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Polyamide 4,6 membranes for the encapsulation of Langerhans islets: preparation, physico-chemical properties and biocompatibility studies

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Porous polyamide 4,6 membranes developed as semi-permeable and biocompatible membranes for the encapsulation of pancreatic islets were prepared by precipitation in water. Different membrane morphologies were obtained by varying the molecular weight of polyamide 4,6, the solvent evaporation time and the composition of the casting solution. Each membrane was submitted to differential scanning calorimetry and water flux measurements to study the total water content and the different kinds of water in correlation with its permeability performances. Their biocompatibility was first evaluated by a surface protein adsorption test. Of the various membranes, the one prepared by immersion in water after 5 minutes of solvent evaporation, of 15% KS200 polyamide 4,6 solution added with 1% of polyvinylpyrrolidone K30 seems to be the most promising. This membrane is characterized by a low adsorption of proteins, a high hydraulic coefficient and an asymmetric structure. Such a membrane represents a good candidate to be an efficient immunological barrier. It also exhibits good glucose and insulin diffusion properties. Moreover, rat islets cultivated on its surface were not affected by its presence and no important cell adhesion was noticed when implanted in mice. This membrane exhibits most of the properties suitable for the islet encapsulation with a view to developing a bioartificial pancreas.

1. Introduction

Diabetes mellitus is one of the major diseases in western countries. Present therapy of type 1 diabetes mellitus with insulin injections cannot completely prevent the non-physiological fluctuations in blood glucose level which cause the later associated complications, including blindness, kidney disease, need for amputations and reduced life expectancy [1]. In rodents it has been shown that transplantations of healthy islets of Langerhans, which regulate insulin delivery, overcome these problems [2]. In humans, islet transplantation has not yet been successful because of immunorejection [3–6]. Since the availability of human donor islets is very limited, the feasibility of islet transplantation depends on animal islets, although this means transplantation across a major immunological barrier.

The use of a semipermeable membrane, which permits sufficient diffusion of glucose and insulin to and from the islets, but which prevents the cells of the immunological system from migrating to the islets (immunoisolation) might be a solution to this problem [7].

During the last few years, several groups have performed transplants of encapsulated islets with various kinds of membrane (alginate poly(L-lysine), cellulose acetate, agarose, poly(2-hydroxyethylmethacrylate), polycarbonate, polyvinylchloride acrylic copolymer. …) [8–14]. These experiments have shown that in some cases, allo- and xeno-transplantations of encapsulated islets permit normoglycemia for up 2 years in diabetic rats. However, an important fibrosis reaction occurred on the surface of implanted membranes limiting their performances. The most important issue remains the membrane material biocompatibility, i.e. the ability of the polymer to elicit a minimal inflammatory reaction in the host's tissues, resulting in the permanent acceptance of the implant without altering its function.

We have chosen aliphatic polyamides as membrane material. These polymers are of growing importance. It is expected that aliphatic polyamides will be used over a much broader field because of their favourable mechanical properties and chemical stability. Furthermore, among aliphatic polyamides, polyamides 4,6 are the most hydrophilic if we consider the number of

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amide groups along the polymer chain in comparison with the other polyamides. However, to date, no scientific papers have reported the use of such aliphatic polyamides for the development of cell macroencapsulation membranes.

Membranes were obtained by the immersion precipitation process. A polymer solution is cast on an adequate support, such as a thin film, and subsequently immersed in a non–solvent bath, thereby inducing diffusion–controlled phase separation. For this technique different factors (choice of polymer, choice of solvent and non-solvent, composition of the initial solution, evaporation time, composition of coagulation bath, etc.) have a major effect upon membrane structure. By varying one or several of these parameters porous as well as non-porous membranes can be prepared.

The aim of this work was:

1. to apply the immersion precipitation technique to the preparation of porous flat polyamide 4,6 membranes,
2. to study the water permeability of these membranes in correlation with their structure and to evaluate the surface protein adsorption in order to explore the potential of the immersion precipitation process as a possible tool to produce a polyamide 4,6 membrane for the encapsulation of islets of Langerhans,
3. to test their permeability to glucose and insulin and to estimate their *in vitro* and *in vivo* biocompatibility.

