Study of the relationship between applied transmembrane pressure and antimicrobial activity of lysozyme

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Abstract

During the processing of biomolecules by ultrafiltration, the lysozyme enzyme suffers conformational changes, which can affect its antibacterial activity. Operational conditions are considered ones of the principal responsible for the modifications, especially when using the same membrane and molecule. The present study demonstrates that, the same cut-off membrane (commercial data) can produce different properties on the protein after filtration, due to their different pore network. The filtration of lysozyme, regardless of the membrane, produces a decrease in the membrane hydraulic permeability (between 10–30%) and an increase in its selectivity in terms of observed rejection rate (30%). For the filtrated lysozyme, it appears that the HPLC retention time increases depending on the membrane used. The antibacterial activity of the filtrated samples is lower than the native protein and decreases with the increase of the applied pressure reaching 55–60% loss for 12 bar which is not reported in the literature. The observed results by SEC-HPLC and bacteriological tests, suggest that the conformation of the filtrated molecules are indeed modified. These results highlight the relationship between protein conformation or activity and the imposed shear stress.

1 Introduction

In the last two decades, the application and processing of proteins in food, biological, pharmaceutical and chemistry industries have been increased due to their applications such as food supplement (whey protein) [1, 2], as hormones, vaccines, antibiotics, antibodies and enzymes in biopharmaceutical [3], among others. Obtaining high quality proteins to delivery to the final consumers is a major concern of the industries and, for this, processing them is necessary. Some of used unitary operations are purification, concentration, mixing, pumping, heating, and delivery. These procedure steps can induce protein denaturation and as consequence the loss of biological activity. [4, 5]. The commonly technique used to guarantee good quality of products is to add additives, preservatives, buffer, etc. However, these additional components can induce side effects.

Chromatography and membrane separations are commonly used for the purification of proteins during the processing [6]. The use of each is based upon the process and economic constrains. Using chromatography techniques, proteins can be purified considering their size, shape, total charge, hydrophobic groups at the surface and the binding capacity with the stationary phase. They are widely used due to their high degree of purity and recovery of protein [7]. However, chromatography is expensive, difficult to scale up or work in batch operations [6].

Membrane processes, on the other hand, are a greater alternative. Their advantages include low energy consumption, high selectivity, continuous possibility of separation, mild conditions of operation and the possibility to avoid any additional chemicals (additives). Another important feature is the possibility to adjust and vary the membrane properties in order to be the best fit for the process. [8]. However, membrane processes are susceptible to fouling, that is, the deposition of undesired particle on the membrane pores or surface, decreasing the permeate flux and limiting the mass transfer. Commonly,

membrane fouling presents simultaneously inorganic/organic, particulate, and microbial fouling. [9]. Various studies address the problem and propose different solutions such as: use a hydrophilic membrane [10], cross flow filtration [11], low-temperature plasma treatment [12] or increase feed solution velocity to limit polarization layer [13]. Beside the purification step, in order to produce a large quantity of purified proteins, the solution must be concentrated.

Ultrafiltration (UF) is a common choice for membrane process as it can be used concomitantly for protein concentration, buffer exchange, desalting, protein purification and viruses and bacteria clearance [14]. It is a pressure driven process for molecules with a molecular weight from 500 $g.mol^{-1}$ to 500 $kg.mol^{-1}$ and diameter of 1-100 nm. [15]. Considering these values, it can retain solutes such as nanoparticles, colloids, and large molecules. Besides that, depending on the feeding solution, it is possible to choose different membrane materials varying from organic to inorganic ones. Inorganic membranes, for example made of ceramic material, are more stable than organic ones at high temperature and pH conditions and that is the reason of their higher price. [16].

