies from

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other fields. We hope that this thesis will contribute inspiring other researchers to extend further this technology to new fields.

Dissemination

Publications

Saghazadeh, S.; Zhang, S.; Lefèvre, D.; Le Beulze, A.; Jonas, A. M.; Demoustier-Champagne, S. A Universal Method to Transfer Membrane Templated Nano-objects to Aqueous Solutions, *Langmuir* 2015, 31, 7264-7273.

Zhang, S.; Demoustier-Champagne, S.; Jonas, A. M. Quantitative Collection and Enzymatic Activity of Glucose Oxidase Nanotubes Fabricated by Templated Layer-by-Layer Assembly, *Biomacromolecules* 2015, 16, 2382-2393.

Zhang, S.; Vlémincq, C.; Ramirez Wong, D.; Magnin, D.; Glinel, K.; Demoustier-Champagne, S.; Jonas, A. M. Nanopapers of Layer-by-Layer Nanotubes, submitted.

Zhang, S.; Demoustier-Champagne, S.; Jonas, A. M. Mats of Layer-by-Layer Assembled Enzyme Nanotubes for Bio-catalysis, in preparation.

Oral communication

Zhang, S.; Jonas, A.; Demoustier-Champagne, S. Nanoconfined enzyme nanotubes for biocatalysis. PhD students' day, Louvain-la-Neuve, Belgium, 22/05/2015.

Poster communications

Zhang, S.; Jonas, A.; Demoustier-Champagne, S. Nanoconfined enzyme nanotubes for biocatalysis. IAP/PAI P7/05 Functional Supramolecular Systems, Spa, Belgium, March 17, 2014.

Dissemination

Zhang, S.; Jonas, A.; Demoustier-Champagne, S. Nanoconfined enzyme nanotubes for biocatalysis. Annual meeting of the Belgian polymer group, Ghent, Belgium, May 19-20, 2014.

Zhang, S.; Demoustier-Champagne, S.; Jonas, A. Enzymatic catalysis confined in free nanotubes. IAP/PAI P7/05 Functional Supramolecular Systems Annual Meeting, Louvain-la-Neuve, Belgium, September 19, 2014.

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