

LMAPR2012 - POLYMERS FOR ADVANCED TECHNOLOGIES

Laboratory : Hard templating, layer-by-layer assembly and
biofunctionalization

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1 Methodology

1.1 Nanotubes and membrane manufacturing process

1.1.1 Layer-by-layer assembly

To create the nanotubes, nano-porous polycarbonate (PC) membranes are used as a template. The tubes are created using a layer-by-layer assembly method. Layers are added one by one to create nanotubes with a defined number of layers. Alternating layers of polyethylenimine (a positively charged polyelectrolyte) and laccase (a negatively charged protein) are deposited. A cross-linking agent is added during the process to enhance interaction between the polyethylenimine and laccase layers, this agent being glutaraldehyde. Two washing steps are also done during the different immersion to clean the excess of solutions, the washing solution is a MES buffer.

The assembly procedure for one cycle :

The first step is to immerse the membrane in the PEI solution for 3 minutes. Then wash the membrane for 30 seconds in the MES buffer. The second layer is added by immersion of the membrane in the laccase solution for 3 minutes. The membrane is then immersed in the glutaraldehyde solution for 3 minutes. A second wash is then done for 3 minutes. At the end, membranes are stored in the MES buffer.

1.1.2 PC membrane dissolution and nanotubes filtration

The membrane now needs to be dissolved to obtain the nanotubes. These nanotubes are collected by filtration. The dissolution and filtration procedures are described in detail :

First, dissolve the membrane with the nanotubes in 10mL of CH_2Cl_2 . Take a syringe and add a metalised PET membrane in it, this membrane will catch the nanotubes from the solution of CH_2Cl_2 . Then split the solution containing the nanotubes through the PET membrane. When all the syringe is empty, remove carefully the PET membrane and keep this membrane for the SEM experiment.

1.2 Enzymatic assay

The objective of the enzymatic assay is to compare the enzymatic activity in a flow state with a UV-VIS spectroscope. When ABTS is oxidised by the laccase molecule, the radical form of ABTS absorbs light at 420 nm. ABTS is a chemical compound used to study the kinetics of certain enzymes.

The procedure is :

Fill first a syringe with the ABTS solution. Place then a membrane in the metallic cell and connect this cell to the syringe. Place the syringe on the syringe injector device which will press the solution out at a certain flow rate. Start the injection of the ABTS solution in an Eppendorf for 3 minutes at a flow rate of $0.2 \text{ mL} \cdot \text{min}^{-1}$. Next, measure the absorbance at 420 nm and based on the absorbance values, calculate the amount of ABTS converted which will give the membrane activity.

1.3 Membranes and nanotubes visualisation by SEM

SEM operates by directing electron beams onto a sample. The electron gun emits these beams, which then travel down the column of the microscope, undergoing acceleration. As the beams move through the microscope, they pass through various lenses and apertures to focus them. This entire process takes place within a vacuum to prevent any interference, leading to high-quality imaging. The sample is thus scanned by the electron beam. As the electrons interact with the atoms on the sample's surface, they generate signals in the form of secondary electrons, back-scattered electrons, and rays. The microscope's detectors capture these signals, which are then used to produce high-resolution images that are displayed on a computer screen [1].

Two PET membranes were coated with a 10 nm layer of gold. The nanotubes released from the dissolved M3 and M4 membranes were then collected on the PET membranes using a syringe. The nanotubes were collected on the side coated by the gold layer, as gold has good electrical conductivity, which is necessary for SEM observation. The membranes are cut and taped before being placed inside the microscope.

2 Results

2.1 Multilayers assembly

Three different types of nanotube have been created using the layer-by-layer assembly technique. Nanotubes with 2,4 and 8 double layers were created, each double layer containing one layer of PEI and one of laccase. In addition to these two layers, a Glutaraldehyde crosslinking agent is used.

A first membrane M1 is composed of two bilayers, the membranes M2 and M3 are composed of four bilayers and the last membrane M4 is composed of eighth bilayers.

