

# Biological-like vesicular structures self-assembled from DNA-block copolymers

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Electronic Supplementary Information (ESI)

## Experimental part

### Materials

The various nucleotide strands (desalted purity grade) used in the present study are listed in Table 1. They were purchased from Microsynth Laboratory (Balgash, Switzerland).

Shortcut	Strand	Modification
(G <sub>7</sub> A <sub>5</sub> )-resin	5'-GGGAGAGAGAGA-3'	5': Carboxy-C9 3': linked to the phosphoramidite resin
(A <sub>5</sub> G <sub>7</sub> )-resin	5'-AGAGAGAGAGGG-3'	5': Carboxy-C9 3': linked to the phosphoramidite resin
(T <sub>5</sub> C <sub>7</sub> )-resin	5'-CCCTCTCTCT-3'	5': Carboxy-C9 3': linked to the phosphoramidite resin
(C <sub>7</sub> T <sub>5</sub> )-resin	5'-TCTCTCTCTCC-3'	5': Carboxy-C9 3': linked to the phosphoramidite resin

Table 1: Modified oligonucleotides used in this study.

Dichloromethane (DCM) (IR spectroscopy grade) and N,N'-diisopropylcarbodiimide (DIC) ( $\geq 98.0\%$  pure), were purchased from Fluka (Buchs, Switzerland). Ammonium hydroxide (28 % NH<sub>3</sub> in H<sub>2</sub>O) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Doubly-distilled water was used for the preparation of all samples.

Amino terminated poly(isobutylene) (PIB) was kindly provided by W. Meier with a molecular weight of  $M_w = 1742 \text{ gmol}^{-1}$  and a polydispersity index (PDI) of 1.17. Amino terminated poly(butadiene) was produced by A. Müller and coworkers according to the one-pot synthesis route reported by S. Nosov et al.<sup>1</sup>. This synthesis route yields quantitative amounts of amino-terminated 1,4-poly(butadiene), with a narrow molecular weight distribution (molecular weight,  $M_w = 3554 \text{ gmol}^{-1}$ , PDI 1.10).

### DNA copolymer synthesis

The grafting of the polymer to the nucleotide sequence follows the route previously published by F. Teixeira et al.<sup>2</sup>. Briefly, the nucleotide sequences functionalized with a carboxylic group at their 5'-end are linked to a phosphoramidite resin through their 3'-end. The solid supported nucleotide sequences are mixed with a dichloromethane (DCM) organic solution of the amino modified hydrophobic polymers. The reaction is performed in the presence of N,N'-diisopropylcarbodiimide (DIC), an activating agent for the formation of the amide bond between both blocks. Subsequent removal of the non-reacted amino-modified polymers is easily performed by washing the resin with DCM. Finally a cleavage step performed in an alkaline medium releases the polymer-modified nucleotide sequences and non-reacted DNA fragments. Removal of the non reacted nucleotide sequences is then performed by size exclusion chromatography (Sephadex G50, GE Healthcare, Glattbrugg, Switzerland) or by dialysis (Slide-A-Lyzer 10K MWCO Dialysis Cassette, Piercenet, Lausanne, Switzerland). Through this chemical route we synthesized the four following diblock copolymers:

poly(isobutylene)-block-5'-GGGAGAGAGAGA-3' (PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub>), poly(butadiene)-block-5'-AGAGAGAGAGGG-3' (PB<sub>65</sub>-A<sub>5</sub>G<sub>7</sub>), poly(butadiene)-block-5'-CCCTCTCTCT-3' (PB<sub>65</sub>-T<sub>5</sub>C<sub>7</sub>) and poly(isobutylene)-block-5'-TCTCTCTCTCC-3' (PIB<sub>31</sub>-C<sub>7</sub>T<sub>5</sub>).

The four copolymers have the same 58% C-G ratio (i.e: guanosine and cytosine to total composition ratio) and are composed of hydrophobic polymer segments of low glass transition temperature (245 K for PB and 208 K for PIB).

### Self-assembly in aqueous solution

Self-assembly is induced by bulk swelling<sup>3</sup> of 1mg of the block-copolymer in 1mL of doubly distilled water. After shaking over 24 hours at room temperature, the solutions are clarified for further characterization through filtration with Millipore filters (HN 0.45  $\mu\text{m}$ ). The final concentration is then quantified by UV-Vis spectroscopy.

### Chemical analysis of the diblock copolymers

**Nuclear magnetic resonance (NMR).** Solid-state <sup>13</sup>C-NMR was performed on the lyophilized product obtained after dialysis. Cross polarization magic angle spinning (CP-MAS) experiments were performed on a Bruker Avance II 400 spectrometer with a Bruker double channel 4 mm probe. Zirconium rotors were employed at 100.6 MHz using a pulse angle of  $\pi/4$ , a recycling delay of 60 s, a spinning frequency of 4 kHz and high-power proton decoupling during the acquisition.

