Preparation and characterization of Liposomes (Small Unilamellar Vesicles, SUV, made of pure phospholipid molecules and real biological membranes)

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Summary

The main goal of this work was to obtain small unilamellar vesicles and a structural characterization of thin membranes involved in the mechanisms of olfaction in mammals. Uni-lamellar vesicles of small size (nanosomes) were prepared via sonication and filtration of membranes containing olfactory receptors. First we determined the real amount of membranes in the product provided by the biologist from INRA laboratory. After that we performed a comparative study of the membrane fraction from different preparation routes and sample lots, from sample FMP124 and FMP32, respectively, to characterize and compare different amounts and sizes of vesicles. The main methods of research was Static (SLS) and Dynamic Light Scattering (DLS). These methods allowed us to obtain the vesicles sizes and their amount.

Introduction

We mainly used Static and Dynamical Light Scattering techniques. These techniques allow determining the sizes, distributions and the organization of vesicles according to the methods and steps of preparation. Static light scattering is a technique that measures the intensity of the scattered light to obtain for example: Molecular weight, Radius of Giration (root mean square radius), Form and Structure Factor, Virial Coefficient (by measuring the scattering intensity for many samples of various concentrations). Dynamic Light Scattering is also known as quasi-elastic laser light scattering. If the light source is monochromatic and coherent we can observe a time-dependent fluctuation in the scattering intensity. These fluctuations result from Brownian motion of small molecules and the distance between them in the solution is changing with time. In dynamic diffusion we study the intensity at a fixed angle and take into account local fluctuations in concentration over time, not average intensity

over time like in static diffusion. Diffusion of molecules in volume cause spatial fluctuations of the local concentration, associated with spatial fluctuations of the local amplitude of the electric field of scattered light. The interference of waves is the source of fluctuations on the detector and give a value of distribution intensity. If the particles are large and move slowly to their initial location, the signal change slowly over time and the correlation persists long positions (Fig. 1). In the opposite case where the particles are small and move quickly or over long distances, the autocorrelation is lost more rapidly [1-6].

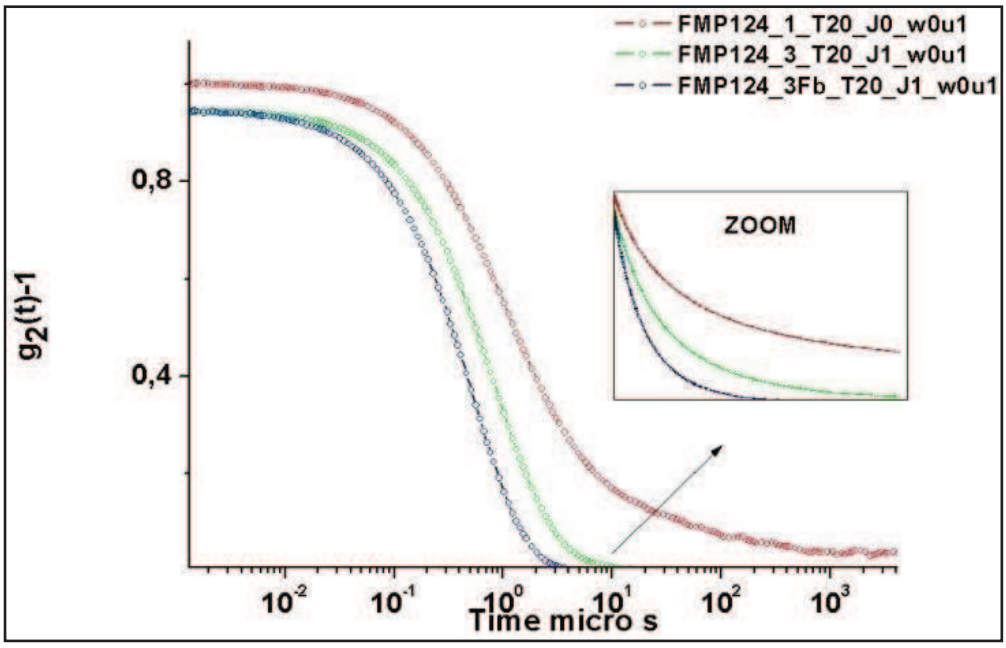

Fig. 1. Correlation curves of the samples FMP132 at different steps.

The received information from the Dynamic and Static Light Scattering techniques can be used in medicine, cosmetology and many other areas.

