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Modification of microfiltration membranes by hydrogel impregnation for pDNA purification

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ABSTRACT: The huge efforts for the achievement of highly purified biomolecules are growing every day. A great number of efficient techniques, such as chromatography, are already available in laboratory for separation processes. However, membrane-based technologies are the best match to assure simplicity, efficiency and easy scale-up procedures. Herein we report the modification of a commercial microfiltration membrane for plasmid DNA purification by agarose gel impregnation. The membrane was characterized by SEM, ATR-FTIR, EDS, contact angle, and porosity measurements. Additionally, the membrane pore radius was estimated from observed rejections of different proteins and with that information the rejection of a 6050 bp plasmid DNA (pDNA) molecule was estimated for different values of flux using a theoretical model of large flexible molecules in membranes with parallel cylindrical pores, which is applicable to pDNA ultrafiltration in conventional membranes, as recently shown in the literature. The experimental results show that the modified membrane has higher pDNA rejections than the predicted by the model, suggesting that the different type of porous structure that a hydrogel has, may have a positive effect on pDNA rejections as compared to other biomolecules with more rigid structures, making this type of modified membranes potential better candidates to be used for the selective recovery of pDNA in this type of bioprocesses. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41610.

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INTRODUCTION

The demand of highly purified biomolecules has triggered the development of new separation technologies. Herein, pDNA has been extensively investigated, to obtain a highly purified molecules for gene therapy applications and DNA vaccine.[1,2] The purification of pDNA has already been achieved through the use of different techniques, including chromatography, enzymatic, and membrane processes.[3–6] Here, microfiltration (MF) and ultrafiltration (UF) membranes have gained special interest, avoiding precipitation of solvents, and centrifugation steps. However, the purification of pDNA, namely from RNA and proteins by ultrafiltration remains a challenge in these type of bioprocesses. Other authors have reported that an improved purification of pDNA, using an UF membrane, can only be obtained when the lysate solution is submitted to extensive periods of incubation.[7]

Currently, there are several UF membranes commercially available that can be used for pDNA purification.[4,5] Considering the size of pDNA molecules, surface modification of microfiltration membranes also arises as a possible choice, offering the possibility of adjusting the desired selectivity in each particular case. Several studies have shown the enhancement of membrane properties such as protein-resistance, biocompatibility, charge, and hydrophobicity through surface modification processes.[8–12] In the present study, an agarose coating was impregnated to a commercial MF membrane with 0.22 μm of nominal pore diameter by an adaptation of a method described in literature.[13] This linear polysaccharide was chosen based on its hydrophilic and thermoresponsive character that allow the production of a porous and simultaneously rigid structure.[14] The deposition of the agarose layer and its impregnation through the porous structure of the MF membrane was expected to provide a substantial increase of pDNA rejection comparing to the nonmodified membrane, and its selective retention was investigated. The MF membrane used in this work was selected based on its cost-effective value, mechanical strength and heat resistance which facilitates the impregnation of a hot solution.[15] The modified

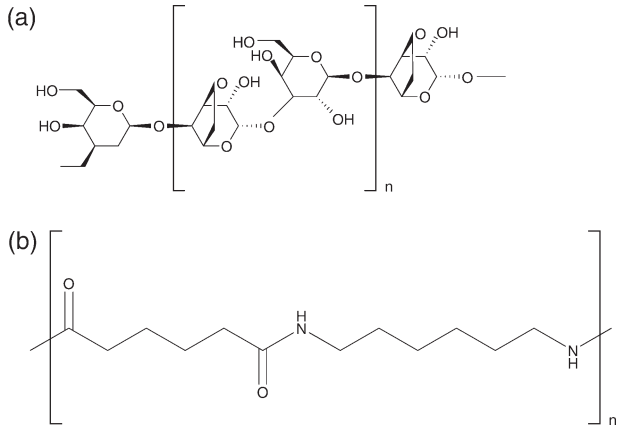

Figure 1. Nylon 6,6 (A) and agarose (B) chemical structure.

MF membrane was morphological and chemically characterized. So far, it is the first time that a modified MF membrane is evaluated for its possible application in a pDNA purification process.

MATERIALS AND METHODS

Materials

Agarose ($M_w = 120{,}000$ Da) was purchased from *Grisp* (Porto, Portugal). Terrific Broth medium for bacterial culture and kanamycin sulfate were obtained from Sigma-Aldrich (Sintra, Portugal). NZYMaxiprep kit for bacterial cell lyses and plasmid DNA purification was acquired from NZYTech and Tris–HCl 10 m*M* IZASA. Microfiltration membranes, Nylaflo (pore diameter of 0.22€µm) from Pall Corporation.