**Fourier transform infrared spectroscopy (FTIR):** Measurements were acquired using a single reflection diamond ATR on a FTIR-8400S spectrophotometer, Shimadzu. Spectra were recorded after 96 scans of 2  $\text{cm}^{-1}$  resolution.

**Elementary analysis.** N, H and C content were measured by combustion using a LECO CHN-900 apparatus with detection limits of 0.001 % for C, 0.01 % for N and H.

**UV-Vis spectroscopy.** Measurements were performed on a Perkin-Elmer Lambda 35 UV-Vis spectrometer. Spectra were recorded between 200 and 400 nm with a slit width of 0.5 nm and a scan rate of 120  $\text{nm sec}^{-1}$ . At low concentration, scattering effects were negligible on absorption. The absorbance follows the Beer-Lambert law:

$$A = \epsilon \cdot l \cdot c \text{ (Equation 1)}$$

where A is the measured absorbance,  $\epsilon$  the extinction coefficient at a given wavelength, l the path length through the solution and c the concentration of the polymer.

Absorbance was then directly correlated to the concentration of polymer-modified nucleotide sequences through a linear regression. Measurements were compared with spectra from the "spectrum prediction tools" developed by A. V. Tataurov et al.<sup>4</sup>

### Self assembly and chain configuration

**Circular dichroism.** Circular dichroism (CD) spectra (200–350 nm) were recorded to determine the impact of the polymer coupling on dichroic properties of the polymer-modified nucleotide sequences. Measurements were performed with an Applied Photophysics Chirascan CD spectrometer. Spectra were recorded in doubly distilled water or NaCl solutions at various concentrations.

**Transmission electron microscopy.** The samples were examined with a transmission electron microscope (Philips Morgagni 268D) at 293 K. Dispersions of polymer-modified nucleotide sequences were negatively stained with a 2 % uranyl acetate solution and deposited on a carbon-coated copper grid. Analysis without staining was performed as well.

**Scanning electron microscopy:** Solutions of polymer-modified nucleotide sequences were deposited on a clean silicon wafer by drop drying. Then the wafer was rinsed three times with doubly-distilled water. This surface was coated with 3 nm of sputtered platinum before being examined by SEM (Hitachi S-4800 FEG, Japan) operated at 5 kV accelerating voltage (1.4 nm resolution at 1 kV accelerating voltage).

**Dynamic light scattering:** The dynamic light scattering experiments were performed using a commercial goniometer (ALV-Langen, Germany) equipped with a He-Ne laser ( $\lambda = 633 \text{ nm}$ ) at scattering angles between 30° and 150°. An ALV-5000/E correlator calculates the photon intensity autocorrelation function  $g^2(t)$ <sup>5</sup>. The samples were prepared by clarifying the solutions through Millipore filters (HN 0.45 and 0.2  $\mu\text{m}$ ) into 10 mm quartz cells. These cells were mounted in a thermostated optical matching vat with a temperature accuracy of  $T = 0.02 \text{ K}$ . The experiments were performed at  $T = 293 \text{ K}$ .

Over the course of a DLS experiment, a time correlation function decaying with time is measured, allowing the determination of the cooperative translatory diffusion coefficient  $D_m$  at a concentration c:

$$D_m = D_0 (1 + k_d c) \text{ (Equation 2)}$$

where  $D_m$  is the z-averaged cooperative translational diffusion coefficient and  $k_d$  the diffusion virial coefficient. The extrapolation to zero concentration yields a diffusion coefficient  $D_0$ , which allows the calculation of the hydrodynamic radius  $R_h$  via the Stokes-Einstein equation.<sup>6</sup>

### Nanoreactor preparation and enzyme activity measurement

## Materials

For the design of biological-like vesicles we used the amphiphile PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub> DNA block copolymer. We induced porosity in these vesicles by the insertion of bacterial OmpF into the polymeric vesicular shell. The OmpF were produced by the BL21(DE3)omp8 Escherichia coli (E.coli) strain<sup>7</sup> and purified as described by A. Graff<sup>8</sup>. Briefly, the BL21 (DE3)omp8 E.coli strain containing the pGompF plasmid were grown in Luria-Bertani Lysogenic Broth at 37 °C under 250 rpm shaking. After 4 hours, OmpF overexpression was induced by adding IPTG 1 mM (isopropyl β-D-1-thiogalactopyranoside). After 6 hours of growth, cell pellets were collected, cells were lysed and OmpF proteins were collected with 3% octyl-POE (ALEXIS company), yielding a solution of OmpF in 3% octyl-POE, 20 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.3. The concentration was adjusted with 20 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.3 buffers to 1 mg mL<sup>-1</sup> for the stock solution.

