

Layer-by-Layer self assembly for nerve tissue regeneration

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Introduction

Even though nerves exhibit a regenerative potential, the recovery of function following a peripheral nervous system injury is poor.

Main obstacles to regeneration:

- inflammatory response → scarring process →
physical barrier to nerve elongation
- presence of tensions (due to adhesions between elongating nerves and surrounding connective tissue) →
contribution to induction of inflammatory response
- non oriented growth of neurites →
failure to establish a functional reconnection with the distal target

Repair strategy for a complete recovery of the physiological nerve function

**Promising solution:
implantable bio-artificial nerve grafts
with which:**

- inhibit activity of profibrotic factors
- avoid formation of local adhesions
- actively guide neurites growth by means of guidance materials

The pro-fibrotic factor Transforming Growth Factor Beta 1 (TGFβ1)

TGFβ family cytokines:

polypeptides strongly involved in the pathogenesis of neuropathies during nerve lesion

TGFβ1:

- pivotal role in the regulation of immune response and inflammation process
- humoral stimulus in scar formation

It has been shown that TGFβ1 neutralisation results in the repair of nerve injuries



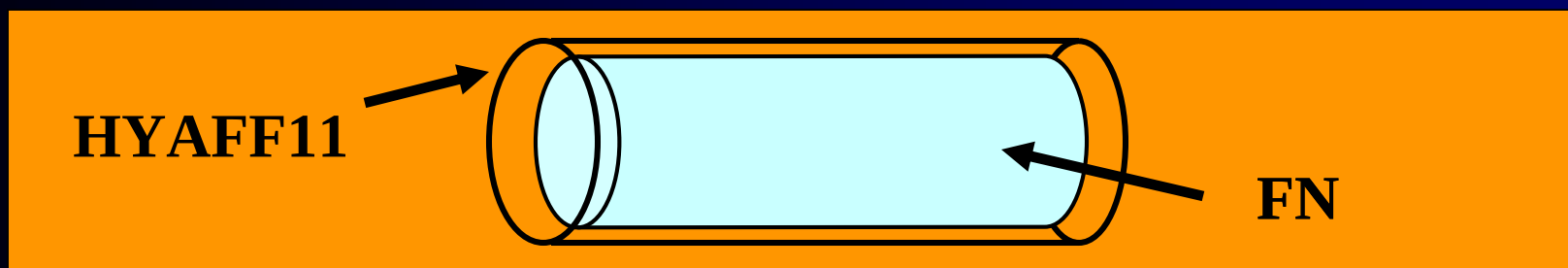
improved

Aim of the work

To nano-functionalise a bioengineered nerve guidance channel with neutralising anti-TGF β 1 in order to set up a tailored device for nerve regeneration purposes.

Nerve guidance channels

- Inner fibronectin core
- Outer HYAFF 11 tube (benzyl ester of the hyaluronic acid)

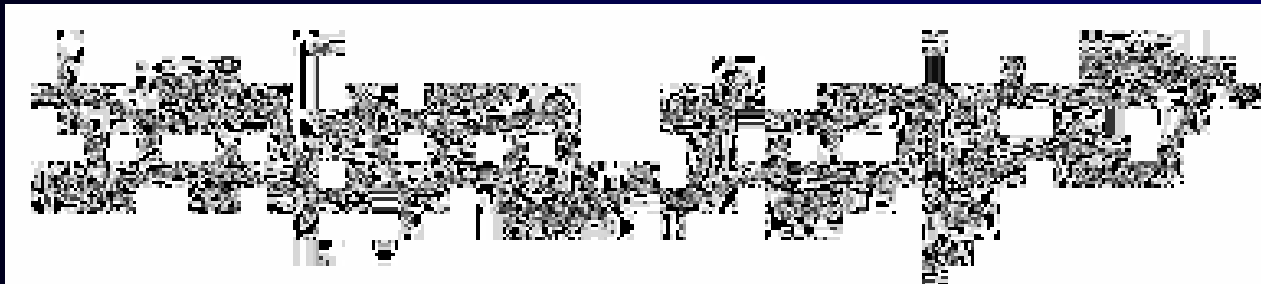


Fibronectin (FN)