The investigated material

It is work with real membrane fractions (FM) from yeast, in which there are olfactory receptors of mammals (RO, humans or rats). Membrane fraction was prepared by biologists from INRA (Paris collaboration). FM also includes the all of the proteins used in the yeasts. Was studied also pure phospholipid liposomes from lipids purchased from Avanti [5] in order to compare with fraction membrane. The main components of the lipid are DPPC mixture (1,2-dipalmitoyl-sn-glycerine-3-phosphocholine). DPPC mixture has the formula $C_{40}H_{80}NO_8P$, molar mass: 734.039 g/mol. In experiment was used several mixtures of phospholipids. These diluted samples were stored in chloroform at a concentration of 25 mg/ml. First we collected the desired amount of molecules in the solution (the quantity we need to hydrate) and evaporated the chloroform. In the next step we added pure water to obtain the desired dilution.

Laboratory apparatus

The experiment was carried out using Thermogravimetric analysis (TGA). It is a technique that allows analysis of the mass variation of a sample as a function of temperature. It is a differential analysis between β alumina in a platinum crucible and the sample considered. The device used in the Laboratory LdOF from the University of Maine contains an oven, a sealed chamber to monitor the atmosphere of the sample with a regulated flux of air, a high precision module weighed (microbalance), a thermocouple for temperature measurement and a computer to monitor and record all data.

The main research techniques used in this work are Static and Dynamic Light Scattering. Equipment (Fig. 2) consists of a laser beam directed onto the sample liquid in a glass tube of 8 mm internal diameter, positioned in a thermostated solution. The laser beam does not undergo refraction because thermostated solution has the same index as glass (toluene or decalin, indices n = 1.4954, 632.8 nm). As a sample *standart* were use solution of toluene. It is necessary for calibrating the scattered intensity. Detector is mounted on a motorized goniometer (where was choose the angle) and measure the scattered intensity. The photomultiplier is connected to optical correlator and the detector is set to retrieve only the broadcast signal of the position of the sample

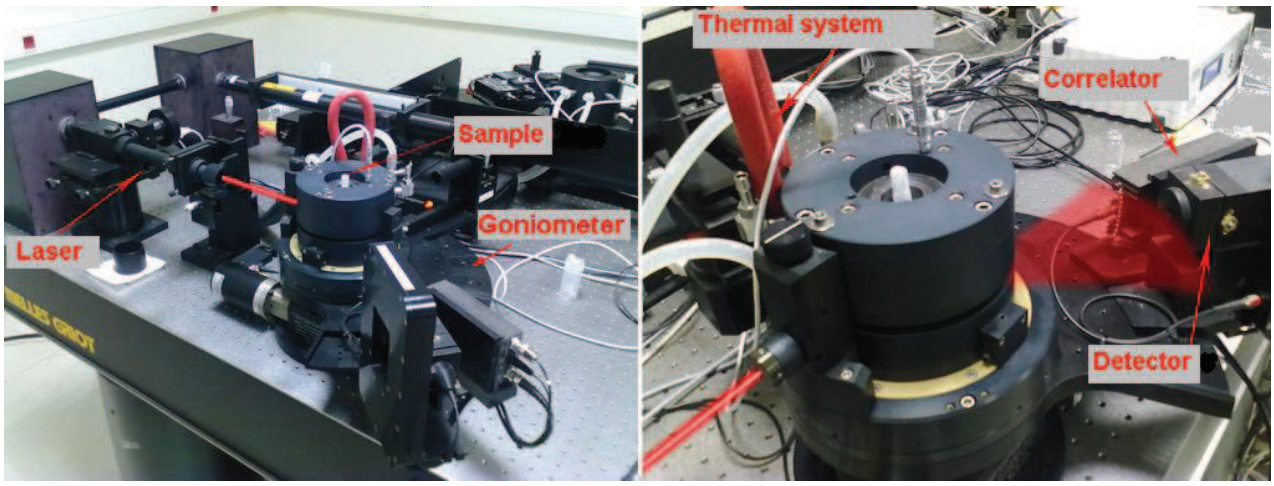

Fig. 2. Pictures of light scattering instrument in PCI Lab at the University of Maine.

It was used supporting technique such as UV-Vis spectrometry. UV-Vis spectroscopy refers to absorption spectroscopy in the ultraviolet-visible spectral region. We can measure the concentrations of absorbing species and it is useful to estimate the proteins concentration that is constant in the membrane fraction used, so that it is a way to measure the total membrane concentration of a solution. It was also tried to use the Thurax technique to homogenise the size distribution of vesicles.