In Fig.1, the 3 different solutions of PEI, laccase and glutaraldehyde can be seen with the two washing steps (W1 and W2).

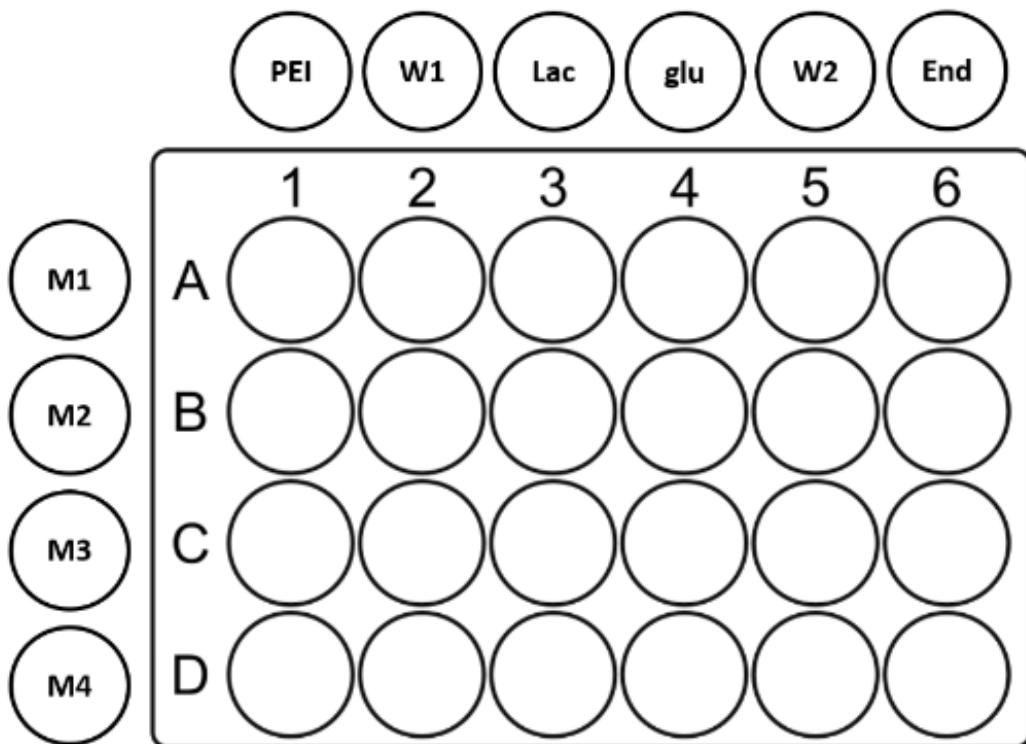


Figure 1: The different sample with the layer solutions.

2.2 Enzymatic assay

The reaction used to determine laccase enzymatic activity involves the oxidation of the substrate ABTS (2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)), which is demonstrated in Fig.2. This reaction is catalysed by laccase, resulting in the formation of oxidized ABTS (which becomes green-blue coloured when it is oxidized), which is the cation radical $ABTS^{\bullet+}$.[2]