- Glicoprotein involved *in vivo* in many aspects of wound healing
- Can be purified from blood plasma and processed into bioengineered materials
- Shown to actively support nerve growth
- Most widely tested form of template in the promotion of tissue repair by contact guidance
- Already taken into account for the release of therapeutic agents showing high potentialities for the development of controlled release systems

HYAFF 11

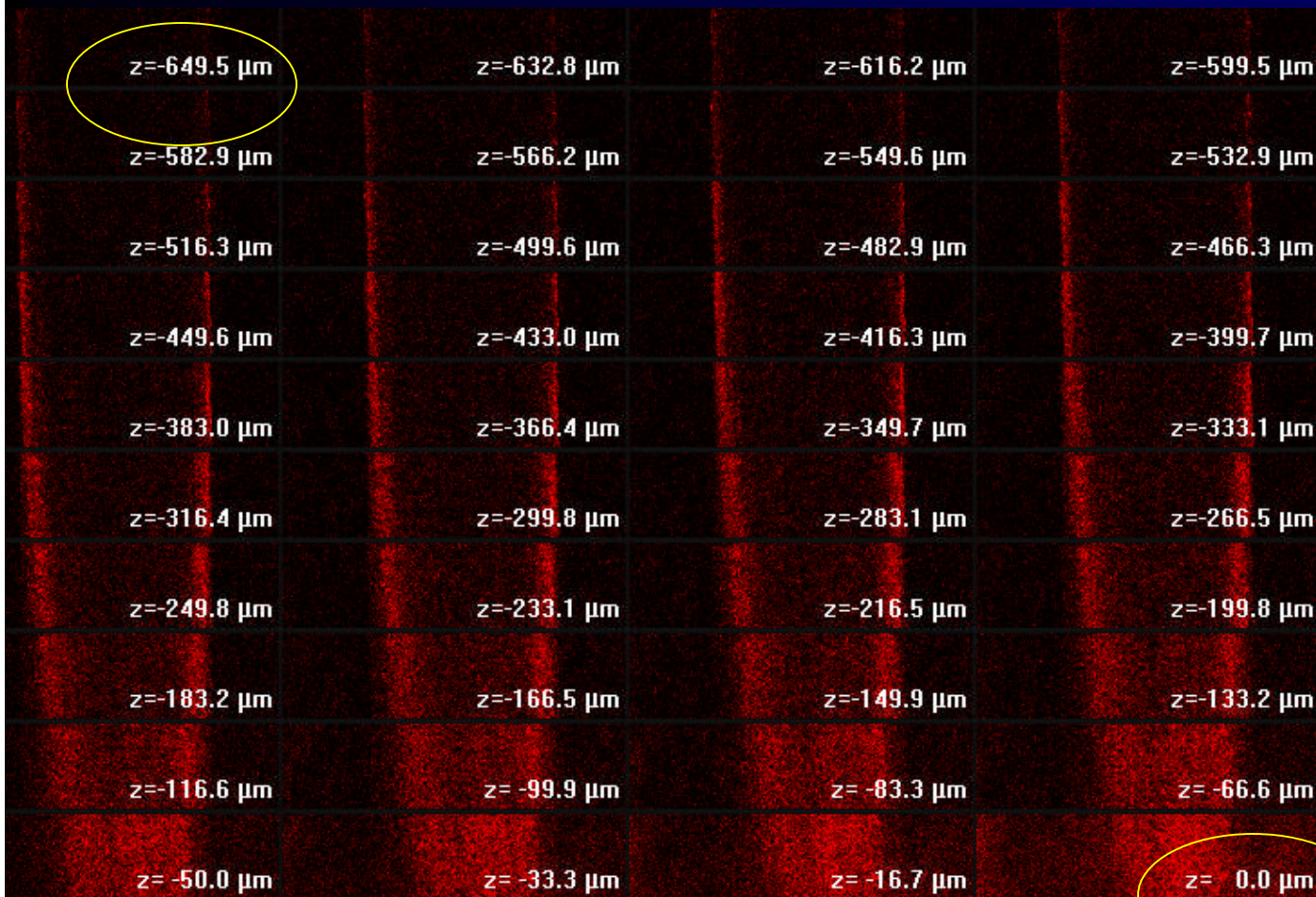
- Benzyl ester of hyaluronic acid, a polysaccharide ubiquitous in soft tissues of higher organisms
- Localised at tissue interfaces and joints preventing mechanical adhesion between connective tissue surfaces
- Currently used to promote tissue regeneration and to reduce postoperative surgical adhesions (a critical factor in peripheral nerve injuries repair)