Analysis procedure

The solutions were prepared of different concentrations of fraction membrane (FM). The solutions have undergone different treatments needed to make liposomes of yeast membranes of small sizes and to study their stability and evolution over time. The analysis procedure was carried out in the following stages:

- Samples sonicated at low temperature (4°C)
- Samples re-sonicated at higher temperature (60°C)
- Sample after filtration. They were filtered with different filter types:
  - Whatman Anatop 25 PLUS 200 nm
  - Micro Sard 200 nm
  - Whatman GMF w/GMF 0.45 μm

In order to homogenize our membrane dispersion and to reduce turbidity, we used the sonication at 4°C to keep membrane proteins intact and at 60°C that might damage the proteins but that gives a high fluidity to all phospholipid chains since this temperature is well above the melting temperature of most of phospholipids molecules. After this steps large aggregates remained in the solutions and might correspond to aggregated small vesicles or larger vesicles. So liposome solutions needed to be filtered to take away aggregates of liposomes which are a problem for light scattering techniques.

For the first experiment we used two different filters that cut above 200 nm (Fa is from Whatman Anatop 25 PLUS 200 nm and filter Fb is from Micro Sard 200 nm) on a 0.1% w/w solution of FMP124 (first sample) after sonication at 4°C for Fa; and after sonication at 60°C for Fb. In order to know how much we loose with filtration and to measure the membrane concentration after filtration, we used UV-Visible absorption of protein membranes. After filtration with Fa, we lost 92.24% of the membrane concentration, and 78.6 % with filter Fb from the same starting solution respectively. This huge lost of material from 200 nm pores filtration shows that a large fraction of vesicles and aggregates have a diameter larger than 200nm. This was confirmed from the fact that the static light scattering curve (SLS, *I/KC = function(q)*) of the starting solution does not reach a plateau at small angles due to the presence of very large objects (red curve 3a), but clearly show a plateau at small angles (small q values) after filtration, once the large aggregates have been removed (Figure 3a). The size distribution measured from DLS shows the presence of large aggregates at sonication at 4°C (red curve 3b).

The DLS curves measured at 30°, 90° and 150° over 300 seconds for the starting sample before filtration were fitted (Fig. 4). For this it was used a REPES algorithm with a regularization parameter (α_{Reg}). This parameter controls the width of authorized peaks in the distribution. The distribution seen at small angles (30°) is much more sensitive to the presence of large aggregates than the ones measured at 90° or 150°.