In UF method, protein retention is very high but the efficiency of ultrafiltration can be affected by the characteristics of the membrane (material, pore size, surface charge, hydrophilicity), the protein solution (concentration, species, pH) and by the operating conditions (pressure, temperature, fluid velocity, etc.). [17]

Nevertheless, during the manufacturing process, the proteins are also subjected to changes in temperature, pH, pressures, to different organic solvents, to shearing, shaking etc. For example, during purification by membrane processes, the protein can be exposed to shear rates between 1000 and 10 000 s^{-1}. [18]. High shear can deform the three-dimensional structure of the protein and affect its inherent biological activity. Charm and Wong reported that by increasing the shear (shear rate 1155s^{-1}), the catalase enzyme suffers a decrease in activity up to 45%. [19]. Bowen and Gan explain the loss of activity by the membrane-enzyme interaction resulting from the shear induced deformation of the enzyme structure during microfiltration. [20]. During filling or packaging, proteins can also be subjected to agitation [21, 22] or shear stress (shear rates up to 20 000 s^{-1} for 20 gauges by 10 cm needle, 0.5 $ml.s^{-1}$ rate) [18].

Lysozyme is a globular protein composed of 129 amino acids sequence and its molecular weight is approximately 14.6 kDa. It comes from hen egg white and is high natural abundant. It presents antibacterial action destroying the bacterial cell wall by polysaccharide hydrolysis, it's also used as active ingredient in antivirus and antitumoral pharmaceutical drugs. [23–25].

Considering the possible influence of protein conformation into its biological activity, the aim of this work is to evaluate the behavior and antibacterial activity of lysozyme when ultrafiltration is used as a method for concentration. Different membranes and applied pressures were tested and antibacterial tests against *Micrococcus Lysodeikticus* were performed to understand the effect of processing conditions into protein applications.

2 Materials And Methods

2.1 Materials

2.1.1 Chemical reagents

Vitamin B12 (98% purity) and Hen egg-white lysozyme (70.000 unit.mg^{-1}) were purchased from Alfa-Aesar and Sigma-Aldrich, respectively.

The evaluated concentrations were obtained by dissolving the desired amount into 4 L of demineralized water (18 MΩ). The concentration of each solution is presented in Table 1. All chemical reagents were used without any further purification.

2.1.2 Membrane

The membrane chosen for the filtration experiments was a mono-channel tubular, bilayer, asymmetric ultrafiltration membrane provided by TAMI Industries. This ceramic membrane is composed for an γ-alumina support and an active layer of TiO_2, with a cut-off of 1 kDa (manufacturer data) and isoelectric point at 6.0. [26]. Three raw membranes were used in this study, called M1, M2 and M3. The two fist ones were also used after a cleaning process (the membrane was completely immersed in demineralized water and left in the oven at 105°C for five consecutive days). These two membranes are called M1Reg and M2Reg.

2.2 Experimental protocol

2.2.1 Filtration of protein solutions and membrane properties

Ultrafiltration tests were carried out in a laboratory pilot-plant provided by Techniques Industrielles Appliquées (TIA, Bolène, France) described in a previous work [27]. The pilot-plant operates in cross-flow mode with high flow velocity (700 L/h) in order to minimize the concentration polarization. Temperature was maintained constant (25°C), while the pressure was varied from 4 to 12 bar. Samples from permeate (part of feed solution which pass through the membrane) and retentate (part of feed solution which is rejected by the membrane) were taken at each pressure for further analysis.

The performances of the membrane were asset by filtration of demineralized water (hydraulic permeability) and filtration of vitamin B12 (selectivity performance) before and after the filtration of lysozyme (molecule of interest). Vitamin B12 has negligible effect on the membrane performances at the concentration used in the present study. Additional tests had confirmed that indeed there is no change in the membrane selectivity after successive filtrations of vitamin B12 (data not shown).

2.3 Method of characterization

2.3.1 UV-VIS analysis

Retentate and permeate solutions were analyzed by UV spectroscopy (Lambda 35, Perkin Elmer Instrument) in order to determine the concentrations and the observed rejection rates. Each solution was analyzed at the wavelengths of 360 nm for Vitamin B12 and 280 nm for Lysozyme, respectively. Rejection rates were calculated using the following equation:

$$R_{obs} = \left(1 - \left(\frac{A_{perm}}{A_{ret}}\right)\right) * 100 \qquad \text{Equation 1}$$

Where: R_{obs} is the observed rejection rate, A_{perm} is the absorbance of the permeate and A_{ret} is the absorbance of the retentate.