Lactoperoxidase (LPO) from bovine milk (lyophilized powder, salt-free, ≥80 units mg<sup>-1</sup> protein, Sigma-Aldrich) was freshly dissolved in sterile NaCl buffer (150 mM) to prepare a stock solution of 10 μM. Tyrosine (reagent grade, ≥ 98%, Sigma-Aldrich) was freshly dissolved to obtain a stock solution of 0.14 mgmL<sup>-1</sup> in sterile NaCl buffer (150 mM). Hydrogen peroxide was diluted to a 16 μM stock solution (stabilized, 30 wt. % in H<sub>2</sub>O, Sigma-Aldrich) in sterile NaCl buffer (150 mM). Proteinase K (BioUltra, for molecular biology, ≥ 30 unitsmg<sup>-1</sup> protein, lyophilized powder, Sigma-Aldrich) was freshly dissolved in sterile NaCl buffer (150 mM) for a stock solution of 20 mg mL<sup>-1</sup>.

## Preparation of biological-like vesicles

Protein insertion, enzyme encapsulation and vesicle formation are achieved simultaneously. To induce self-assembly of PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub>, the most efficient technique, which was used in this study, is bulk swelling. Briefly, a 0.3 mg mL<sup>-1</sup> block copolymer solution was obtained by adding 0.3 mg polymer powder to 1 mL of a 150 mM NaCl solution containing the 10 μM LPO stock solution and 20 μL of the OmpF stock solution while shaking for 6 hours at room temperature. The solution was then filtered three times through a Millex-HV filter, 0.45 μm pore size (PVDF, 33 mm, sterilized, Milipore). This filtration reduces the size and the size distribution by breaking apart existing aggregates.

## Purification

The biological-like vesicles are separated from non-encapsulated enzymes and non-inserted OmpF by centrifugal filtration. This purification was performed by filtration through centrifugal filtration units (Amicon Ultra-0.5, Ultracel-100 Membrane, 100 kDa, Milipore) with a M<sub>w</sub> cut-off of 100 kDa and a spin speed of 4000 rpm. The whole separation procedure was repeated five times, according to Vriezema et al.<sup>9</sup>. At each separation steps, the polymer concentration and enzyme activity were determined for both the purified solution and the retentat.

## Other samples and sample names.

In order to evaluate and discuss the quality of purification and the efficient activity of the biological-like vesicles, two control samples were prepared. First, the same procedure (shaking and purification) was used to prepare only a pure solution of the free enzyme at 10 μM. The second sample consisted of a polymer solution (final concentration of 0.3 mg mL<sup>-1</sup>) that was shaken along with 1 mL of the 10 μM stock solution of LPO, 20 μL of a 3% octyl-POE and 20 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.3, leading to the formation of non-porous self-assembled structures, which are not expected to be enzymatically active, since both the substrate and the product cannot diffuse across the vesicular membrane.

A set of experiments was subsequently performed to determine the protective effect of the polymeric shell on the encapsulated enzyme. These assays, including incubation with proteinase K, were performed by mixing 200 μL of solution obtained after purification with 50 μL of a 20 mg mL<sup>-1</sup> proteinase K for 4 h at 37 °C.

## Polymer and enzyme concentration determination

Polymer and enzyme concentrations were determined by UV-Vis spectroscopy. Measurements were performed on a Perkin-Elmer Lambda 35 UV-Vis spectrometer. Spectra were recorded between 200 and 400 nm with a slit width of 0.5 nm and a scan rate of 120 nm sec<sup>-1</sup> in 10 mm quartz cuvettes. The oligonucleotide concentration linearly follows absorption at 253 nm, which allows an accurate determination of the polymer concentration for dilute solutions. In concentrated solutions, however, scattering effects prevent any linear correlation between the concentration and the absorption. Besides LPO absorbs at 280 nm. The presence of the enzyme thus yields a small contribution to the absorption, easily observable in the free enzyme solution. Nevertheless, the signal associated to LPO is weak compared to that of the nucleotide-based copolymer, which allows the determination of the concentration of the self-assembled structures without significant error. In order to quantify the enzyme concentration, absorption spectra at 280 nm were recorded for solutions of the free enzyme. However, as evoked above for polymer containing solutions, enzyme absorption was hidden by the high intensity of polymer absorption signals. However, the encapsulated enzyme can be detected by enzyme activity monitoring by fluorescence spectroscopy, and concentration determined from the Michaelis-Menten kinetics.