Methods

Modification and Characterization of the Membranes. The Nylaflo membrane is a nylon 6,6 hydrophilic membrane. Herein, a modification on the surface of this membrane was performed using an adaptation of a method already described in literature[13] through the deposition of agarose [Figure 1(B)] to improve pDNA rejection. An agarose solution (2% w/v) at 70°C was poured on to the surface of the microfiltration membrane which was placed on a previously heated glass plate; a second plate was used to form a sandwich. The glass plates were then clamped together, taking care to ensure that no air bubbles were trapped in the gel. After 30 min., the membrane was cooled down to room temperature.[16]

Bacterial Growth, Cell Lysis, and pDNA Purification. The plasmid production procedure was adapted from the literature.[17,18] The 6050 bp plasmid pVAX1-LacZ was amplified in a cell culture of *E. coli* DH5α. The fermentation was carried out at 37°C in 250 mL of Terrific Broth medium, supplemented with 50 µg mL^{-1} of kanamycin. Growth was suspended at the late log phase ($OD_{600\ nm} \approx 10$–11) and cells were harvested by centrifugation. Afterwards, pDNA extraction was performed using an NZYMaxiprep kit. After the extraction and purification, pDNA was stored at 4°C before membrane filtration.

Scanning Electron Microscopy. The morphology of the membranes was analyzed by scanning electron microscopy (SEM). Samples were frozen using liquid nitrogen, freeze-dried overnight, and finally mounted onto aluminium stubs with double adhesive tape and sputter-coated with gold using a Quorum Q150R ES sputter coater. The samples were analyzed using a Hitachi S-3400N scanning electron microscope operated at an accelerating voltage of 20 kV and at different amplifications.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy. Agarose polysaccharide, modified-Nylaflo and Nylaflo membranes spectra were acquired in the range of 4000–500 cm^{-1}, using a JASCO 4200 FTIR spectrophotometer, operating in ATR mode (MKII GoldenGate™ Single Reflexion ATR System). Data collection was performed with a 4 cm^{-1} spectral resolution and after 128 scans.

Energy Dispersive Spectroscopy. In order to determine the percentage of the characteristic elements of the membranes, an energy dispersive spectroscopy (EDS) (Bruker XFlash Detector 5010) analysis was carried out. For that, samples were placed on aluminium stub supports, air-dried at room temperature (RT), and sputter-coated with gold.[19]

Contact Angle. Contact angles of the membranes were determined using a Data Physics Contact Angle System OCAH 200 apparatus, operating in static mode. This assay was performed with two different polar solvents, namely, water and ethylene glycol.

For each sample, solvent drops were placed at various locations of the analyzed surface, at room temperature. The reported contact angles are the average of at least three measurements.

Membrane Porosity. The membrane porosity method has been previously described.[3] Briefly, the total porosity of the membranes was measured through the determination of the amount of ethanol absorbed by the membranes using the following equation:[20]

$$P(\%) = \frac{W_2 - W_1}{d_{\text{ethanol}}\, V_{\text{membrane}}} \times 100 \qquad (1)$$

where W_1 is the weight of the dry membrane and W_2 is the weight of the wet membrane, d_{ethanol} the density of the ethanol at room temperature, and V_{membrane} is the volume of the wet membrane, directly determined by immersion.

Membrane Filtration Tests. All the membrane filtration experiments were performed in a 10 mL stirred cell from Amicon/Millipore, model 8010, according to a procedure previously described in the literature.[4] A modified-Nylaflo membrane was placed in the bottom of the filtration cell, being initially flushed with MiliQ water. Then, water was removed and 10 mL of 10 m*M* Tris–HCl 0.15*M* NaCl (pH = 8.00) buffer were introduced in the filtration cell to determine the hydraulic permeability. This was obtained at a different pressures with compressed N_2 (range 0.025–0.1 bar), measuring the obtained flux. Six permeability measurements were performed with each membrane tested and the average value was considered the hydraulic permeability of each membrane, L_p. To perform the filtration of the pDNA, the remaining buffer in the filtration cell was carefully removed and immediately after that 5 mL of 10 m*M* Tris–HCl 0.15*M* NaCl (pH = 8.00) buffer were placed in the cell with 100 µL of an aliquot of pDNA (recovered by the