2. Materials and methods

2.1. Materials

Two kinds of polyamide 4,6: Stanyl KS200 and Stanyl KS611 of different molecular weights, were kindly supplied by DSM (The Netherlands). Polymers were dried at 50 °C before use. Then they were dissolved in analytical grade formic acid (Prolabo, Rhone Poulenc, France) in a concentration of 15% by weight.

Polyvinylpyrrolidone (PVP) was purchased from Aldrich Chemical (France). PVP1 ($M_w = 40\,000$) and PVP2 ($M_w = 360\,000$) were added to the polyamide 4,6 solution in a concentration ranging from 0 to 5% by weight of the solution

Water, a solvent of PVP, was used as a non-solvent of polyamide 4,6.

2.2. Preparation of the membranes

Polymer solutions were cast on glass plates with a thickness of 300 μm. Immediately, or after 5 minutes of solvent evaporation at 30 °C, the film was immersed in a water bath. After precipitation, the membrane was removed from the glass plate and washed several times with methanol and water.

Eight different membranes were obtained according to this procedure under various conditions of preparation (Table I).

TABLE I Preparation conditions of the eight membranes: initial compositions of the polyamide 4,6 (KS200 or KS611) solutions with or without addition of PVP and solvent evaporation time at 30°C before immersion in water

Membrane	Initial solution composition	Evaporation time (minutes)
1	KS200 (15% wt)	0
2	KS200 (15% wt)	5
3	KS200 (15% wt) +1% wt PVP1	5
4	KS200 (15% wt) + 5% wt PVP2	5
5	KS611 (15% wt)	0
6	KS611 (15% wt)	5
7	KS611 (15% wt) +5% wt PVP1	5
8	KS611 (15% wt) +5% wt PVP2	5

2.3. Physico-chemical characterization of the membranes

2.3.1. Morphology and structure

The morphology of the membranes was examined using a scanning electron microscope (SEM) (Stereoscan 120, Cambridge Instruments Company, France). The samples were cryogenically broken and dried under vacuum. A thin gold layer was sputtered on samples using an E5200 auto sputter coater (Bio RAD, Microscience Division, England).

2.3.2. Water flux measurements

Water flux measurements were carried out on a Millipore filtration cell (Millipore Corporation, USA) connected to a dionized water reservoir with a maximum capacity of 10 l. The effective membrane area was 16.62 cm^2. The system was pressurized with air in the pressure range 0 and 5×10^5 Pa. The flux was determined by measuring the volume of water collected for a known period of time.

The hydraulic permeability coefficient was calculated using the equation [15]:

$$J = K\ (\mathrm{d}P/\mathrm{d}x)$$

where K is the hydraulic permeability coefficient (cm^2/s.bar).
$\mathrm{d}x$ is the membrane thickness (cm).
$\mathrm{d}P$ is the applied pressure gap (atm).
J is the water flux per area unity (cm^3/s.cm^2).
All the values are reported in Table II.

2.3.3. Study of different water states in the membranes

A Perkin Elmer scanning calorimeter was used to measure the phase transition of adsorbed water in the membrane samples.

It has been reported that three kinds of water can exist in polymers and can be studied by means of differential scanning calorimetry (DSC) [16–20]. The three kinds of water are referred to as follows:

(1) *Non-freezing water* or "bound water": this term refers to the water molecules which are bound to polymer molecules through hydrogen bonds and are immobilized. This kind of water shows no endothermic peak in the temperature range 0 to − 70 °C.

(2) *Intermediate water* or "secondary bound water": other water molecules which interact weakly with polymer molecules are referred to as intermediate water. This kind of freezing water has a melting point below 0 °C.

(3) *Free water*: water molecules which do not take part in hydrogen bonding with polymer molecules are called free water because of their greater degree of mobility in comparison with other water molecules. Free water is freezing water showing a melting point at 0 °C. It has a transition temperature, enthalpy and DSC curves similar to those of pure water.

DSC is used for the quantitative determination of the amounts of freezing and non-freezing water.