Structure of HYAFF 11 polymer

ANTI-TGF β 1 absorption by FN cores

- After 96 hours of absorption the most part of Ab appears to be localised on the surface of constructs made of an inner FN core and an outer HYAFF11 tube



Ab distribution in a sequence of focal longitudinal planes (thickness 16 μm) (Excitation: 543 nm; emission at 580 nm)

Sequence of focal longitudinal planes

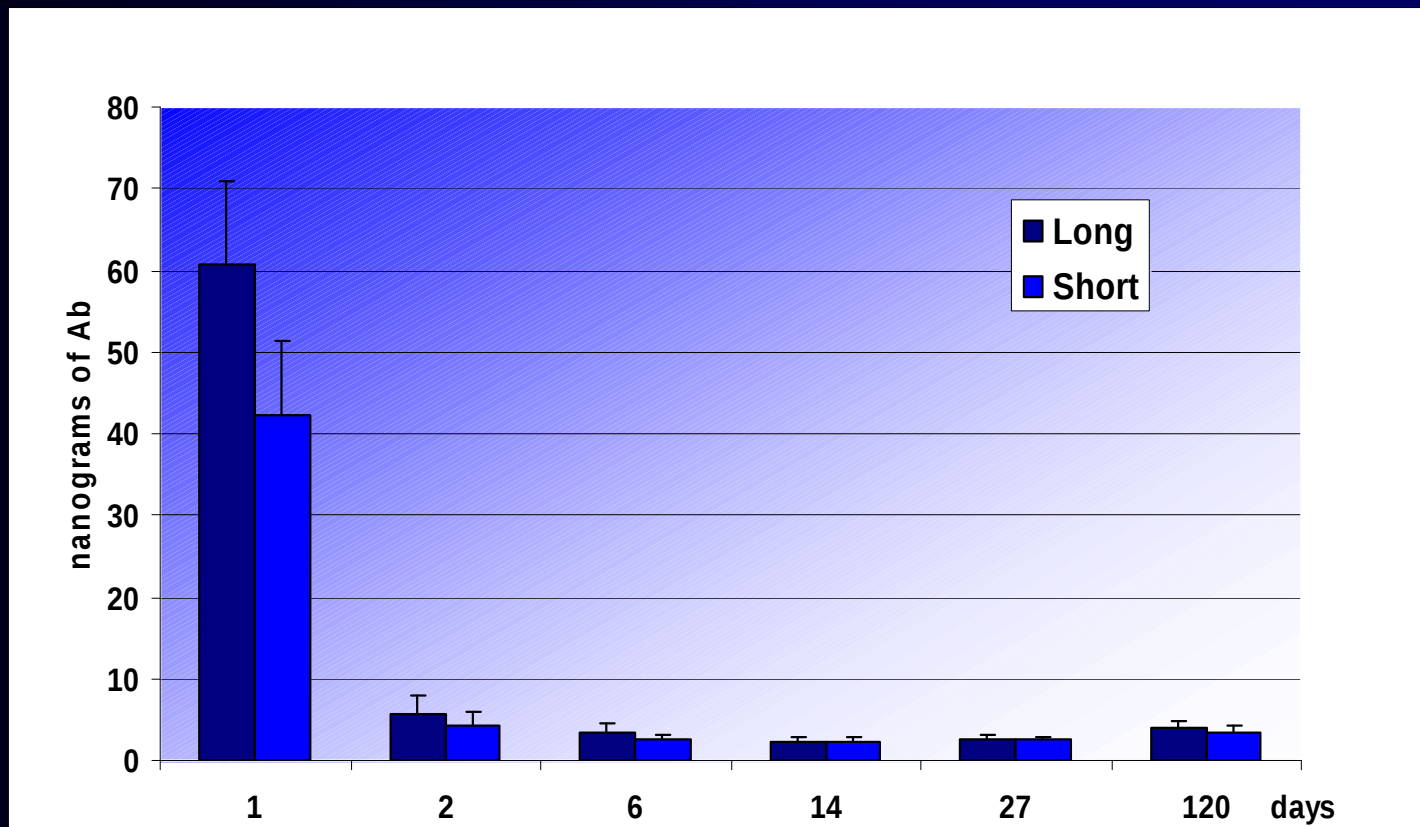
ANTI-TGF β 1 absorption onto HYAFF11

Confocal microscopy analysis has shown that Ab can not be absorbed onto the unmodified HYAFF11 surface.