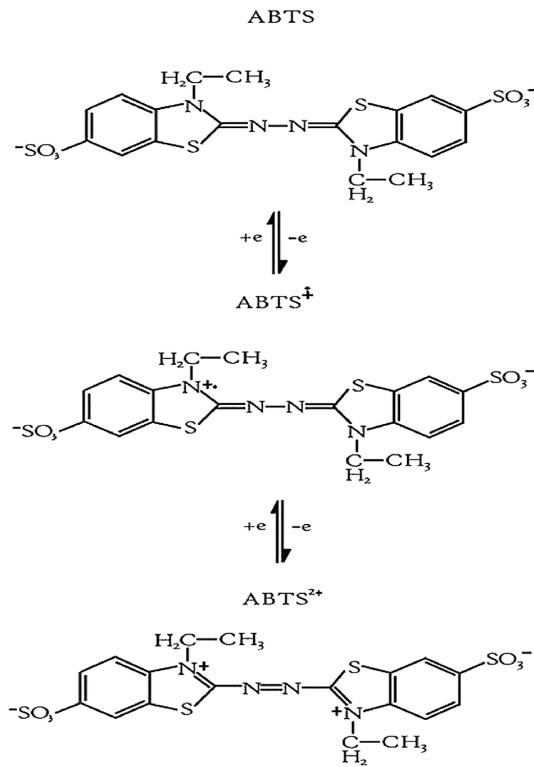


Figure 2: Oxidation of ABTS by laccase.[3]

ABTS is a particularly interesting substrate for quantifying laccase activity because the oxidized form of ABTS exhibits a strong green color, which allows for easy detection and quantification using spectrophotometric methods. The increase in absorbance at 420 nm correlates directly with the enzymatic activity of laccase. Furthermore, ABTS is stable in solution and has good solubility in aqueous buffers, making it suitable for enzymatic assays. Additionally, ABTS is specific to laccase, providing a reliable measure of laccase activity without interference from other enzymes or substrates.

The formation of cation radical $\text{ABTS}^{\bullet+}$ was followed by absorbance measured at 420 nm, in known concentrations of $\text{ABTS}^{\bullet+}$ (C_{ABTS}) in order to generate a calibration curve (Fig.3) that establishes a linear relation between the absorbance measured and the concentration of the substrate.

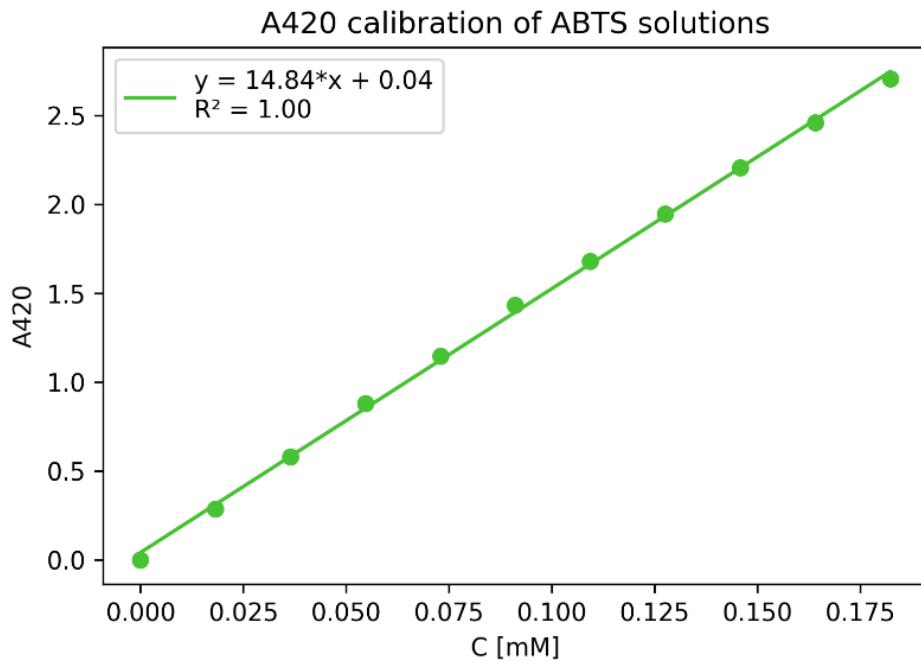


Figure 3: Calibration curve to convert A420 into C_{ABTS} .