2.3.2 SEC- HPLC analysis

HPLC (High-Performance Liquid Chromatography) studies were performed with Agilent 1100 Series chain equipped with an UV detector and a quaternary pump. The column used for separation of proteins in the range of 400 000 to 4000 g.mol^{-1} was a 9.4 x 250 mm Zorbax Bio Series GF-250 column (Agilent).

The buffer solutions for the mobile phase were prepared by dissolving 1 tablet of phosphate buffer saline (PBS from Sigma-Aldrich), 0.1% wt sodium dodecyl sulfate (SDS purchased from SIGMA-ALDRICH) and 0.005% wt sodium azide (NaN_3) in 200 mL of demineralized water. The pH is 7.4 at 25°C. The concentrations are 0.01 mol.L^{-1}for phosphate buffer, 0.0027 mol.L^{-1} for potassium chloride and 0.137 mol.L^{-1} for sodium chloride.

100 µL of lysozyme solution was injected in the column and analyzed at the wavelength of 280 nm. The analysis was performed for 15 minutes, with a flow rate of 1.0 mL.min^{-1} and a constant temperature of 25°C.

2.3.2.1. Data treatment

Five imposed components (Gaussian shape, same full width at half maximum) was used to fit the chromatography peak profile. Each peak corresponds to a population of different hydrodynamic volume. The area of the peaks was considered positive and, then, curve fitting was performed by iterative least-squares calculation. Only the two major peaks (highest area of the 5 components) were considered for peak profile. The peaks were denominated as A and B, with A being the population with a lower hydrodynamic volume than B.

2.3.3 Validation of HPLC/UV-VIS calculation

Using Eq. 1 and according to the information from Huang *et al.* [28], the rejection can also be calculated using the areas of the retentate and permeate obtained in HPLC experiments. In order to verify the results, the rejection rates obtained by the two methods were compared. The results from both techniques are in agreement (Supplementary data - Table S1).

2.3.4 Antibacterial activity of lysozyme analysis

Lysozyme antibacterial activity on the *Micrococcus Lysodeikticus* (ML) bacterial strain was studied using a microplate absorbance reader apparatus (MultiSkan FC from Thermo Fisher Scientific) and a 96 well microfiber sterile plate from Thermo Fisher.

Micrococcus Lysodeikticus (ML) lyophilized cells were purchased from SIGMA-ALDRICH. These tests were carried out according to Toro et al. [29] and Lee et al. [30]. The assay started adding 20 µL of lysozyme solution in a well with 200 µL of bacteria culture (0.3 $mg.mL^{-1}$). The procedure was repeated twice with the same bacteria culture and the complete assay was performed two more times with different cultures to assess the reproducibility of the measurement. The solutions were stirred for 30 seconds before measurements and were incubated at 30°C throughout the measurement.

Turbidity modification (ΔA_{450nm}) was measured at 450 nm for 10 minutes with intervals of 15 seconds and the collected data was plotted as a function of time.

A pre-study was conducted for optimizing the method (investigation and results analysis) to study the protein antibacterial properties. Following the article of Prasad et al. [31], the absorbance, the logarithm and the reciprocal of lysozyme absorbance against the bacteria substrate over time were plotted against time. The linearity for activity-time function was observed only in the plot of 1/Absorbance (1/A) as a function of time. (data not shown). This indicates that the reaction between the lysozyme and the *Micrococcus Lysodeikticus* is a second order reaction.

The reaction rate was estimated from the slope of the $1/A_{450nm}$ versus time graph and activity of the lysozyme samples was calculated using Eq. 2.

$$A_u = \frac{\frac{\text{Slope}}{[LSZ]}}{[ML]} \qquad \text{Equation 2}$$

Where: A_u is the activity of the lysozyme, Slope is the slope from $1/A_{450nm}$ vs. time graph, *[LSZ]* is the concentration of lysozyme in the well in $mg.mL^{-1}$, *[ML]* is the concentration of Micrococcus Lysodeikticus in the well in $mg.mL^{-1}$.

Normalization was done using the reference lysozyme sample (untreated lysozyme) value to give an index of activity (Eq. 3).

$$I_{Au} = \frac{A_u}{A_{u_{reference}}} \qquad \text{Equation 3}$$

Where: I_{Au} is the index of activity, A_u , $A_{u\ native}$ are the calculated activities of the treated and the reference lysozyme, respectively.