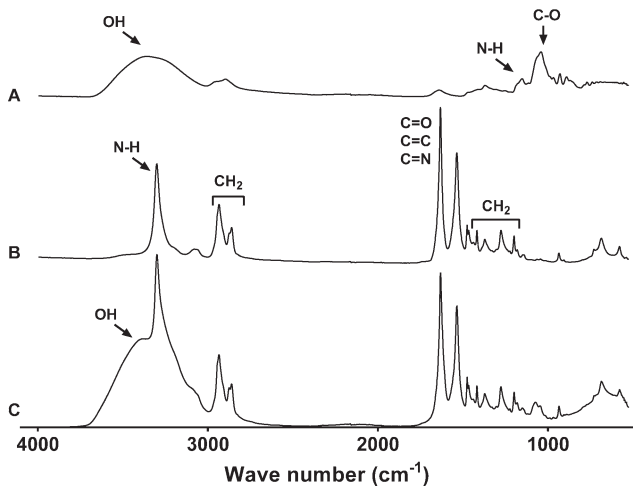

Figure 2. FTIR spectra of agarose powder (A), Nylaflo membrane (B), and modified-Nylaflo membrane (C).

method mentioned in ****section 2.2.2). A continuous filtration of the content of the cell was performed by applying constant pressure and four samples of 0.5 mL of permeate were collected; these four samples correspond to the total permeate collected in each run.

Plasmid DNA Assay. Plasmid concentration was determined by Ultraviolet/Visible Spectroscopy at 260 nm. The absorbance of the initial pDNA solution (feed) and the final concentrate were determined in each run (as well as the absorbance of the four consecutive permeate collected samples previously mentioned). The 10 m*M* Tris–HCl, 0.15*M* NaCl (pH = 8.00) buffer was used as the reference solution for measuring the absorbance due to the pDNA.

RESULTS AND DISCUSSION

Membrane Characterization

Functionalization of the Membrane with Agarose was Verified by FTIR Analysis. Figure 2 shows the characteristic peaks of agarose (A), specifically, at 3359 cm^{-1} (—OH stretching) hydroxyl group, 1042 cm^{-1} (C—O stretch) C—O groups of sugar molecules, 1636 (N—H) and 929 cm^{-1} (vibration of

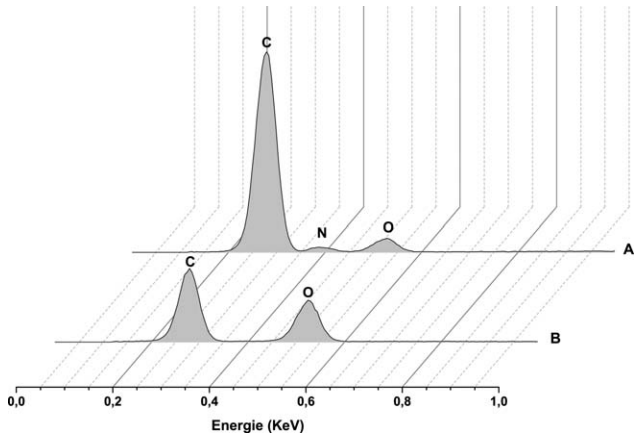

Figure 3. EDS spectra of Nylaflo membrane (A) and modified-Nylaflo membrane (B).

Table I. EDS Analysis of the Membranes

Samples	C (at %)	O (at %)	N (at %)
Nylaflo	72.06	14.18	13.75
Modified-Nylaflo	57.41	42.59	-

C—O—C bridge of 3,6-anydro-L-galactopyranose), in accordance with other reports.[21,22] The FTIR spectra of the Nylaflo membrane was also acquired with and without agarose surface modification, Figure 2(C) and (B), respectively. These results only show that the hydroxyl group peak of agarose at 3359 cm^{-1} is presented in the modified sample. To further verify that agarose impregnation has occurred, an EDS analysis was also performed.

Figure 3 shows that the amount of chemical elements at the surface of the un/modified Nylaflo membrane. Herein, it can be concluded that the surface modification was achieved, once the rate of oxygen elements (characteristic from agarose) increased in the modified membrane (Table I).

Moreover, SEM images were also acquired for further characterization. Here a cross section SEM image shows an agarose layer over the surface of the Nylaflo membrane (Figure 4).