The membranes, immersed in distilled water, were wiped with paper to remove any remaining surface water and sealed hermetically in stainless steel pans. Each sample weight was about 3–6 mg. The samples were cooled to − 30 °C at a rate of 4 °C/min and then the DSC measurement for each sample was taken from − 30 °C to 20 °C at a heating rate of 4 °C/min. The heat of melting of the freezing water (intermediate and free water) was determined from the area under the endothermic curve and was calibrated using pure distilled water as a standard as described in Mansor and Malcolm's work [19].

After DSC measurements, the sample pans were pricked with a pin to remove water from the samples. The samples were completely dried at 50 °C for several days and weighed, giving the total water content of the membrane (Table III).

2.3.4. Protein adsorption on the surface of the membranes [21]

In vitro, a protein coating of the membranes was performed by incubating a circular fragment of each membrane (surface: 700 mm^2) for one day at 37 °C in a CMRL 1066 medium supplemented with 10% foetal calf serum (GIBCO, Cergy Pontoise France). Protein adsorption on the membrane surface was evaluated by washing the membranes three times in a 1 M NaCl solution (500 µl) for 10 minutes each. Protein concentrations in each solution were measured by the method of Lowry *et al.* [22]. The results represent the total amount of released proteins in the three baths.

2.4. Biological evaluation of the membranes

2.4.1. Permeability to glucose and insulin

Of the eight membranes, only one (characterized by its optimum hydraulic permeability, suitable structure and low level of protein adsorption), was submitted to biological evaluation.

The ability of the membrane to allow the diffusion of glucose and insulin was studied *in vitro* at 37 °C, in an incubator, using a diffusion chamber coated with silicone (SIGMACOTE, St Louis, USA). The chamber had two compartments, A and B, with respective volumes of 3 and 8 ml separated by the membrane (surface: 113.10 mm^2). The chamber was filled with CMRL 1066 medium supplemented with 10% foetal calf serum.

At time zero, glucose was added to compartment A, the initial concentration being 3.7 g/l. Aliquots (100 µl)

TABLE II Determination of the hydraulic permeability coefficient K, from water flux J measurements. Δp is the applied pressure gap. t_{ev} is the evaporation time before immersion in water. Results are means of at least three experiments

Membrane	Δp (bar)	J ($\times 10^5$ $cm^3/s.cm^2$)	Thickness ($\times 10^4$ cm)	K ($\times 10^8$ $cm^2/s.bar$)
KS200				
1. $t_{ev} = 0'$	★	★	104	★
2 $t_{ev} = 5'$	4.1		45	9
3. +1% PVP1 $t_{ev} = 5'$	0.57		38	1773
4. + 5% PVP2 $t_{ev} = 5'$	0.52		69	9500
KS611				
5. $t_{ev} = 0'$	★	★	126	★
6. $t_{ev} = 5'$	4	7	57	10
7. +5% PVP1 $t_{ev} = 5'$	4	19	40	19
8. +5% PVP2 $t_{ev} = 5'$	1.1	270	56	1375

★As the water flux for membrane 1 and membrane 5 was too high without applying any pressure, K was not determined for these membranes.

TABLE III Total water contents of the membranes determined by weighing and expressed in percentages of the wet membrane weight

	KS200				KS611			
	$t_{ev} = 0'$	$t_{ev} = 5'$	+ 1% PVP1 $t_{ev} = 5'$	+ 5% PVP2 $t_{ev} = 5'$	$t_{ev} = 0'$	$t_{ev} = 5'$	+ 5% PVP1 $t_{ev} = 5'$	+ 5% PVP2 $t_{ev} = 5'$
Membrane	1	2	3	4	5	6	7	8
Total water content	76	61	66	61	75	54	68	63

Total water content = [(wet membrane weight − dry membrane weight)/wet membrane weight] × 100.
Results are expressed as means of at least two experiments.

of compartment B medium were taken at 0, 10, 20, 30, 45 and 60 minutes. After sampling, in order to maintain the volume of solution constant in compartment B, an adequate volume of CMRL 1066 supplemented with 10% foetal serum calf serum (100 µl) was added. Glucose concentrations were measured by the method of Hugget and Nixon [23].