2.3.4.1 Statistical analysis

In the current study, statistical test was performed with two pair t-test using OriginPro 2019 software. A confidence level of 95% was selected to estimate the significance and a difference of statistical significance was defined for $p < 0.05$.

3 Results And Discussion

3.1 Filtration study

Ultrafiltrations of lysozyme were carried out with raw membranes and regenerated ones following the protocol defined in § 2.**2.2**. All of tested membranes have a commercial cut-off of 1 kDa. The initial properties, hydraulic (filtration of demineralized water) and selectivity performances (1st filtration of vitamin B12), of the chosen membranes for this study are presented in Fig. 1.

It can be observed that the membranes M1 and M2 have similar initial hydraulic permeability properties as M1Reg and M2Reg. However, the selectivity of M1Reg and M2Reg are slightly higher than the raw membranes. This suggests that the membrane cleaning used in this study manages to restore the hydraulic performances of the membrane, but not the selectivity. The results show that the membranes have an apparent different pore size distribution (different rejection rates of vitamin B12). The three raw membranes have no lysozyme filtration history, while the others have performed lysozyme filtrations before cleaning process. In a previous study [4], it was observed that during the ultrafiltration of lysozyme regardless of the membrane used, there is a decrease in the hydraulic performances of the membrane, while there is an increase in the selectivity. This behavior is related to a phenomenon of protein adsorption. Thus, considering all above, it can be presumed that a part of lysozyme is irreversibly adsorbed during filtration. While the membrane is cleaned, the irreversible adsorbed lysozyme is not removed, which might explain the increase in the rejection rate of vitamin B12.

M3 is a raw membrane with the same commercial cut-off as the other membranes used in this study (M1 and M2). However, during filtration tests, it was observed that it possesses different initial membrane properties. It has almost 50% less initial hydraulic permeability and registered higher initial selectivity by 16 percentage points. This suggests that M3 has a different pore size distribution. Given its initial properties, this membrane is considered to have a lower real cut-off than the ones.

The different properties of the membranes tested are also highlighted by the selectivity towards of lysozyme (molecule of interest) filtration. With M1 or M2, the rejection rate of the first lysozyme solution filtrated is around 80%. With M1Reg or M2Reg, the rejection rate of lysozyme is 90–95%. With M3, the rejection rate is 98%. Regardless of the membrane used, after filtration of lysozyme there is still an increase in selectivity and a decrease in permeability, suggesting adsorption of lysozyme inside the pores.

3.2 Conformation and antibacterial activity of filtrated solutions

The normalized chromatograms of permeate samples obtained when using membrane M1 and/or M2 are presented in Fig. 2.

The results show that, when lysozyme is filtrated with a membrane M1 there is relatively no change in the hydrodynamic volume of the proteins after filtration regardless of the pressure used (Fig. 2 left). The same results are obtained with membrane M2. Additionally, neither the population densities suffer great changes (Fig. 2 right). The population densities of the permeate are the same as the retentate and the reference sample. The reference sample (untreated lysozyme solution) is characterized by two populations with different hydrodynamic radius. The two major populations used to fit the chromatogram peaks are termed A (65%) and B (35%). There can be three equally probable explanations for these SEC-HPLC results. First, the shear stress is not sufficient to provoke any noticeable changes in the lysozyme solutions after filtration with these two membranes. Second, lysozyme can be denatured while passing through the pores, but regains its initial conformation once there is no stress applied (reversible denaturation) in the permeate solution. The case of reversible denaturation of lysozyme due to shear was observed by Ashton *et al.* [32] using a Taylor-Couette flow cell. According to their study, after the shear constraints are stopped, lysozyme recovers its initial conformation. Third, it might be possible that just a small number of proteins is denatured due to the passage into the little pores compared to the greater protein amount that passes through the big ones. In the current case, this membrane pore size distribution has a majority of pores with diameters around 2 nm, with some pores surpassing even 4 nm [33]. Lysozyme has a Stokes radius of 2 nm, thus, it manages to pass through the pores surpassing its dimension, without being forced to change its three-dimensional folding structure. The shear forces in the bigger pores are not sufficiently high to produce any irreversible changes in the lysozyme molecule after filtration.