Antibody release per time interval

Over a period of 4 months:

- Only 23% of the Ab was released
- Major Ab release in the first 24 hours
- Little amounts of Ab released in the days after



Preliminary conclusions

The technique used (passive functionalization) to insert Ab into the construct does not offer the possibility to control absorption and release processes

Layer-by-Layer Self Assembly Technique

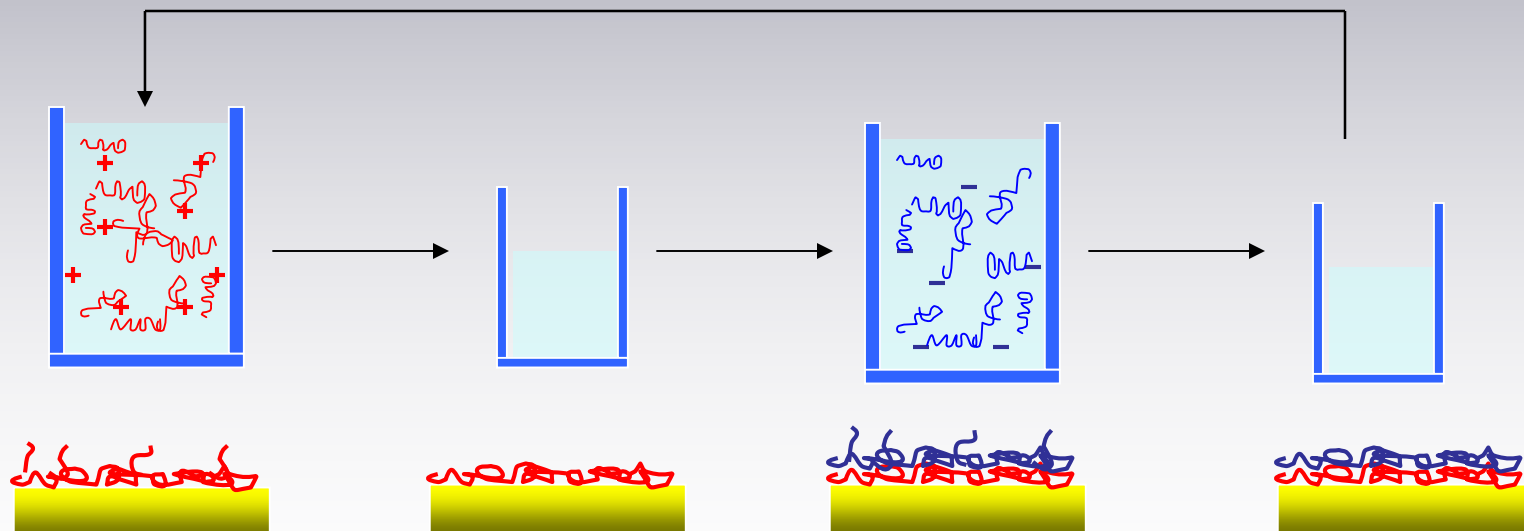
Film assembly by alternate absorption of linear polyanions and polycations