## Enzyme activity monitoring and concentration determination from the Michaelis-Menten kinetics measurement by fluorescence spectroscopy

Two assays were performed: In the first experiment the activity of the LPO was followed by the conversion of tyrosine in dityrosine when H<sub>2</sub>O<sub>2</sub> is present as a cosubstrate. Enzyme activity was monitored by mixing 50 μL of biological-like vesicles or non-porous structures or free enzyme with 150 μL of H<sub>2</sub>O<sub>2</sub> stock solution, 50 μL of the tyrosine stock solution and 200 μL of NaCl buffer (150 mM pH 6.5). The time course of emitted fluorescence signal at 415 nm was recorded with a Jasco F-P-773 spectrofluorimeter (excitation at 316 nm<sup>10</sup>). The assay was conducted for different enzyme concentrations to qualitatively estimate the equivalent concentration based on the activity of the free enzyme and nanoreactors. The second enzymatic assay aimed at determining the Michaelis-Menten kinetics parameters<sup>11</sup>. In this assay, the

reaction speed is determined as a function of the tyrosine concentration varying from 0.014 to 0.049 mg mL<sup>-1</sup>. This kinetics enables the determination of the enzyme parameter: the affinity constant for the substrate and the enzyme  $K_m$ , and the turnover constant of the enzyme (i.e. the processing speed of the enzyme)  $K_{cat}$ . Those values are characteristic of the enzyme-substrate system. The reaction involving one substrate, one enzyme and one product, is a first order reaction. From this kinetics we can extract the Michaelis-Menten equation<sup>11</sup>. Knowing the reaction speed ( $V$ ), using different initial substrate concentration ( $[S]_{ini}$ ), we can determine  $K_{cat}$ ,  $K_M$  and  $[E]$  (enzyme concentration) by fitting the obtained hyperbolae, or graphically with the Lineweaver-Burk representation<sup>12</sup>. The reaction was performed with a fixed value of  $H_2O_2$  (1.6  $\mu M$ ) and the reaction between tyrosine and LPO was considered as a pseudo first order reaction due to the fixed value of  $H_2O_2$ , allowing the determination of  $K_m$ ,  $K_{cat}$  and concentration. Kinetics parameters were recorded for the free lactoperoxidase at 0.5  $\mu M$  in NaCl (150 mM, pH 6,5) and for the self-assembled structures.

#### Calculation of enzyme encapsulation efficiency:

After determining the constant  $K_m$  on the basis of calibration curve based on free lactoperoxidase, the Lineweaver-Burk<sup>13</sup> calculation (Equation 3) allows to determine the enzyme concentration corresponding to the measured activity (measurement providing  $v_i$  and  $v_{max}$ ).

$$\frac{1}{v_i} = \left( \frac{K_M}{v_{max}} \times \frac{1}{[S]} \right) + \frac{1}{v_{max}} \text{ (equation 3)}$$

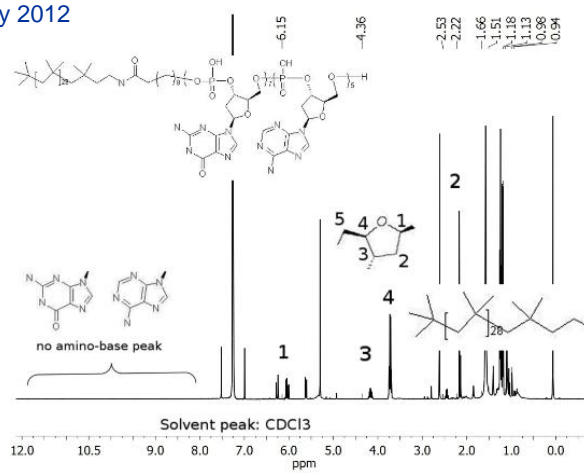
In a subsequent step, the polymer concentration can be calculated on the basis of the Beer-Lambert law from UV-vis measurements, as described for polymer quantification. Then the encapsulation efficiency of 1mg mL<sup>-1</sup> of polymer is calculated, on the basis of a linear correlation with the experimental values, by applying the following equation:

$$E_{eff} = \frac{C_m^{LPO} \times V_{sample}}{C_{init}^{LPO} \times V_{init} \times C_m^{Polymer}} \times 100 \text{ (equation 4)}$$