Whereas no changes have been detected on the chromatograms for raw membranes, the permeate solution filtrated with membranes M1Reg and M2Reg exhibits a delay in the retention time of 0.4 minutes comparing to the reference due to filtration (Fig. 3 left). This indicates that there is a decrease in the apparent hydrodynamic volume of the lysozyme after filtration, which can be related to a breakage of intramolecular interactions and consequently, a change of hydration (denaturation) or a cleavage of disulfide bridges. [34]. Nevertheless, the increase in the applied pressure in filtrated lysozyme does not appear to cause any change in the hydrodynamic volume. On the other hand, the population densities change for the filtrated samples. In Fig. 3 right it is observed that the A population decreases from 65%

(retentate and reference sample) to 10% and besides that, A population continues to decrease with the increase of pressure. In the current case, the two membranes (M1Reg and M2Reg) have a different apparent pore size distribution with smaller pores when compared to M1 and M2. Thus, lysozyme is forced to pass through the pores by the applied pressure. Due to its dimensions and the pore size, the molecules are affected by a combination of interface interactions (e.g. electrostatic, dielectric, etc.) and shear forces.

Lysozyme filtrated with the lower real cut-off membrane (M3), also shows the same change in the hydrodynamic volume (data not shown). The shift in the retention time is to higher values and is constant regardless of the applied pressure. The samples show mainly a B population (99%) independently of the applied pressure. In this case, the forces acting upon the molecules manage to force lysozyme into a complete change of population, with the A population almost completely reduced. The behavior of the permeate samples (shift in retention time to higher values, decrease of A population) can be considered similar with the behavior of the samples filtrated with M1Reg and M2Reg.

Thus, it can be concluded that it is the real cut-off / pore size, which contributes to the behavior of the lysozyme after filtration. Shear and interactions between proteins and surface also modify the conformation of the filtrated lysozyme. The changes in the hydrodynamic properties suggest that indeed the filtrated molecule (permeate) is not the same as in the feeding solution (retentate).

Literature presents different studies that show no changes in hydrodynamic properties between the permeate and the retentate. The studies of Wan *et al.* [35] and Ghosh *et al.* [36] report no changes in the retention time between the permeate and the retentate (using ultrafiltration and different cut-off membranes). Nevertheless, the conditions used by them are favorable for not forcing the molecule to change in order to pass through pores and the cut-off of the membranes used in their studies are higher (30 kDa) than the dimensions of the lysozyme (14.3 kDa). On the other hand, Belmejdoub *et al*. [37] argues that in ultrafiltration (membrane 150 kDa), the permeate samples of lysozyme are different from the retentate (total recirculation of the permeate). The changes were associated with the stress in the pores, the physico-chemical interactions with the membrane material and the actual crossing through the pores. According to the study of Marieke van Audenhaegue et al., the cut-off of the membrane is mainly responsible for the modifications of the protein after filtration (ultrafiltration of α-lactalbumin 14.2 kDa. [38] With membranes having smaller cut-off, the shear stress inside the membrane pore increases. In the study of Portugal et al. [39], it is concluded that there is a ratio between the protein size and the membrane pore size which influences the behavior of the molecule. If the pore size is bigger than the protein dimensions, shear stress influence is decreased, and any other physicochemical condition can be considered as responsible for the modifications observed in the molecule. If the pore size is much smaller than the protein, then the structural modifications occurring to the molecule could be attributed to shear stress.

Depending on the membrane used for the filtration, the antibacterial activity of lysozyme is different. For the membranes M1 and M2, the permeate samples (Fig. 4 (a)), as well as the retentate ones, show

statistically the same activity against Micrococcus Lysodeikticus comparing to the reference sample (untreated lysozyme solution), regardless of the operating conditions. For the membranes M1Reg and M2Reg (Fig. 4 (b)), the retentate shows no modification of antibacterial activity due to filtration and on the other hand, the permeate solutions show a different behavior in activity. For them, the antibacterial activity appears to decrease with the increase of pressure. The change in activity is statistically significant ($p < 0,01$) at a pressure higher than 8 bar and it decreases by 60% for 12 bar applied pressure.