1° Step: Immersion of the support in polycation solution

2° Step: Wash the support in buffer solution

3° Step: Immersion of the support in polyanion solution

4° Step: Wash the support in buffer solution



Layer-by-Layer Self Assembled multilayers

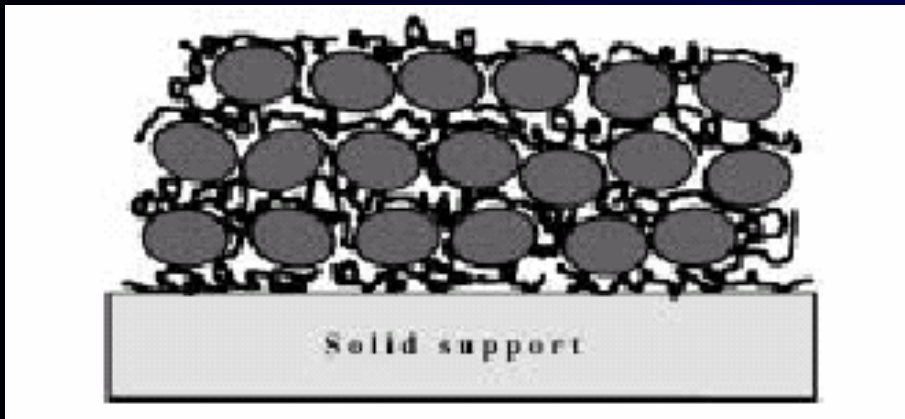
Layer constituents

- Synthetic polyelectrolytes
- Inorganic nanoparticles
- Lipids
- Ceramics
- Biopolymers

Multilayer properties

- Predetermined thickness ranging from 5 to 1000nm
- Precision 1 nm
- Definite knowledge of molecular composition
- Proteins enhanced structure stability

Schematic representation of the protein-polyion multilayer



The immobilization of proteins in multilayers preserves them from microbial attack

Applications

Current

Biocompatible surface coverage.

Enzyme immobilization to increase bioreactors efficiency

Dye casting on optical elements

Potential

Nanobiosensors

Nanoreactors

Drug delivery (nanocapsules)

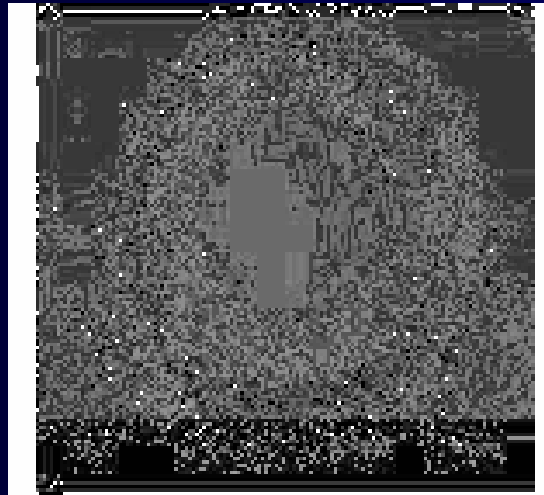
electronics

Materials and Methods

- HYAFF 11 tubes have been bioactivated with specific antibodies by the Layer by Layer technique
- Antibody (IgG): Monoclonal anti-human Transforming Growth Factor Beta 1 (anti-TGF β 1)
- Anionic species: poly(styrenesulfonate)
- Cationic species: poly(dimethyldiallylammonium) chloride and poly-D-lysine
- Quartz Crystal Microbalance to monitor the assembly process
- Analysis of the self assembled layers by Scanning electron microscopy (SEM) and by Atomic force microscopy (AFM)

Optimisation of the assembly procedure

- Quartz Crystal Microbalance (QCM) monitoring of multilayer growth represents the first stage of the assembly procedure elaboration since it allows the step by step monitoring of the process.
- The elaborated assembly procedure is then used onto the desired surface.



Electrode of Quartz Crystal Microbalance

Quartz Crystal Microbalance

Piezo-electric crystals (e.g. quartz) vibrate with a characteristic resonant frequency under the influence of an electric field. The resonant frequency changes as molecules adsorb on the crystal surface. A direct relation between the frequency shift, mass, and thickness of the deposited layers can be obtained using the following equations.

$$\Delta F = k_1 \Delta M/A \quad \text{and} \quad \Delta F = k_2 \Delta T/A$$

ΔF : resonant frequency shift (Hz); ΔM : mass shift (ng); ΔT : thickness shift

k_1 : constant depending from crystal density and shear modulus; k_2 : constant depending from k_1 and from the density of the protein/polyion film A: adsorbing surface area (cm²)

Lvov Y. et al, Langmuir 1997, vol. 13, p. 6195; Sauerbrey G., Z. Phys., 1959, vol. 155, p. 206).