The diffusion and adsorption of insulin on the membrane were studied using ^{125}I-labelled insulin as a tracer. Labelled insulin was A_{14} monoiodo-insulin (Amersham, UK) with a specific activity of 2000 µCi/mmol. Diffusion of ^{125}I-labelled insulin was determined according to a method similar to the one described above. At time zero, regular porcine insulin and ^{125}I-labelled were added to compartment A to reach a final concentration of 1.33 mU/ml and an activity of 19 875 cpm/ml. Aliquots of 100 µl were taken from compartment B at 0, 10, 20, 30, 45 and 60 minutes. Activity was measured in each aliquot with a γ counter (Auto Gamma Packard, USA).

2.4.2. Biocompatibility of the membrane

In vitro the biocompatibility of the membrane was evaluated by cultivating pancreatic rat islets on its surface. Pancreatic islets were isolated from adult male Wistar rats by a standard collagenase digestion [24], and hand-picked with the help of a dissecting microscope. The 25 isolated islets were cultured on the membrane surface (surface: 700 mm^2) for up to 2 weeks in 2 ml of CMRL 1066 medium supplemented with heat-inactivated 10% foetal calf serum, 30% L-glutamine and 1% gentamycin, at 37 °C in a humidified atmosphere of 5% CO_2 in air. The morphological state of the pancreatic islets was examined daily by phase contrast microscopy.

In vivo the biocompatibility of the membrane was evaluated by implanting a circular fragment of the membrane (surface: 700 mm^2) in the peritoneal cavity of male adult mice (IOPS Caw, Iffa Credo, L'Arbresbe, France). A fragment of the membrane was deposited in the right *ileal fossa* of six anaesthetized mice by means of a small laparotomy. One month after implantation, the mice were sacrificed. The membranes were removed, dried, coated with gold and examined by SEM.

2.4.3. Statistical analysis

For protein adsorption, mean values $\pm$ SEM were calculated for each membrane ($n = 6$). Comparisons between the eight membranes were performed using a one–way analysis of variance followed by a Newman–Keuls test. Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Physico-chemical characterization of the membranes

3.1.1. Influence of the kind of polyamide 4,6

The structure of membrane 6 (Fig. 1e) seems to be denser than that of membrane 2 (Fig. 1b). On the cross-section of KS611 polyamide 4, 6 membrane the volume occupied by macrovoids appears to be less important (Fig. 1e). Apart from these remarks, the morphologies of both membrane cross-sections are very similar.

The surface aspect of the side in contact with the air during the process, is nearly the same for both membranes: few pores are noticed between the crystalline units of the polyamide 4, 6 (Fig. 2a).

The KS200 and KS611 membranes prepared without PVP exhibit nearly the same hydraulic permeability coefficient (Table II).

Membrane 6 contains less freezing and non-freezing water than membrane 2 (Fig. 3). So it is logical that it contains less water (membrane 6 contains 54% of total water versus 61% for membrane 2) (Table III). The most important difference between the two membranes is the amount of non-freezing water: 28 g for membrane 6 versus 44 g for membrane 2 (Fig. 3).

As illustrated in Fig. 4, the amount of proteins which can be desorbed from the KS200 polyamide 4, 6 membranes was significantly lower than that from the KS611 polyamide 4, 6 membranes, except for one KS611 polyamide 4, 6 membrane prepared without PVP and precipitated after 5 minutes of solvent evaporation at 30 °C (membrane 6).

Without addition of PVP, the structure of the membranes made of KS200 and KS611 are relatively similar. When PVP is added, the structure and the morphological evolution of membranes made of KS200 and KS611 polyamide 4, 6 appear identical.

Membranes 2 and 6 exhibit comparable water permeability performances. For both kinds of polyamides 4, 6 there is a strong increase of the hydraulic permeability coefficient when PVP is added.

The membranes made of KS611 exhibit higher amounts of desorbed proteins than those of KS200.

3.1.2. Influence of the solvent evaporation step

The following remarks are available for both KS200 and KS611 polyamide 4, 6 membranes.