For M3 (Fig. 5), there is also a statistical decrease in antibacterial activity with pressure increase. At 12 bar pressure, lysozyme loses 55% of its activity. Currently, as observed in the literature, there is not any study showing a correlation between applied pressure and a decrease of protein activity after filtration process.

Lesnierowski *et al*. have studied the effect of applied pressure, temperature, pH and ultrafiltration time on antibacterial activity of lysozyme. [40]. They did not observe a change due to the pressure increase, but only due to a change of pH.

In the present study, there was no complete loss of lysozyme activity. This suggests that there is no complete denaturation of the lysozyme after filtration, either that there is a non-enzymatic mechanism which accompanies the enzymatic mechanism of lysozyme against Gram-positive bacteria. The hypothesis of a non-enzymatic mechanism is discussed usually when lysozyme is denatured by temperature. According to Ibrahim *et al.* [41], lysozyme can act independently of the enzymatic mechanism by electrostatic interactions between the positively charged lysozyme and the negatively charged bacteria. Also, the intrinsic structural motifs of the enzyme can induce the lysis of bacteria.

The changes in hydrodynamic properties and antibacterial activity are related to the operating conditions and to pore size distribution of the membrane used. The results suggest that the filtration with a low cut-off membrane causes sufficiently high shear stress in the pores to change protein conformational structures and modify its inherent bactericidal activity.

4 Conclusion

A relationship between the hydrodynamic parameters, protein conformation and cell lysis activity of lysozyme was investigated in this work by ultrafiltration experiments performed at different pressures, SEC-HPLC analysis and antibacterial tests.

With the lower “real” cut-off membranes, the filtrated protein suffered modifications in the hydrodynamic volume and a decrease in its antibacterial activity, indicating a significant denaturation. For the membrane with the higher cut-off, no significant difference was observed between filtrated and non-filtrated solutions (chromatography and antibacterial activity investigations). These results show the relationship between the protein properties and shear forces in the pore of the membrane. The antibacterial activity loss (up to 60% at 12 bar) was directly related with the increase of pressure (shear forces in the pore) that is never reported in the literature. The membrane’s real cut-off should be taken into

account when processing proteins especially in the pharmaceutical industry. Besides that, the choice of operating conditions for the optimization of the process should also be considered.

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Conflicts of interest

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Availability of data and material

Not applicable.

Code availability

Not applicable.

Ethics approval

Not applicable.

Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Authors Contribution

Conceptualization: AP and PD; methodology: SM, AP and PD, experimental work: SM, data analysis: SM, AE, AP and PD, writing-original draft preparation: SM, AE, AP and PD: supervision: AP and PD.

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Table

Due to technical limitations, full-text HTML conversion of Table 1 could not be completed. However, the table can be downloaded and accessed in the Supplementary Files.

Figures

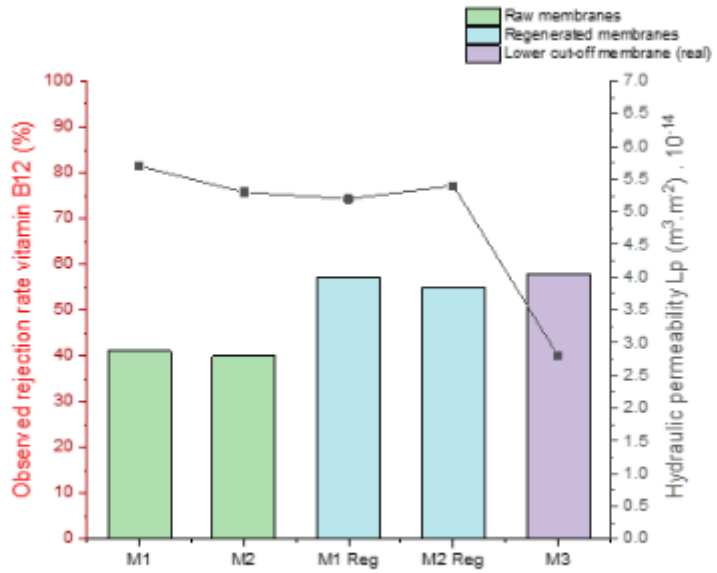

Figure 1

Initial membrane performances: hydraulic permeability Lp (point) and selectivity (columns) for the studied membranes (TAMI membranes, commercial cut-off 1 kDa)