Optimized Assembly procedure

Working conditions:

- Phosphate Buffer Saline solution (PBS) 0.01 M + NaCl: 0.01 M
- pH: 7.4 taking into account the IgG isoelectric point (pI: 6.8)
- 4°C for the assembly of PDL and anti-TGFβ1 to prevent denaturation
- Room Temperature for the assembly of poly(styrenesulfonate) and poly(dimethyldiallylammonium) chloride

Three precursor bilayers (to provide a uniform surface for subsequent Ab absorption, *Lvov et al, J. Am. Chem. Soc. 1995, vol. 117, p. 6117*):

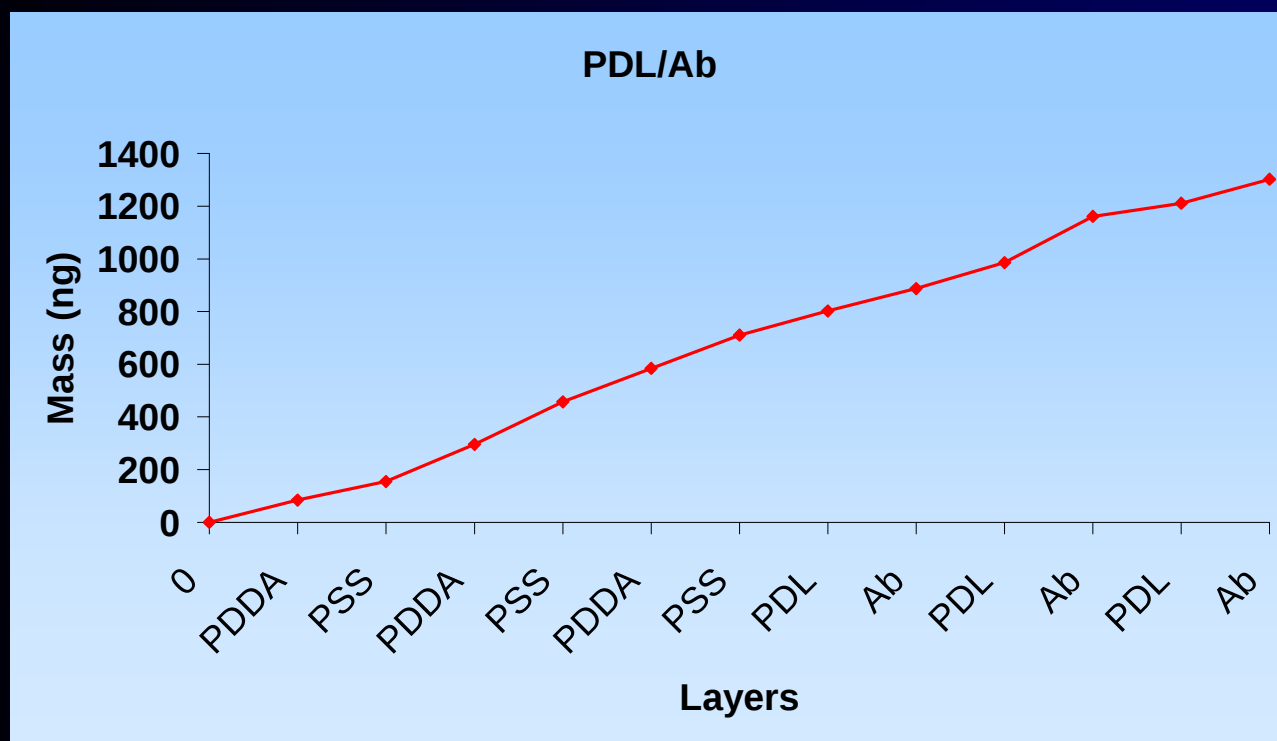
- poly(styrenesulfonate) (PSS), absorption time: 10 min, 3 mg/ml
- poly(dimethyldiallylammonium) chloride (PDDA), absorption time: 10 min, 2 mg/ml

Three bioactive bilayers:

- poly-D-lysine (PDL), absorption time: 30 min, 0.5 mg/ml
- anti-TGFβ1 (Ab), absorption time: 1 hour, 20 μg/ml

Assembly on piezoelectric crystals

A linear film mass increase with the number of assembly steps indicated a successful procedure.