In addition, the top view of the Nylaflo membrane surface with/without modification were also analyzed [Figure 5(A,B), respectively]. The results revealed that different surface morphologies were obtained after the modification process.

As can be seen the Nylaflo membrane surface showed to be porous [Figure 5(A)], with much larger pores than the nominal 0.22 μm are observed, indicating a wide pore size distribution. After modification with agarose the pores could not be observed anymore [Figure 5(B)].

Contact angles in two different solvents were obtained in order to evaluate the hydrophobic character of each membrane, as can be seen in Table II. Herein, the Nylaflo membrane showed

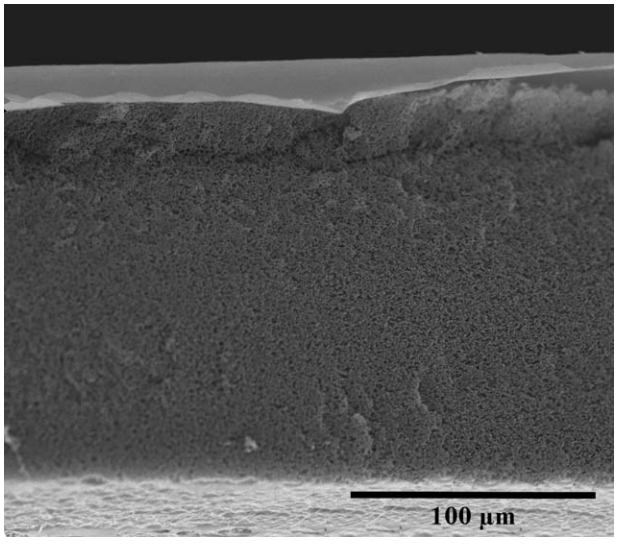

Figure 4. SEM cross-section image of the modified-Nylaflo membrane.

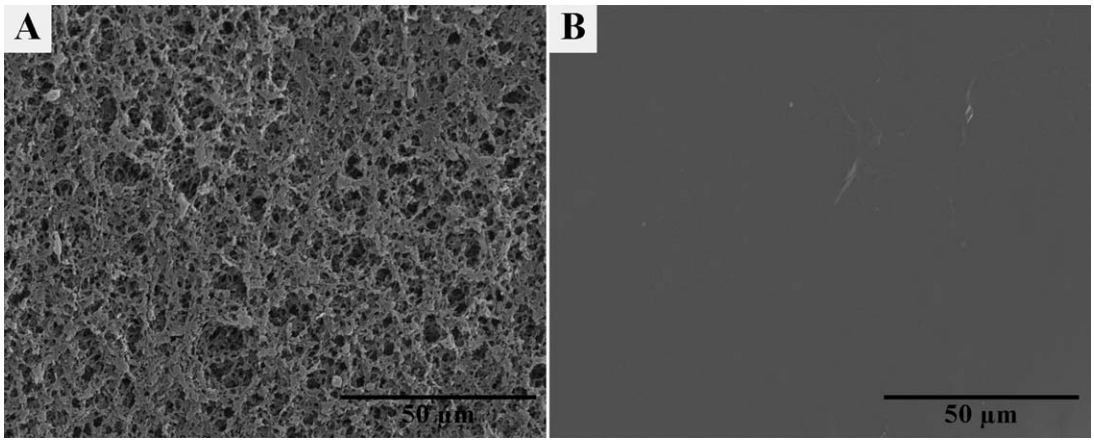

Figure 5. SEM images of the surface of Nylaflo membrane (A) and modified-Nylaflo membrane (B).

to be the most hydrophilic, presenting low contact angles in both the solvents used to perform the assay. For the modified-Nylaflo an increase on the contact angles was observed, however the hydrophilic character of the membrane was essentially kept, which is important to prevent the occurrence of fouling phenomena during the purification process, namely due to protein adsorption.

The porosity of the membranes, determined gravimetrically, is analyzed in Figure 6. As can be seen, the porosity of the modified-Nylaflo membrane decreased to a much lower value than the original Nylaflo, as previously observed in SEM images (Figure 5).

The results obtained in the permeability tests are summarized in Figure 7. As can be seen, the Nylaflo membrane has L_p values near 11,000 L h m^{-2} bar. By turn, the modified-Nylaflo membrane shows a high decrease on L_p values when compared with the original Nylaflo. It is worth to note that the L_p values of the modified-Nylaflo are typical of an UF membrane.