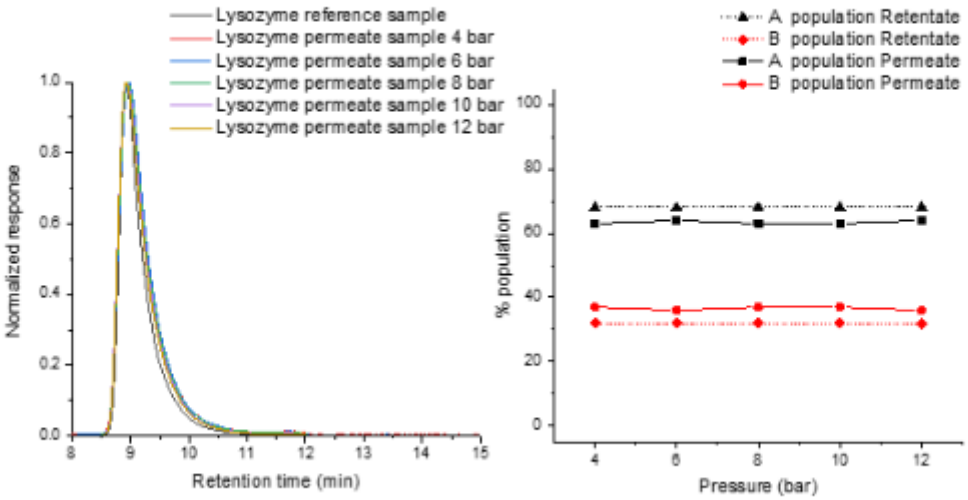

Figure 2

Normalized chromatograms of permeate samples (left) and population distribution (right) for M1 membrane

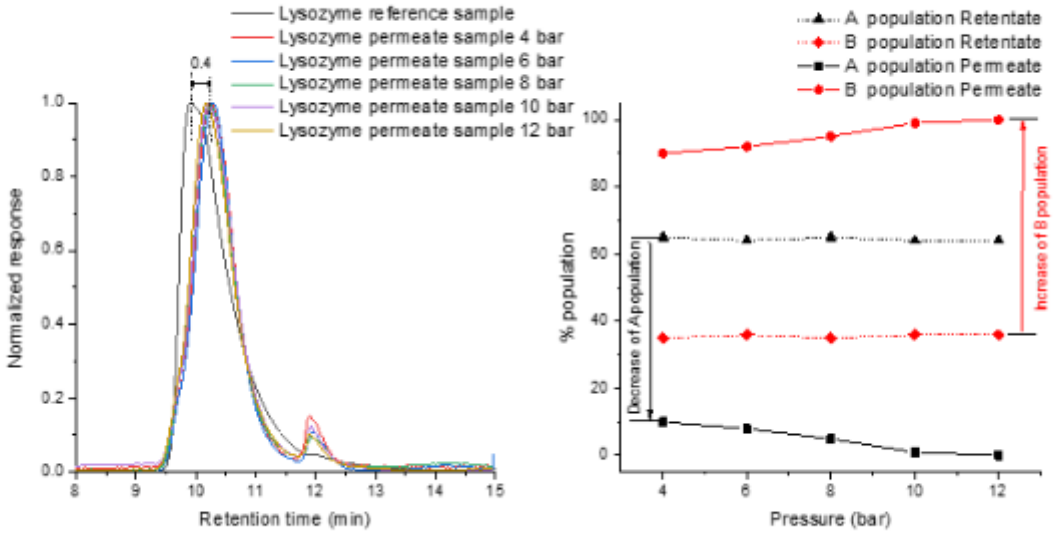

Figure 3

Normalized chromatograms of permeate samples (left) and population distribution (right) for M1Reg and/or M2Reg membranes