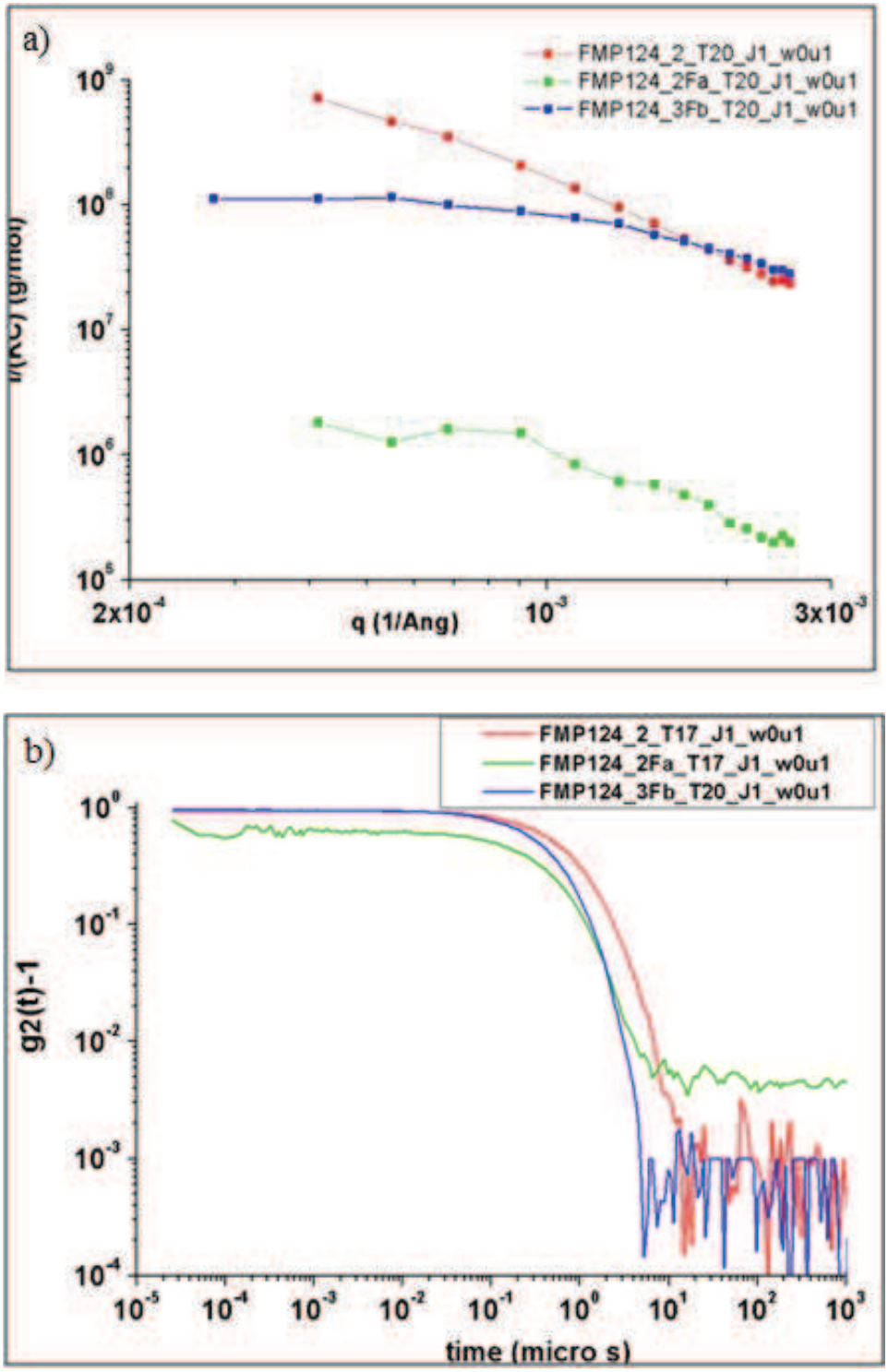

Fig. 3. Static light scattering (SLS) curves of the three solutions compared in experiment 1 (red is the starting solution, green after filtration with filter Fa, and blue with filter Fb (a), correlation curves measured from DLS at 90° of the same samples (b).