Table 1: Measures of the absorbance at 420 nm (A420) and concentration of $ABTS^{\bullet+}$ (C_{ABTS}), in the control solution-pure water-(A), and after passing through membrane M1(B) and M2(C).

| Measure | A1 | A2 | A3 | B1 | B2 | B3 | C1 | C2 | C3 |
|-----------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| A420 | 0.0441 | 0.0435 | 0.0447 | 1.3502 | 1.3732 | 1.4275 | 3.1537 | 3.1592 | 3.1278 |
| C_{ABTS} (mM) | 0.000276 | 0.000236 | 0.000317 | 0.088288 | 0.089838 | 0.093497 | 0.209818 | 0.210189 | 0.208073 |

For each solution (pure water-A; ABTS solution after passing through M1-B; and ABTS solution after passing through M2-C) the absorbance was measured three times.

The C_{ABTS} in Table 1, was calculated using the equation of the curve presented in Fig.3 and the values of the absorbance of the respective table.

The mean values of C_{ABTS} , in mM, relative to the membranes M1 and M2 (subtracting the mean value of the control-0.000276) are 0.090265 and 0.209084, respectively.

The laccase activity for each membrane was measured as the amount of ABTS (μmol) oxidized per minute and cm^2 of the membrane, through the formula: $C_{ABTS} / (\text{time of flow} * \text{membrane surface area})$, giving 3 minutes as the time of flow of ABTS through the membrane and 0.95 cm^2 as the membrane surface area.

Taking this formula into account, the values of laccase activity measured in membranes M1 and M2 were 31.67193 and 73.36281, respectively. These results are in line with what was expected since M1 contains 2 layers of laccase and M2 has 4 layers of the enzyme, being expected a larger activity of laccase in the last membrane (M2) and a lower value in M1 membrane, due to having less concentration of laccase that can oxidize ABTS.

2.3 Membranes and nanotubes visualisation by SEM

During the SEM laboratory, we were unable to make correct observations of our M3 and M4 samples. There was a shielding or charging effect during our observations, and the images were blurred and unusable as can be seen in Fig.4. The charging effect or shielding effect is the phenomenon of the reduction of the force of attraction of the nucleus on the outermost valence electrons due to the presence of the inner shell electrons. Due to their non-conductive nature, the surface of non-conductive materials acts as an electron trap. The resulting accumulation of electrons on the surface is called “charging” and creates extra-white regions on the sample, which can influence the image information [4]. The cause of this effect is certainly due to the poor dissolution of the polycarbonate membranes in the solvent. Large pieces were observed where the nanotubes were supposed to be. The dissolution would therefore have gone wrong and the nanotubes still present in the membrane would have been deposited on the surface of the PET membrane.

Fig.4 shows dark discs corresponding to the pores in the PET membrane. The white filaments correspond to undissolved pieces of the polycarbonate membrane. The white cloud around these filaments is therefore the charge effect and makes it impossible to observe any nanotubes.

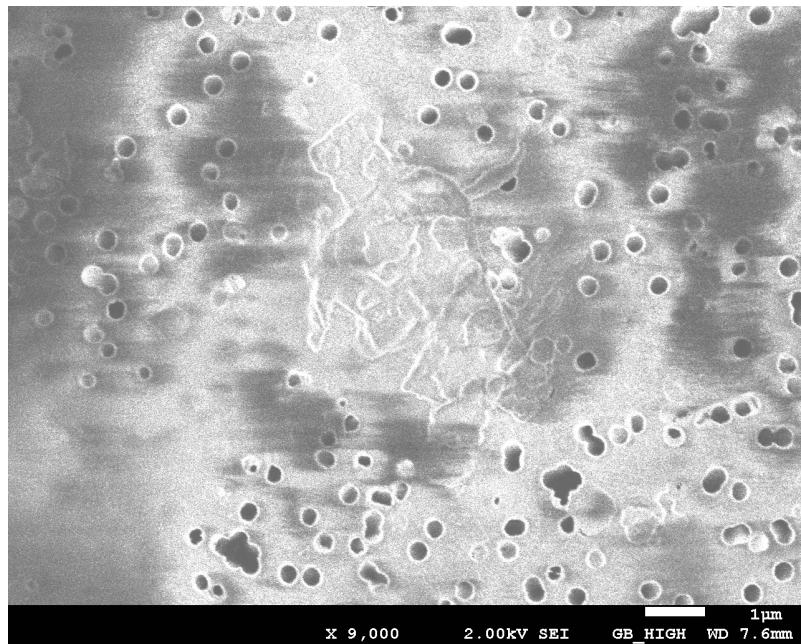


Figure 4: Scanning electron microscope view of M3 or M4 nanotubes deposited on a gold coated PET membrane.