The effect on the ultimate membrane morphology by introducing an evaporation step before immersion in the coagulation bath, can be clearly observed by means of scanning electron microscopy (SEM). When the solution film is immediately immersed in water, the observed membrane cross-section (Fig. 1a) presents a cellular morphology containing large open pores. The thickness of the membranes prepared without solvent evaporation is about twice as much as the thickness of those precipitated after 5 minutes of solvent evaporation (Table II). It should be noted that the membrane surface in contact with air during preparation presents a large number of pores between the crystalline units. Membranes obtained by immediate precipitation were very brittle because of their very open structure.

After 5 minutes of solvent evaporation, the structure is completely different (Fig. 1b). The membrane presents macropores or smaller pores in only one part of their cross-section. The pores are between the crystalline units of the polyamide 4, 6 which are large nonporous spheres called spherulites. The thickness of the

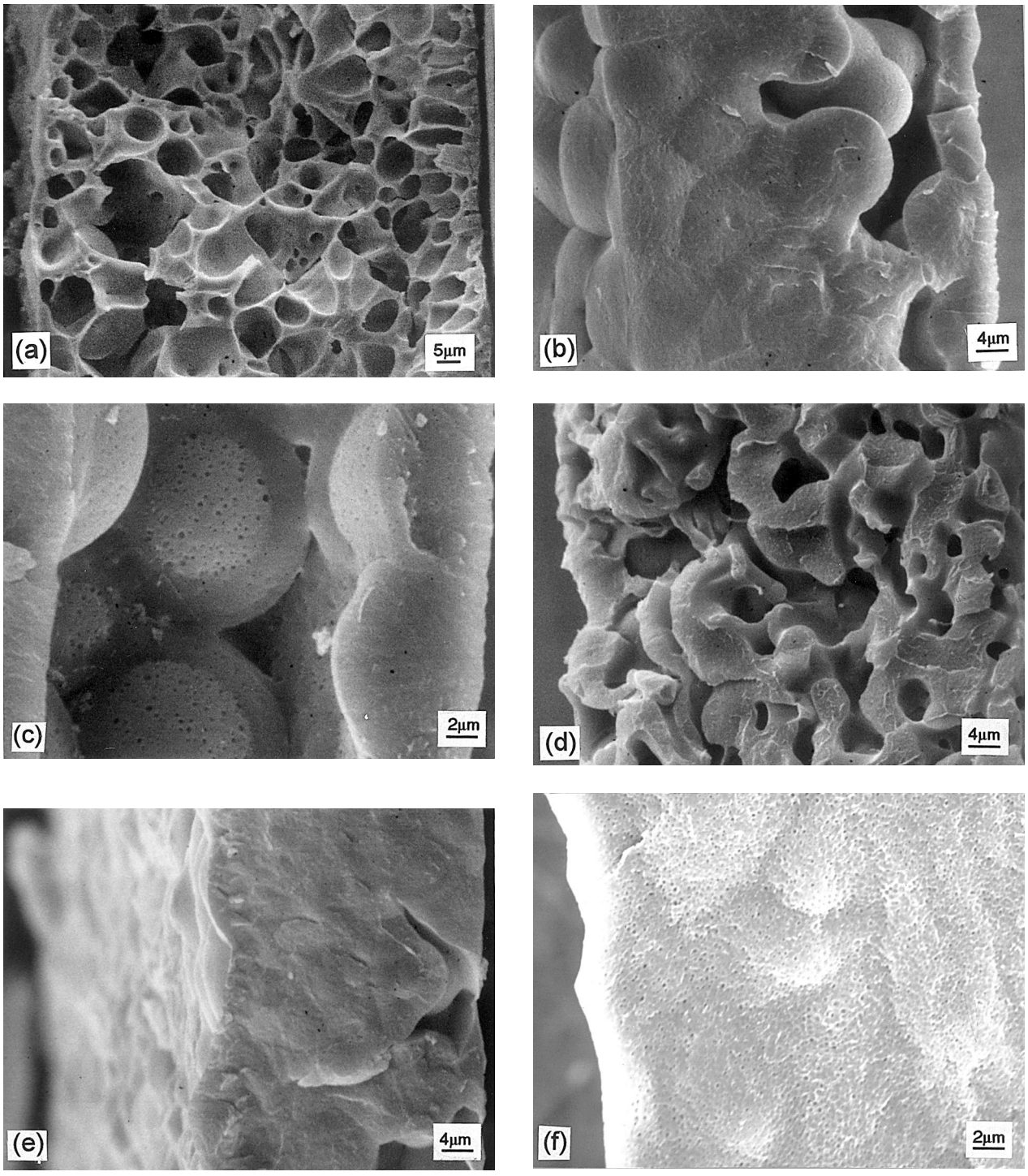

Figure 1 SEM micrographs showing the morphology of KS200 polyamide 4,6 membranes: (a) membrane 1: note the characteristic cellular morphology made up of large open pores; (b) membrane 2: note the importance of the macropore volume on the right part of the micrograph. The arrow indicates a spherulite, crystalline element of the membrane structure; (c) membrane 3: note the porosity of the spheres which constitute the membrane