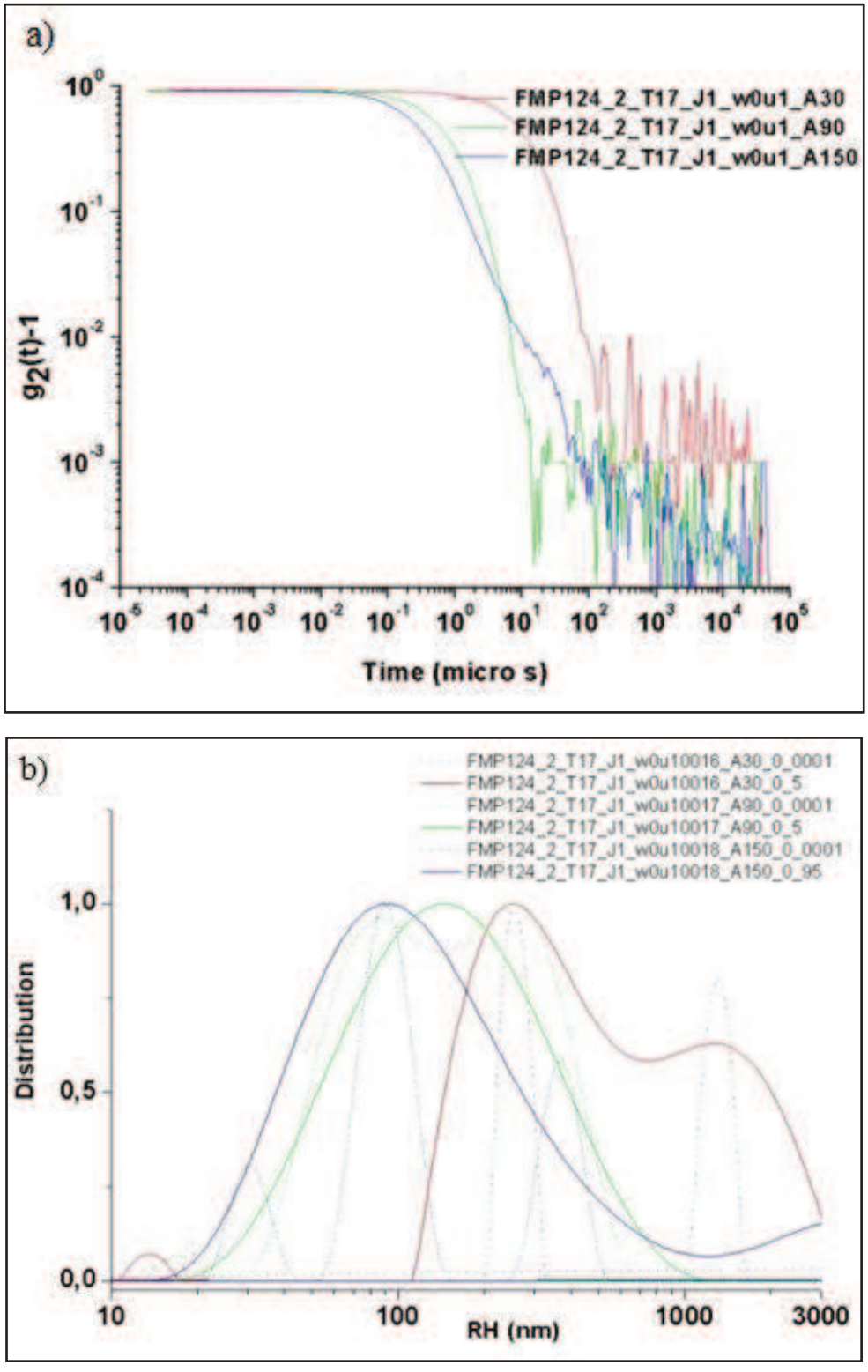

Fig. 4. Correlation curves (DLS) measured at 30° (red), 90° (green) and 150° (blue) of the starting solution of experiment one (a), distribution curves obtained from the analysis of the DLS curves with same colors (REPES algorithm with regularization parameter of 0.0001 for dotted curve and 0.5 or 0.95 for solid lines) (b).

Because of large scattering objects, we cannot see the small ones that scatter less, since scattering for vesicles is proportional to the mass of vesicles. The molar mass of vesicles M_{Ves}, increases with the square of the radius. To know below which angle

the size distribution curves become representative of the sample, one should be measuring the DLS curve on the SLS plateau that appears for monodisperse solutions (in our case – filtered solution). After filtration with 200 nm pores, the distribution curves measured at different angles are more similar and all show a population around 100-120 nm (Fig. 5).