To check the expected pore size reduction, the pore radius of the modified membranes was estimated from the rejections of reference proteins using the theory of hindered transport of spherical solutes in liquid-filled pores,[23] more specifically using the method described as the symmetric pore model, SPM, in Ref. (24). The SPM enables the immediate determination of the membrane pore radius from the intrinsic rejections of a reference solute of known hydrodynamic radius, r_s, and the L_p of the membrane.[24] Intrinsic rejections were calculated from the observed, by estimating the mass transfer coefficient of the proteins in the concentration polarization layer, using the correlation proposed by Opong and Zydney.[25] The proteins used in this work and their relevant properties are summarized in Table III. The observed rejections and the corresponding estimated values of pore radius are indicated in Table IV. The absence of fouling in these tests was checked by measuring the hydraulic permeability of the membranes before and after filtration. The average obtained pore radius was 33 nm, which is substantially

Table II. Contact Angles of the Membranes

Membranes	Water	Ethylene glycol
Nylaflo	28.03° ± 3.81°	16° ± 0.07°
Modified-Nylaflo	32.67° ± 5.74°	23.53° ± 4.66°

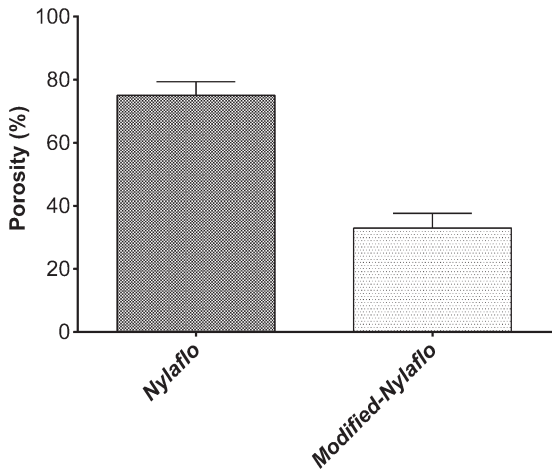

Figure 6. Porosity of the membranes.

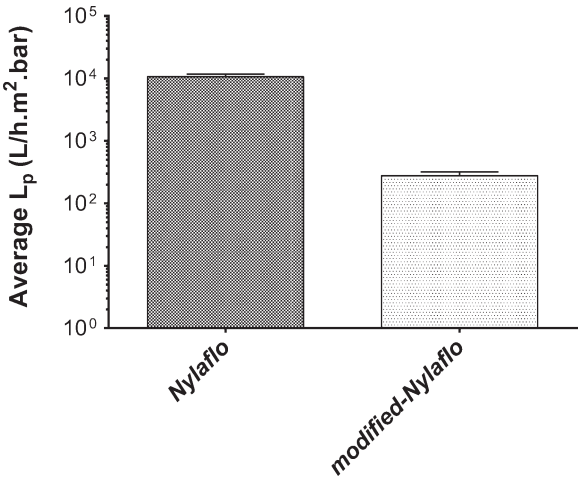

Figure 7. Hydraulic permeability of the different membranes tested, $T = 25°C$.

Table III. Selected Properties of the Proteins Tested

Protein	M_w (kDa)	r_s (m)	D_s (m^2 s^{-1})	Ref.
BSA	67	3.55×10^{-9}	6.95×10^{-11}	26,27
γ-Globulins	158	5.59×10^{-9}	4.42×10^{-11}	26,27

lower than the nominal value of the pore radius of the nonmodified membrane, which is 110 nm.

Plasmid DNA Experiments

Biomolecules separation is important in biology, medicine, and chemistry.[28,29] Herein, pDNA a flexible biomolecule was used in this study. After membrane characterization quantification of pDNA rejection was carried out (Figure 8). As can be seen, at low fluxes the modified-Nylaflo membrane presented near 100% of pDNA rejection and the rejection decreases as the flux is increased, despite the fact that the hydrodynamic radius of the plasmid used in these tests, pVAX1-LacZ, is near 83 nm (value estimated from the diffusion coefficient of the plasmid[30] using the Stokes–Einstein equation), i.e., much higher than the pore radius of the membrane. This type of behavior has been previously reported in literature for pDNA[4,5,31–33] and can be interpreted as a result of flow-induced deformation of the molecular structure of these macromolecules which leads to their permeation through membranes with narrow pores.