A blank PET membrane used for the deposition and observation of nanotubes can be seen in Fig5. Such a polymer membrane needs to be sputter coated with a conductive element in order to be observed without charge effect as explained previously. The most commonly used metals for metallising a non-conductive surface are gold, platinum, palladium and silver. These elements are used for their high electronic conductivity. For Energy Dispersive Spectroscopy (EDS) analysis, carbon can be used. These elements are applied to the samples using a sputter coater [5][6][4].

Fig.5 shows several views of the PET coated membrane. A wider view at the top of the figure and closer views below. The higher resolution image was used to determine the pore size, which is around 400 nm. The other two images, with lower resolution, show that the distribution of pores on the membrane is not totally uniform. Many pores overlap and some areas contain few or no pores. The pores alone have a well-defined circular shape.

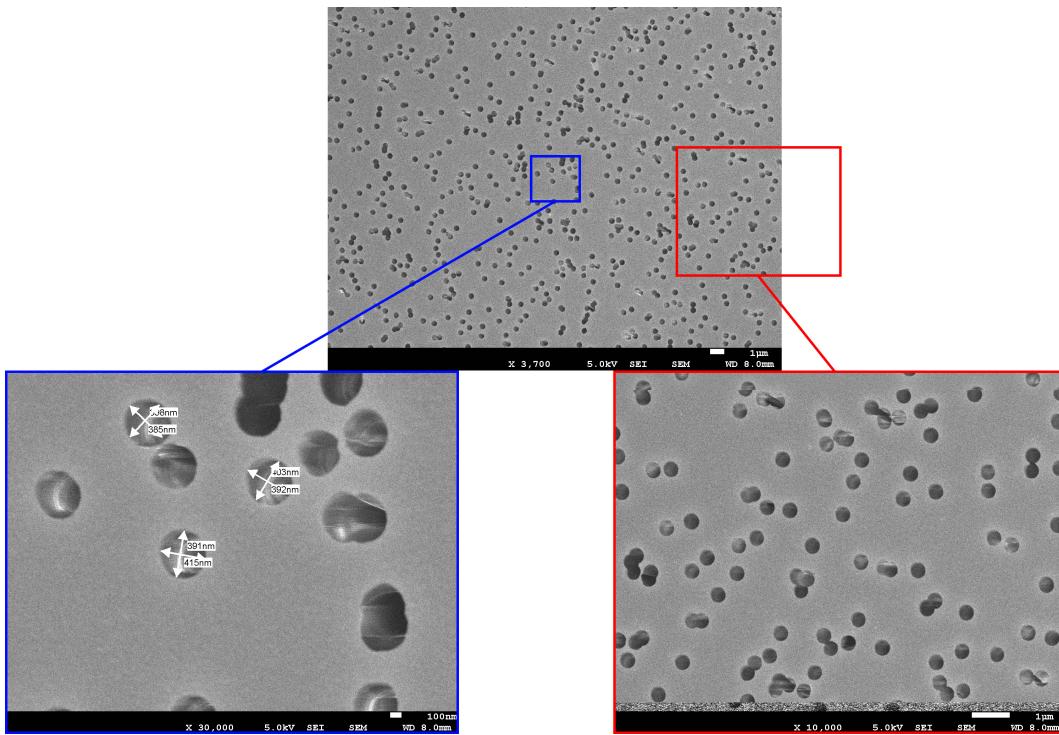


Figure 5: Scanning electron microscope of pristine porous PET membrane for nanotubes deposition.