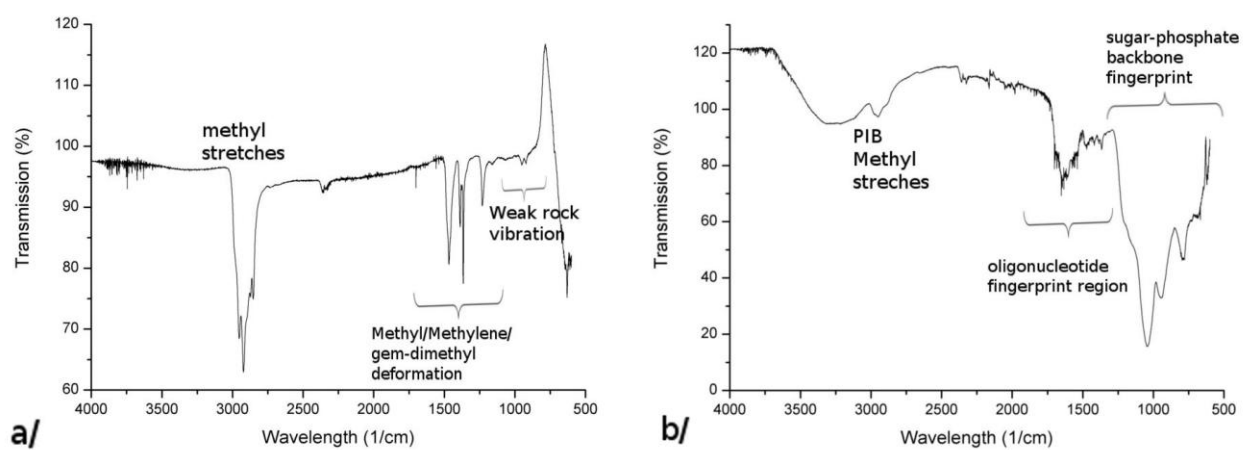
In this equation:  $E_{eff}$  is the encapsulation efficiency (% per milligram of polymer),  $C_m^{LPO}$  is the measured concentration of lactoperoxidase in the sample of volume  $V_{sample}$ .  $C_{init}^{LPO}$  is the concentration of the lactoperoxidase stock solution.  $V_{init}$  is the initial volume of the solution in which the concentration  $C_m^{Polymer}$  was measured.

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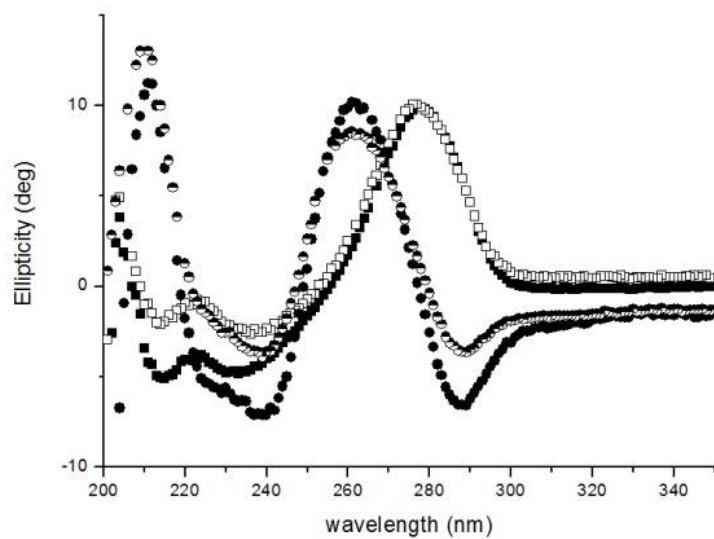
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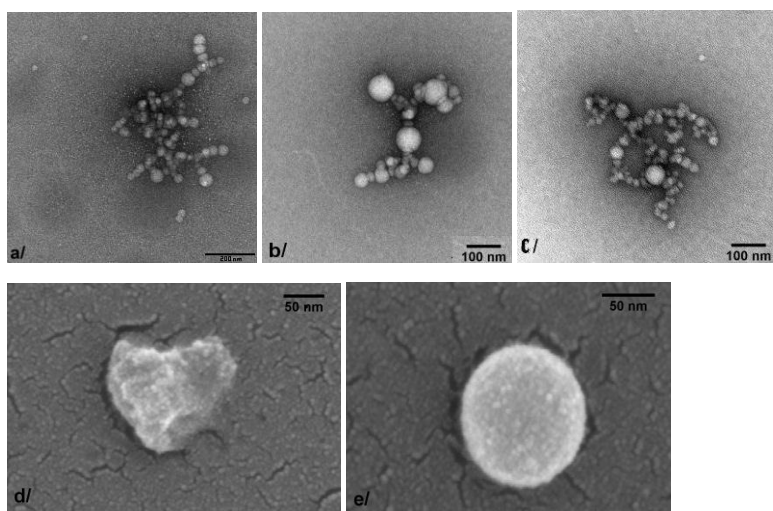
**Figure 1:** <sup>1</sup>H-NMR spectrum of PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub> obtained in deuterated chloroform. Peaks were assigned to the poly(isobutylene) block and the oligonucleotide sugar backbone. No peak could be assigned to the nucleotides, probably due to the self-assembly of structures in which the nucleotide sequences are embedded in a poly(isobutylene) environment