structure; (d) membrane 4: note that the structure is completely different from that observed in (a) (b) and (c) micrographs; and the morphology of KS611 polyamide 4,6; (e) membrane 6: the volume occupied by the macropores is smaller than that observed in micrograph (b); and (f) membrane 7: note the high number of small pores observed.

membrane is considerably reduced when the solvent evaporation step is introduced in the process. This step induces densification of the membrane structure especially in the top layer, leading to the improvement of its mechanical strength by lowering the size and number of pores.

When the water flux measurements were performed on membranes prepared without the solvent evaporation step, the water flux was so high that it was impossible to measure correctly the water volume which had flown through the membrane in a given time. The obvious effect of the solvent evaporation step on the membrane water permeability is a strong reduction of the hydraulic permeability coefficient K through the membrane (Table II). For membranes 2 and 6, the hydraulic permeability coefficients are low and almost the same.

The introduction of the solvent evaporation step in the preparation of membranes from solutions of KS200 and KS611 polyamide 4, 6 induces a notable

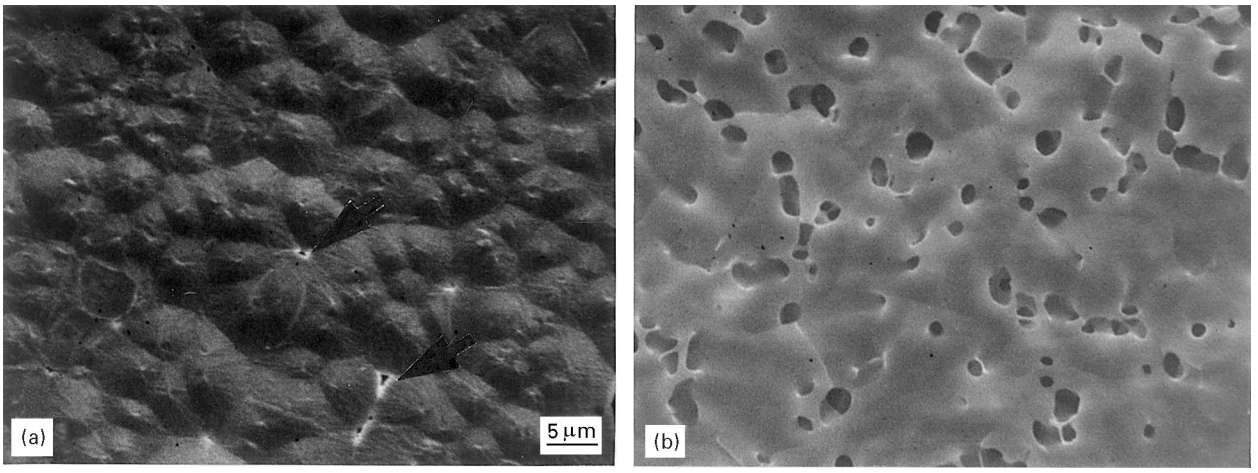

Figure 2 SEM micrographs showing the surface aspect of (a) membrane 2: note that the membrane surface is very dense and that few pores are noted between the spherulites (arrows indicate pores) and (b) membrane 4: note the high surface porosity.