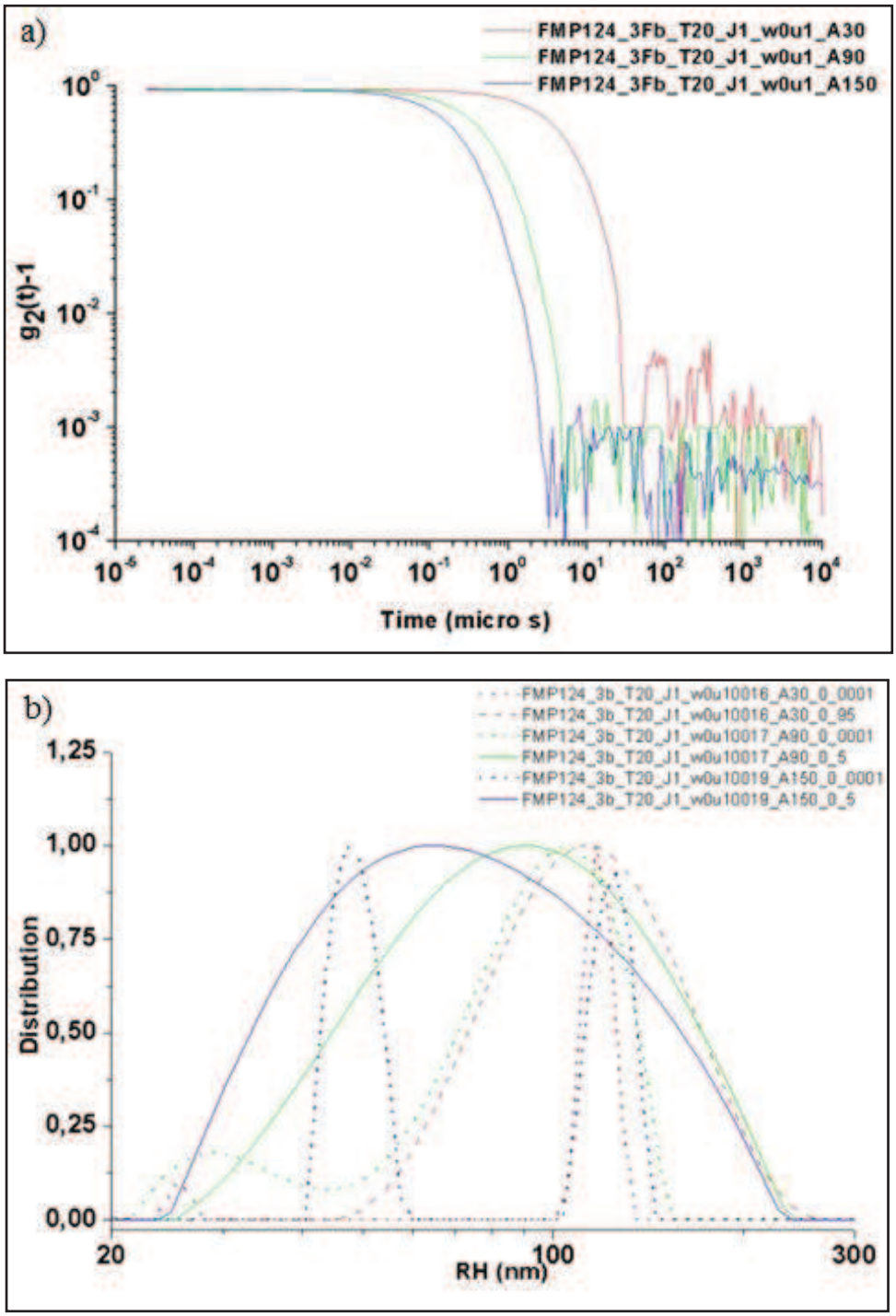

Fig. 5. Correlation curves (DLS) measured at 30° (red), 90° (green) and 150° (blue) of the solution after filtration with filter Fb (a), distribution curves obtained from the analysis of the DLS curves with same colors (REPES algorithm with regularization parameter of 0.0001 for dotted curve and 0.5 or 0.95 for solid lines) (b).

The blue curve measured at larger angle, that is less sensitive to large objects of the real population, gave a distribution of small vesicles, the so-called nanosomes (47 nm). The fact that we can see their presence, is a proof that the number of large aggregates or vesicles is very low in the solution after filtration, so nanosomes do not aggregate after filtration and are stable.

Also was estimated the size of the nanosomes from the static light scattering using the Guinier analysis. The construction obtained from the blue curve of Figure 3a. From the slope fitted at smaller angles, we measured a mean gyration radius of 63.4 nm.

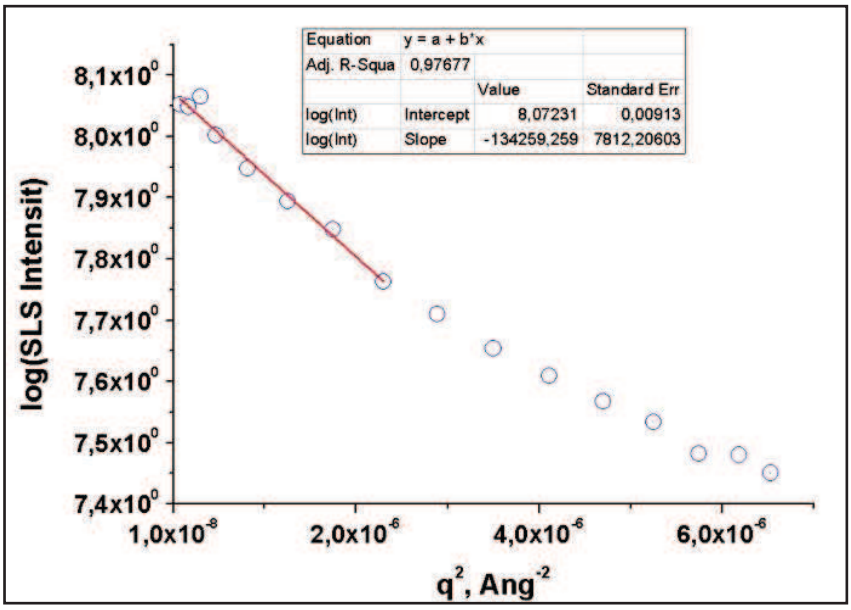

Fig. 6. Guinier construction on the Static light scattering (SLS) curve of sample FMP124_3Fb_T20_J1_w0u1 (blue curve of Figure 3a).