**Figure 2:** FTIR spectra of (a) amino-modified PIB<sub>31</sub> and (b) PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub>. Both spectra were recorded on air background. Most significant regions are highlighted to provide the fingerprint of the polymer-modified oligonucleotide

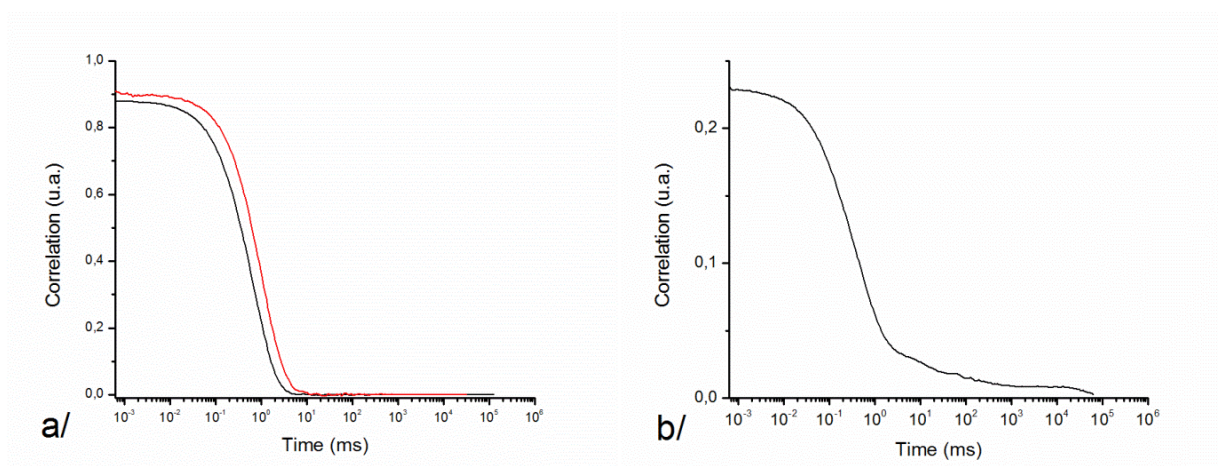


**Figure 3:** CD spectra of PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub> (black circle), PB<sub>65</sub>-A<sub>5</sub>G<sub>7</sub> (half black circle), PIB<sub>31</sub>-C<sub>7</sub>T<sub>5</sub> (black square) and free C<sub>7</sub>T<sub>5</sub> (white square)

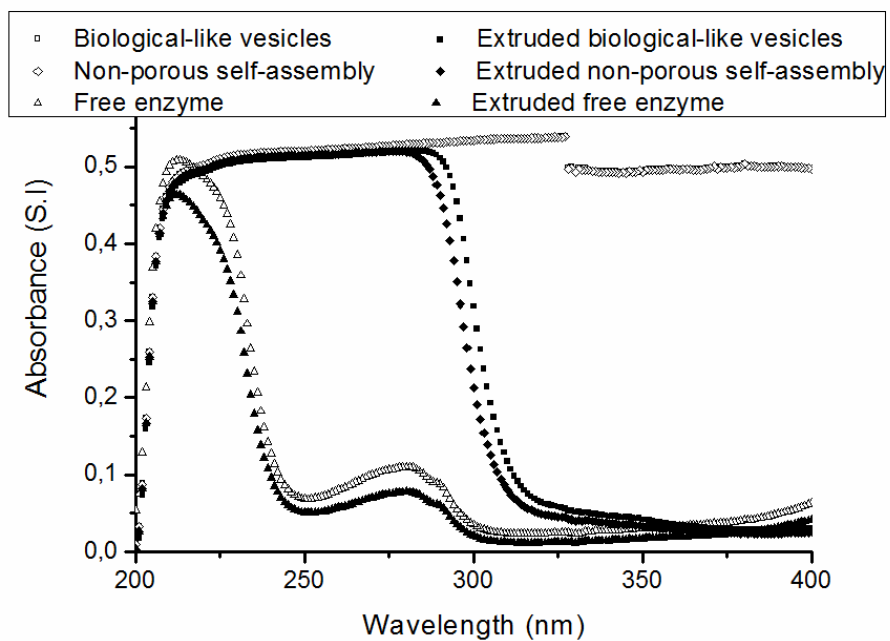


**Figure 4:** TEM of a) and b)  $\text{PB}_{65}\text{-T}_5\text{C}_7$  and c)  $\text{PIB}_{31}\text{-C}_7\text{T}_5$  d) SEM of a single  $\text{PIB}_{31}\text{-A}_5\text{G}_7$  structure. e) Collapsed structure in the high vacuum of SEM (the cracks are those of the very thin coating performed prior to imaging)