The observations in Fig.6 come from a laboratory carried out during a previous year. Three views are available, a more distant view at the top and two views with better resolution at the bottom. In the top view, the pores of the PET membrane are visible as grey or black discs. Some of them are surrounded by a white halo, which could once again correspond to charge effects, but in this case they are localised. Grey aggregates and filaments can also be seen in the figure. These correspond to nanotubes that have collapsed onto the membrane following the deposition step. The aggregates would therefore be a stack of nanotubes, while each filament represents a nanotube spread over the membrane surface.

Zoomed-in views allow us to determine the diameter of the nanotubes present. The diameter measured was between 109 and 139 nm. These results would therefore come from the observation of nanotubes produced by layer-by-layer assembly in a membrane with a pore diameter of around 110-130 nm. However, it is impossible to determine the thickness of the membrane used, as none of the nanotubes are fully visible. The size of the nanotubes should normally correspond to the thickness of the membrane used.

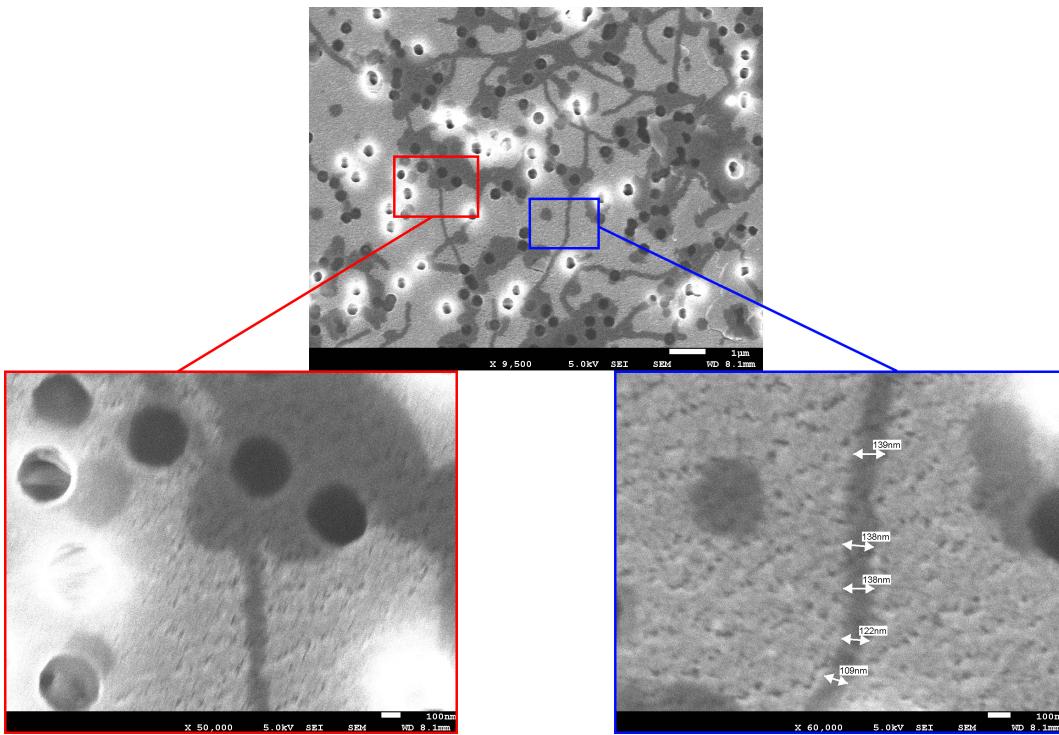


Figure 6: Scanning electron microscope of 8 layers nanotubes deposited on a gold coated PET membrane, images retrieved from T. Delire's work.