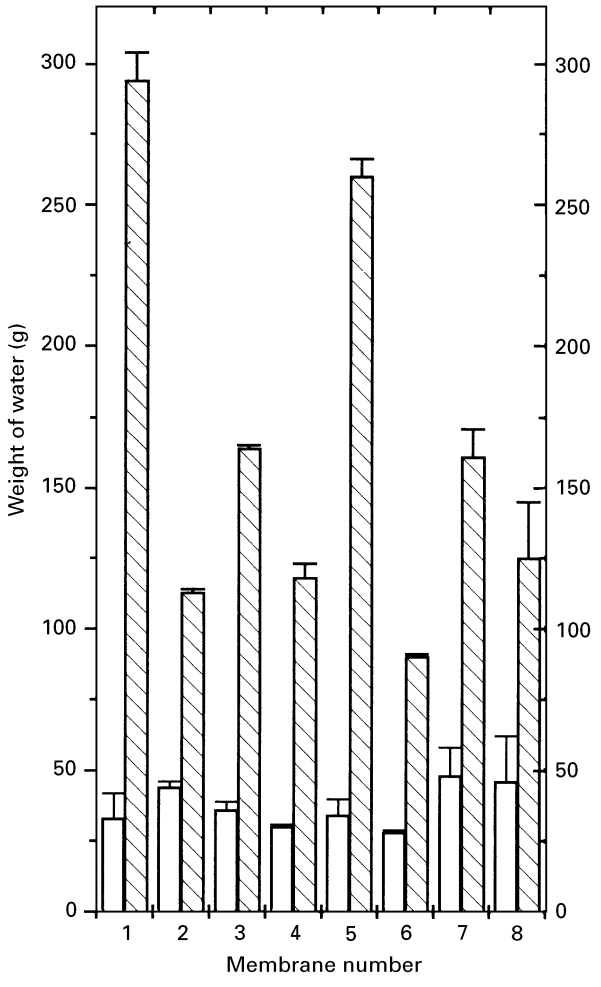

Figure 3 Weight of non-freezing water (□) and freezing water (▨) for each one of the eight membranes. Weights are means of at least two experiments and given in g per 100 g of dry membrane.

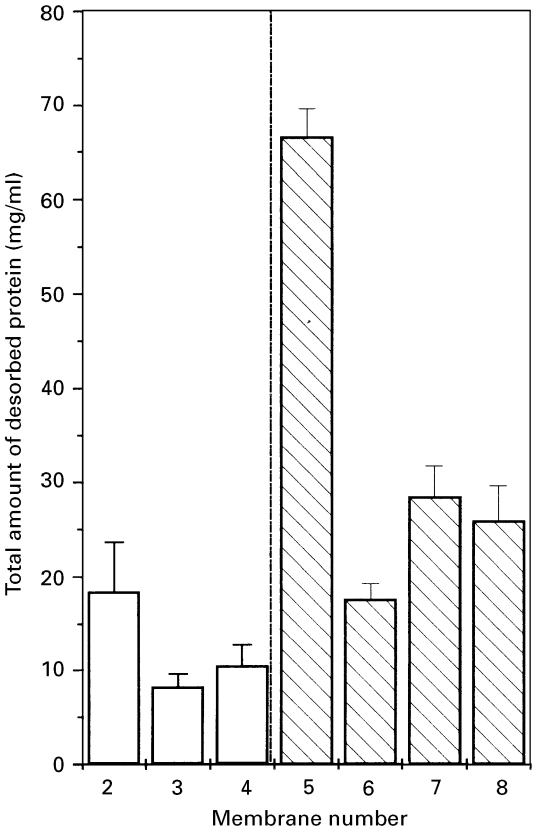

Figure 4 Amounts of desorbed proteins from the membranes 2 to 8. Membrane 1 was not submitted to an adsorption test because it was too brittle. KS200 (□) and KS611 (▨) polyamide 4,6. Results are expressed as means ± SEM of six experiments.

diminution of the total water content in the membrane that must be related to the decrease of the amount of freezing water (Table III and Fig. 3). The amount of non-freezing water remains approximately the same with or without solvent evaporation (Fig. 3).

The decrease of water flux through the membranes and the water contents are in agreement with the morphological evolution observed when a 5 min solvent evaporation is introduced in the membrane preparation process.