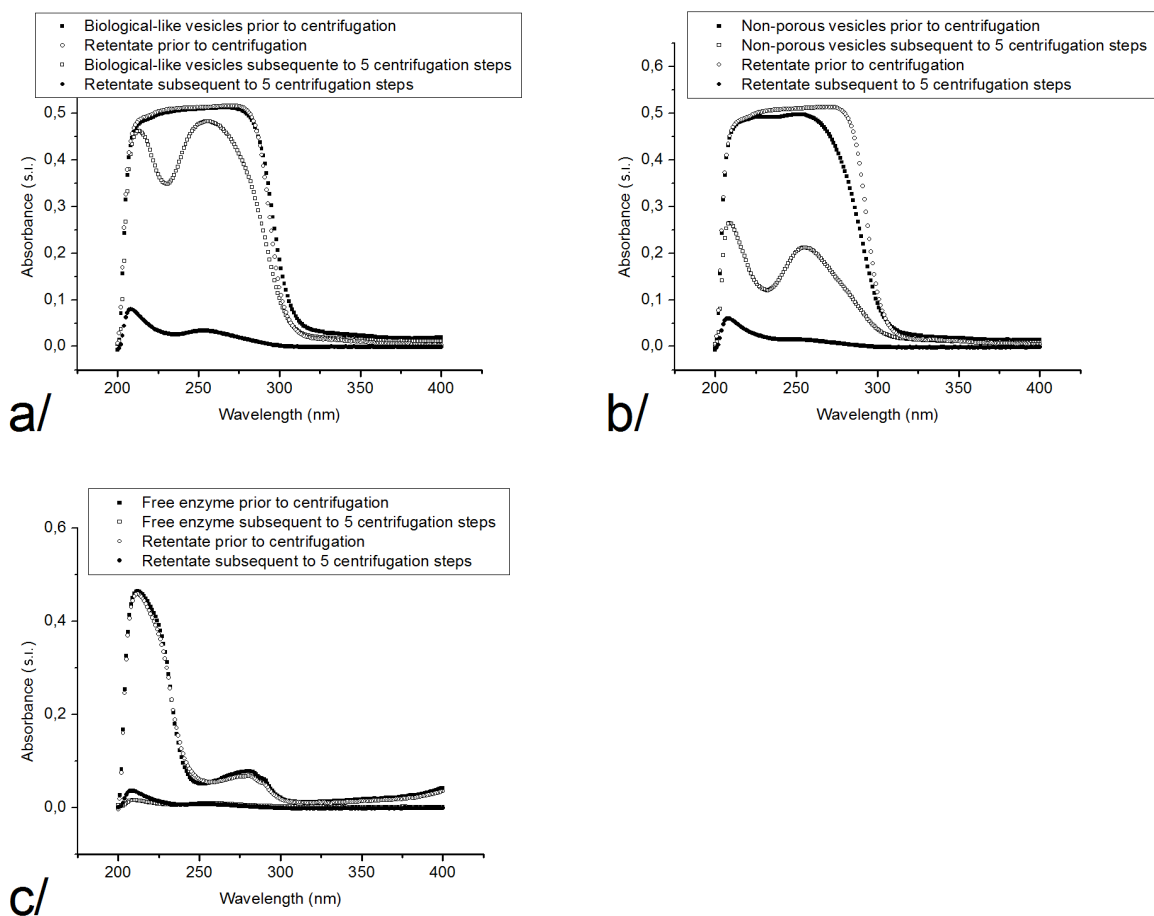




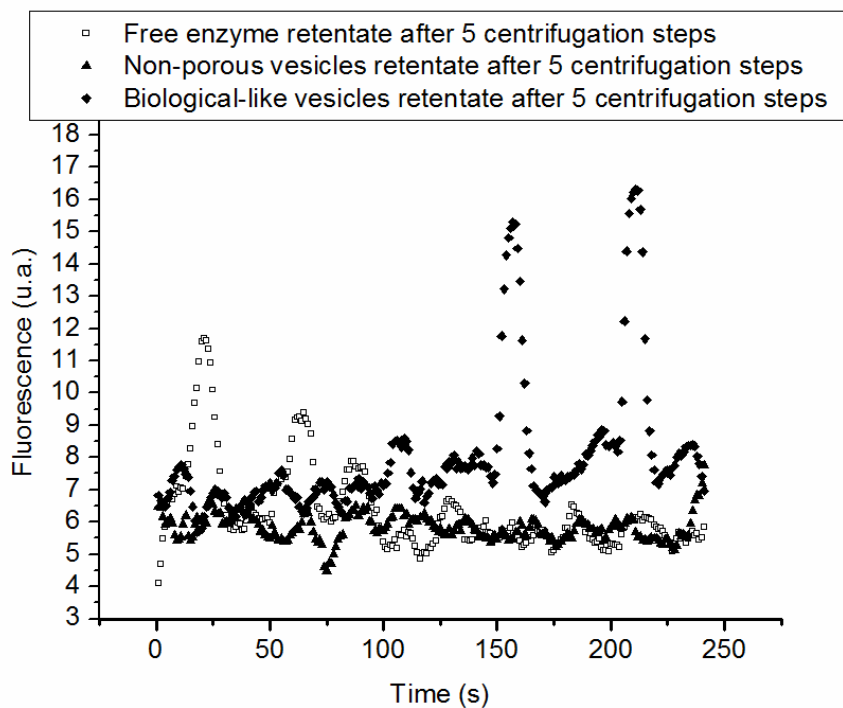
**Figure 5:** Dynamic light scattering: a) Correlation function corresponding to the diffusion of self-assembled structures from PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub> (black line) and PB<sub>65</sub>-A<sub>5</sub>G<sub>7</sub> (red line). The correlation function is measured at 90° after sequential filtration through 0.45 and 0.2 μm pore size membrane filters. b) The correlation function measured at 150° for PIB<sub>31</sub>-G<sub>7</sub>A<sub>5</sub> after filtration at 0.45 μm, highlights the polydispersity of the system and the necessity to extrude down to 0.2 μm.



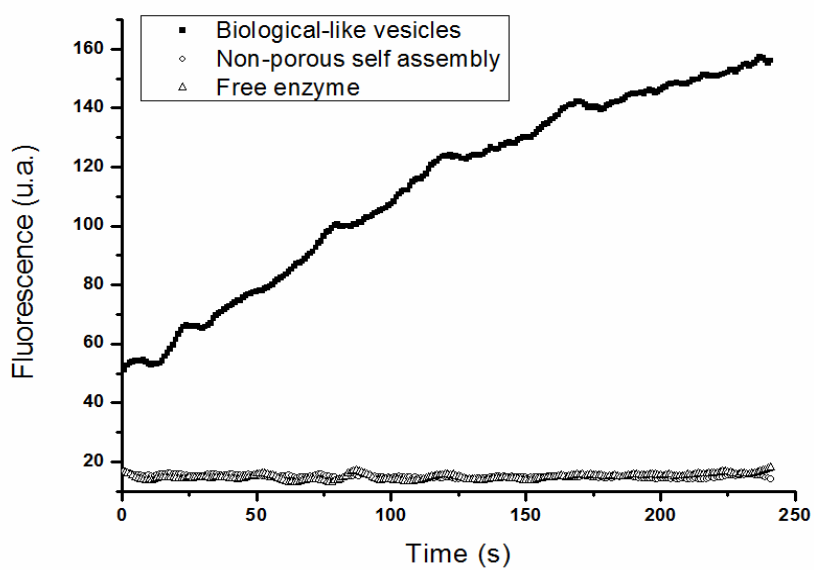
**Figure 6:** UV-Vis spectra before and subsequent to Millex-HV extrusion, for the three solutions of biological-like vesicles, not permeable self-assemblies, and the free enzyme. These spectra highlight the ability of extrusion to eliminate large polymer aggregates without affecting the enzyme concentration. There is an overlap of the signals from the non-porous and biological-like vesicles prior to extrusion.



**Figure 7:** UV-Vis spectra recorded over the course of centrifugal purification for a) biological-like vesicles, b) non-porous self-assembly and c) free enzyme. The decrease of signal after 5 centrifugation steps, combined with the disappearance of signal in the retentate confirm the elimination of free enzyme, free OmpF and structures of smaller size from the solution of biological-like and non-porous vesicles. Similarly the disappearance of signal from the free enzyme solution after 5 purifications confirms the ability of centrifugal filtration purification to eliminate non-encapsulated enzyme.



**Figure 8:** Fluorescence spectroscopy (emission at 415 nm) displays the enzymatic activity recorded for the centrifuged fraction subsequent to 5 centrifugation steps of the free enzyme, the biological-like vesicles and non-porous self-assemblies. The absence of significant enzymatic activity confirms the efficiency of centrifugal filtration purification (note that we are at the detection limit of the technique to demonstrate the absence of activity).



**Figure 9:** Enzymatic activity of LPO on tyrosine measured after incubation of biological-like vesicles, free enzyme and non-porous self-assemblies in the presence of proteinase K. The strong activity of biological-like vesicles highlights the protective role of the polymer wall against the influence of proteinase K.