Knowing the r_p value of the membrane and the radius of gyration, r_g, of the pDNA molecule, Morão *et al.*[33] have recently shown that one can accurately estimate observed rejections of this type of flexible molecules in the case of several conventional (asymmetric polymeric) ultrafiltration membranes.[4,5,33] This model was originally established for linear molecules, namely linear dextran and linear double-stranded DNA and then adapted to supercoiled plasmid DNA. The model assumes the occurrence of total permeation of large flexible macromolecules provided that they can be sucked into the pores and this only occurs if the molecule has a favorable configuration and orientation when approaching the pores; then the probability of permeation, thus the intrinsic sieving coefficient, can be estimated from the ratio r_g/r_p. In order to estimate observed sieving coefficients one needs, also, to estimate the concentration polarization of the macromolecule and for the purpose one can use the correlation obtained by Opong and Zydney[25] as described by Morão *et al.* in Ref. (33).The theoretical curve shown in Figure 8 was calculated by this method using r_g=90 nm; the r_g value depends on the ionic strength of the solution and was estimated following the method proposed in Ref. (33). In this case, however, the observed rejections appear to be significantly higher than predicted, strongly suggesting that the structure of the hydrogel layer affects the retention of pDNA molecules, by significantly increasing it. A possible explanation for this phenomenon may be that, although the porosity of the modified membranes decreases in respect to the nonmodified, the experimental values obtained are still very high; in fact, near 30% of porosity is a very high value for the membrane porosity of an ultrafiltration membrane, considering that conventional ultrafiltration membranes have typical porosities in the range of 2–7%.[34,35] The effect of the porosity at the membrane surface, which can be identified with the ratio of the pore area to the membrane area, on the rejection of a large flexible molecule like pDNA can be significant, considering that flow-induced deformation is expected to decrease as the porosity increases, due to less suction effects. In the model used for the calculations, which is the only one available in literature for the prediction of sieving coefficients of pDNA, the effect of the membrane porosity on the degree of molecular deformation is not considered; thus, it is possible that the deviations between the experimental results and the theoretical predictions are due to this effect.

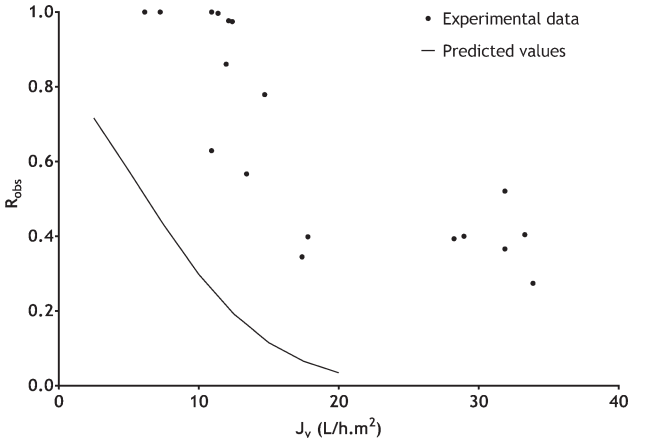

Figure 8. Predicted and observed rejections of plasmid pVAX1-lacZ by the modified-Nylaflo membrane.

Table IV. Observed Rejections of BSA and γ-Globulins at 760 rpm, 25°C at the Indicated Values of Transmembrane Pressure (Protein Concentrations: 0.3 g L^{-1})

Protein	p (bar)	J_v (L h^{-1} m^{-2})	R_{obs}	R_m	r_p (nm)
BSA	0.05	5.3	0.046	0.057	34
	0.10	10	0.054	0.080	28
γ-Globulins	0.10	9.8	0.072	0.12	36

CONCLUSIONS

In this study a commercial 0.22 μm microfiltration membrane was modified by impregnation of a layer of agarose gel in order to obtain significant rejection of a pDNA molecule with interest for gene therapy and DNA vaccines applications. The modified membrane presented characteristics of an ultrafiltration membrane, namely in terms of hydraulic permeability and pore size, but a very high porosity compared to conventional asymmetric polymeric ultrafiltration membranes. The modified membrane has shown 100% of pDNA rejection at low values of flux, but the rejection decreases as the flux increases, due to flow-induced molecular deformation, a phenomenon also observed with other ultrafiltration membranes, as reported in the literature. However, the results suggest that less deformation may occur in the case of the modified membranes tested here, leading to a very positive effect in terms of selective retention of pDNA molecules. This fact and also the simplicity of the modification

procedure makes this type of modified membranes potential candidates for practical applications in pDNA purification.

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