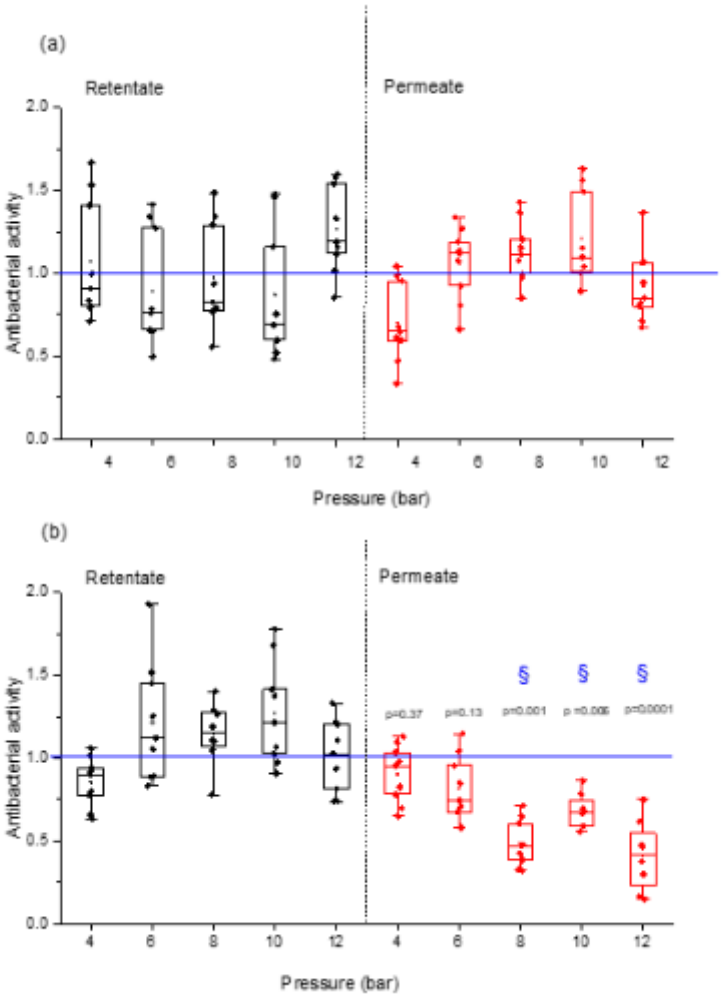

Figure 4

Lysozyme antibacterial activity for retentate (black) and permeate (red) solutions for (a) raw (M1 and M2) and (b) M1Reg and M2Reg; § represents the significant difference for $p<0.05$ with respect to lysozyme reference (untreated lysozyme), blue line represents the lysozyme reference

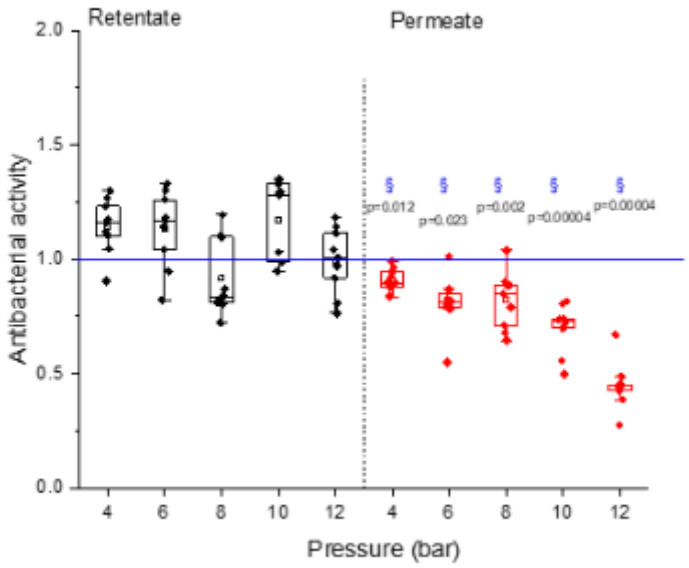

Figure 5

Lysozyme antibacterial activity for retentate (black) and permeate (red) solutions for lower cut-off membrane M3; § represents the significant difference for $p<0.05$ with respect to lysozyme reference (untreated lysozyme), blue line represents the lysozyme reference

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx
- Table1.docx