For the second set of experiments, we wanted to increase the quantity of small vesicles prepared, so we filtered after sonication at 60°C the FMP32 (second sample) lot that was prepared at smaller membrane concentration (w = 0.026 % w/w so at 0.266 mg/ml). The first used a filter with large pores (Fc, Whatman GMF w/GMF 0.45 μm) and then we filtered a second time (after Fc) with the 200nm filter Fa (Whatman Anatop 25 PLUS 200 nm) since it is more selective than Fb.

The SLS curves show that sonication homogeneized the sample (black to red curve) but that large membrane aggregates are already present just after dilution and steering for few hours (black curve). One can see the disruption of large aggregates from sonication from the reduction of long correlation times between the black and red DLS curves of Figure 8b.

Filtration at 0.45 μm gave the green SLS curve that is more flat than the red one before filtration and the size of the nanosomes estimated from the static light scattering using the Guinier analysis is equal to 84.2 nm. Large aggregates were lost with this filtration and 33.65% of the membrane material was lost (measured from UV-Vis at 272.5 nm absorption level) so we obtained a solution at 0.1765 mg/ml (0.01765% w/w). Filtration at 200 nm gave another 59.35% lost of material (green curve to blue),

corresponding to a 73.1% total lost from the starting solution (red to blue curve). Thus, the two filtration gave a 0.072 mg/ml nanosomes solution at the end (so w = 0.0072% w/w). From the Guinier analysis were obtained on filtrated samples, radii of 84.2 nm and 58.6 nm from filtration with Fc (green curve on the plot 7a) and after second filtration with Fa (blue curve on the plot 7a), respectively. It was compare the results of the two experiments, in terms of proteins concentration levels (measure from UV-Visible absorption) and SLS scattering analysis.