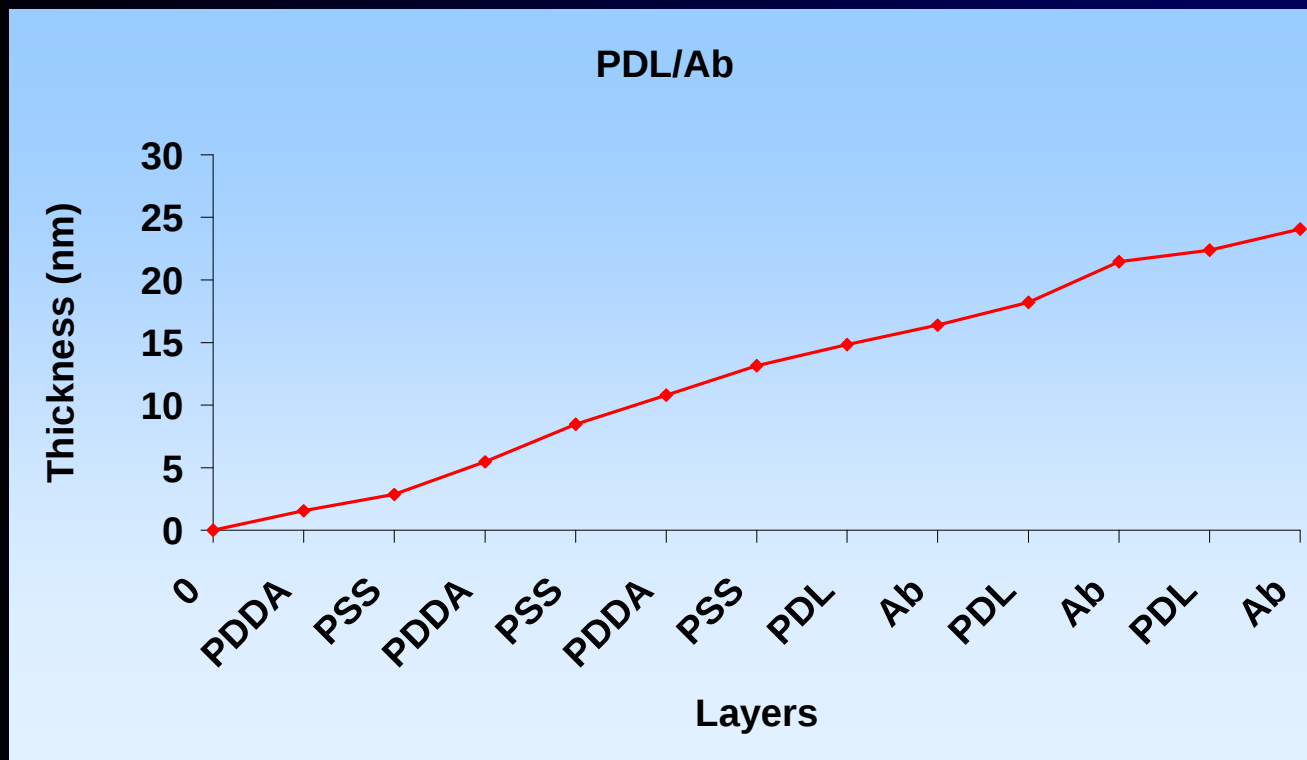


Average Ab layer
mass 117 ng

Mass shift of QCM resonator for the architecture

$(\text{PDDA}/\text{PSS})_3 + (\text{PDL}/\text{Ab})_3$

Assembly on piezoelectric crystals



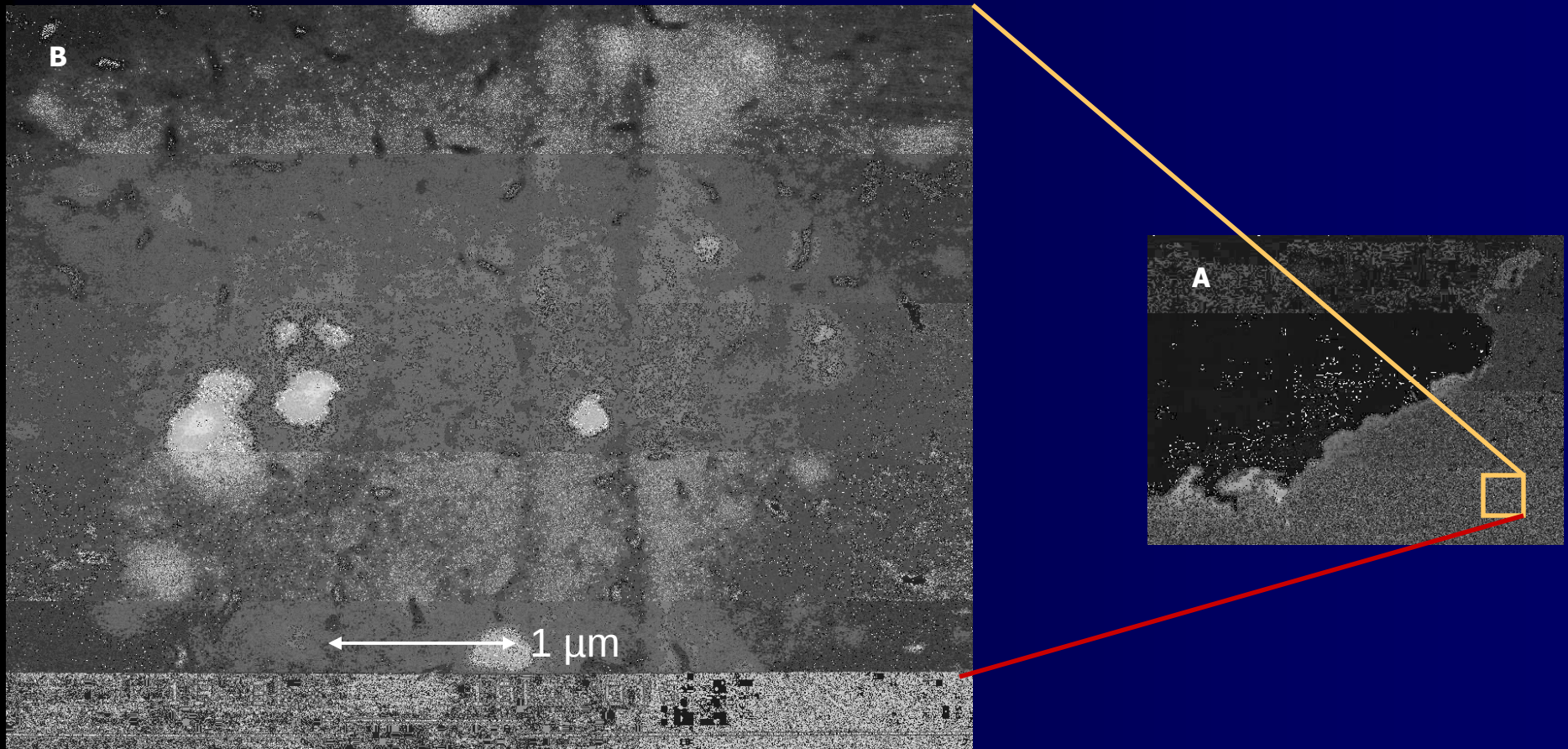
Average Ab layer
thickness= 2.16 nm

Thickness shift of QCM resonator for the architecture

$(\text{PDDA/PSS})_3 + (\text{PDL/Ab})_3$

SEM Analysis

Scanning electron micrographs of
(PDDA/PSS)₃/(PDL/anti-TGFβ1)₃ film on HYAFF11



A: Scratch on a LBL modified HYAFF11 surface
B: detail of the LBL multilayer onto HYAFF11

Conclusions

- Ab has been self-assembled on HYAFF11
- The multilayer growth has been characterized.
- The multilayer morphology has been characterized.
- A nanobioactivated nerve guide can be developed on the basis of this work.