The latest observations shown in Fig.7 come from a sample whose origin is not known to the authors of the report. According to the information provided, the samples contain nanotubes composed of eight layers. This information is potentially confirmed by the fact that nanotubes can be observed. In the images, the pores of the support membrane are barely discernible, although the top left image shows more or less circular deposits that could be impurities deposited on the membrane. The nanotubes also appear less crowded and more dispersed, and their diameter can be determined using the image on the bottom right. The diameter is between $0.48 \mu\text{m}$ and $0.52 \mu\text{m}$, which leads to the conclusion that the membrane used to form the nanotubes has pores with a diameter of around 500 nm. Once again, the length of the tubes cannot be determined graphically using the SEM images.

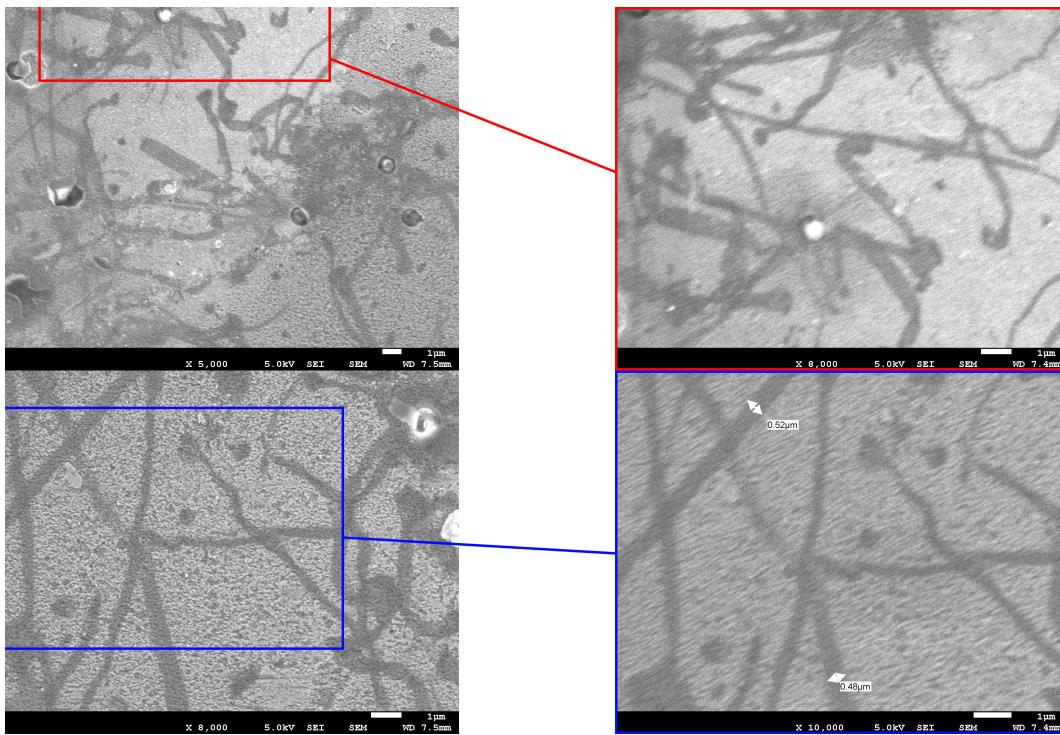


Figure 7: Scanning electron microscope of 8 layers nanotubes deposited on a gold coated PET membrane, sample origin is unknown.

To conclude this section on SEM observations, let's return to what we could or should have observed using microscopy of samples M3 and M4. The membrane we used to synthesise the nanotubes is 25 μm thick and has pores with a diameter of 400 nm. The nanotubes corresponding to this membrane should theoretically have a length identical to the thickness of the membrane. If it had been possible to measure the diameter of the tubes using SEM images, this diameter should have been of the order of 400 nm.

If dissolution had gone well, it is highly likely that nanotubes could have been observed in the nanotubes composed of eight layers (sample M4), as this type of observation has already been made. Sample M3, corresponding to four-layer tubes, would have been less likely to give significant results, as the tubes are technically less well formed with so few layers.

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