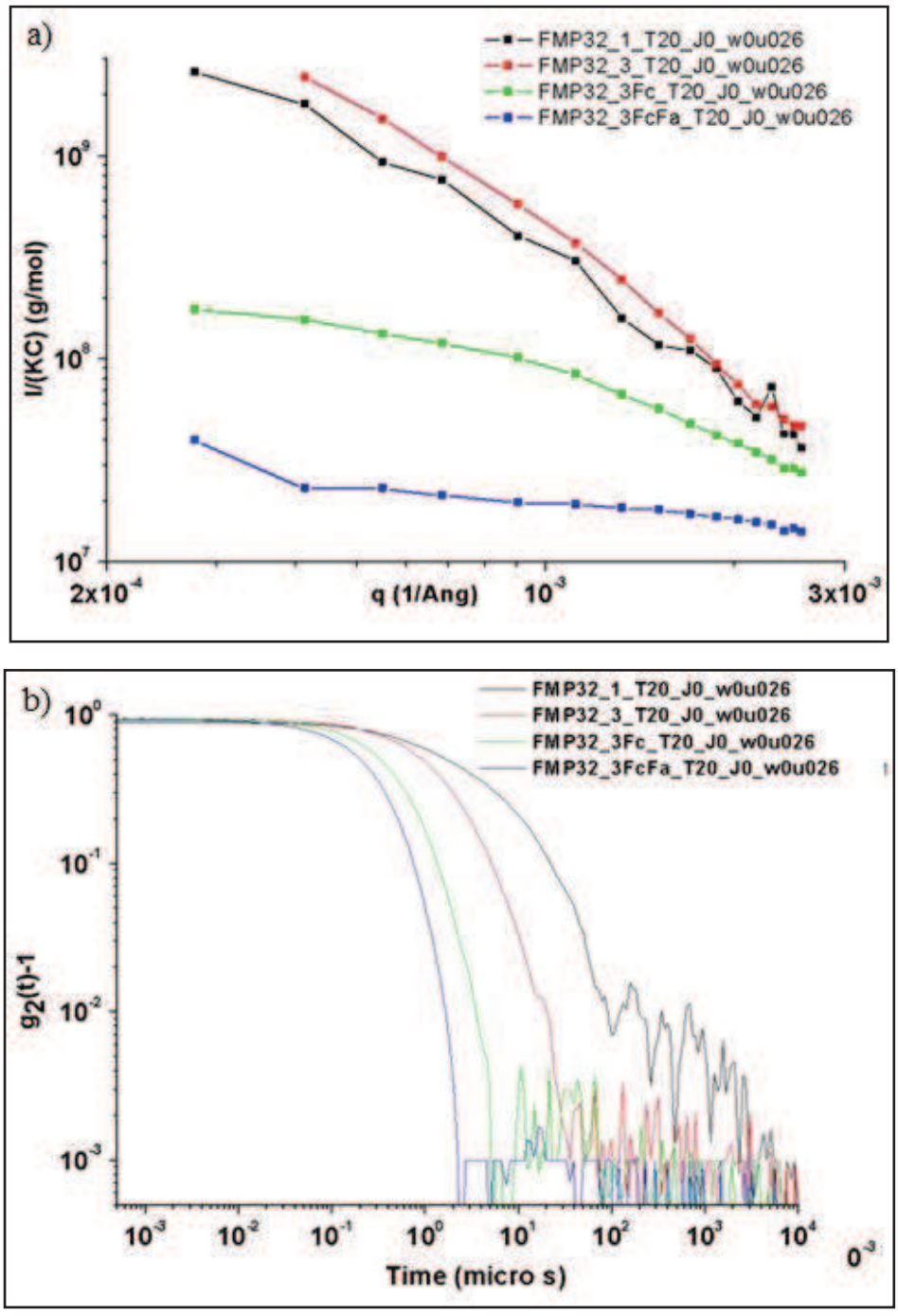

Fig. 7. Static light scattering (SLS) curves of the samples FMP32 at different steps (black is the starting solution, red after sonication at 60°C, green after sonication at 60°C and filtration with filter Fc (450nm), and blue after sonication at 60°C with filtration with Fc and after Fa (200nm)) (a),correlation curves (DLS) measured at 90° from same samples (b).

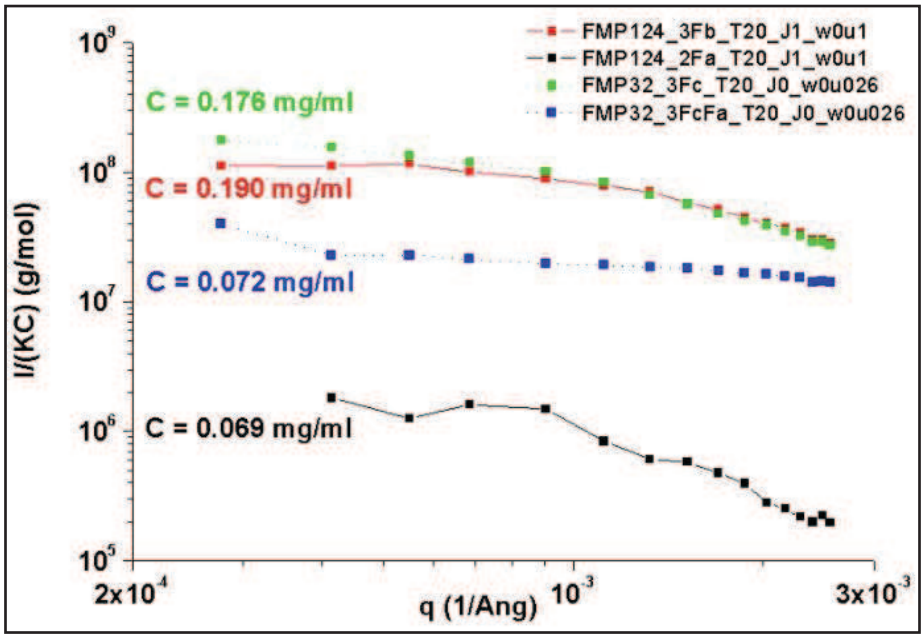

Fig. 8. Static light scattering (SLS) curves of filtrated samples, from FMP124 (black filtered with Fa and red with Fb) and from FMP32 (green filtered with Fc and blue refiltered with Fa).

Table 1. The values of the radius of gyration.

Sample	R_g (nm)
FMP124_3Fb_T20_J1_w0u1	63.4
FMP124_2Fa_T20_J1_w0u1	Not SUV
FMP124_3Fc_T20_J0_w0u026	84.2
FMP124_3FcFa_T20_J0_w0u026	58.6

Conclusion

As showed that stable solutions of small unilamellar vesicles of natural membranes can be obtained from simple preparation routes (ultracentrifugations of yeast and extraction of membrane done by biologists, dilution, sonication and filtration steps).

Commonly known that phospholipids nanosomes may have many applications. These can be used to obtain an experimental system adapted to scattering experiments and to deposition on a solid electrode to prepare olfactory receptors in a natural membrane.

The size distribution of the nanosomes elaborated at sufficient concentration is surprisingly narrow. It was showed the use of combining several techniques such as ATG combined to UV-Vis spectrometry to measure the absolute membrane concentration, including solutions after filtration steps. Light scattering techniques (DLS and SLS) were used to determine the sizes and distribution of the small unilamellar vesicles.

Acknowledgements

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Literature

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