When the membranes were prepared without solvent evaporation before immersion, the membranes were very brittle, which made it impossible to perform an adsorption test on membrane 1. However, the quantity of proteins which could be desorbed from the membrane prepared with KS611, decreased significantly when an evaporation time was introduced. After 5 minutes of evaporation only 17.56 ± 1.65 mg/ml of protein

could be desorbed whereas this value increased to reach 66.49 ± 3.07 mg/ml for the membrane prepared without evaporation time. Furthermore, the protein desorption from both KS200 and KS611 polyamide 4, 6 membranes was similar after 5 minutes of solvent evaporation and reached, respectively, 18.37 ± 5.28 mg/ml and 17.56 ± 1.65 mg/ml for the two membranes (Fig. 4).

The 5 minutes of solvent evaporation before immersion in water induce the formation of denser membranes and so, less brittle than without solvent evaporation. This structural evolution of the membranes leads to an important reduction of the hydraulic permeability coefficient and of the total water content. As the weight of non-freezing water remains the same with or without solvent evaporation a decrease of freezing water weight is observed.

The introduction of the solvent evaporation step induces an important decrease of the amount of desorbed proteins for the KS611 polyamide 4, 6 membranes.

3.1.3. Influence of the addition of PVP

KS200 polyamide 4, 6 membranes. The addition of 1% by weight of the final solution of PVP1 to a 15% by weight KS200 polyamide 4, 6 solution, results in membrane 3 which presents a relatively thick dense top layer (this dense top layer is called "skin" and its thickness is about 7 μm) (Fig. 1c). Under this skin, large porous spheres can be observed. The macropores between the porous spheres still occupied an important volume. The main difference with the morphology of membrane 2 (Fig. 1b) is due to the appearance of small pores in the polymer spheres (for membrane 2 the spherulites appear dense without pores). Membrane 3 surface porosity is not very different from that of membrane 2 (Fig. 2a): few pores are noticed on the membrane surface. The structure of membrane 4 (Fig. 1d), obtained by precipitation in water of a solution containing 5% by weight of the final solution of PVP2, is completely different from those observed for membrane 2 (Fig. 1b) and 3 (Fig. 1c). It cannot be described as a juxtaposition of spheres, as for the others. The structure is more homogeneous and the pores are embedded in a continuous polymer matrix.

The surface porosity is much higher because of the presence of large pores (Fig. 2b) (the pore diameter is in the order of a few micrometres).

The hydraulic permeability coefficient K calculated for membrane 3 is about 200 times higher than that obtained for membrane 2 (Table II). The hydraulic permeability coefficient of membrane 4 is about 10 times higher than the coefficient of membrane 3 and about 1000 times higher than for membrane 2 (Table II).

The amount of total water in membrane 3 is lower but not significantly different from that calculated for membrane 2 (Table III). The amount of non-freezing water is slightly lower. The amount of freezing water is higher but in the same ratio as the amount present in membrane 2 (Fig. 3). Membrane 2 and membrane 4 present the same total water content (Table III), about the same weight of freezing water but membrane 4 has a lower weight of non-freezing water (Fig. 3). The total water content of membrane 4 is lower than that of membrane 3 (Table III). The amount of non-freezing water as well as that of freezing water is a little lower for membrane 4 than for membrane 3 (Fig. 3).

The amount of proteins which can be desorbed from membrane 3 and 4, synthetized, respectively, with addition of 1% of PVP1 and 5% of PVP2 is reduced as compared to membrane 2 prepared without PVP (Fig. 4). For membranes 2, 3 and 4, protein desorption values were, respectively, 18.37 ± 5.28 mg/ml, 8.18 ± 1.46 mg/ml and 10.42 ± 2.34 mg/ml.

The lowest amount of protein was observed on membrane 3.

KS611 polyamide 4, 6 membranes. Membrane 7, obtained from a KS611 polyamide 4, 6 solution containing 5% by weight of PVP1, is characterized by a homogeneous microporous structure. Few macropores are present along its cross-section (Fig. 1f). Membrane 7 is thinner than membrane 6 (Table II). The surface aspect and the surface porosity of the membranes appear very similar.

The structure of membrane 8 resembles that of membrane 4, also obtained with a solution containing 5% by weight of PVP2 (Fig. 1d): large pores are embedded in a continuous matrix of polyamide 4, 6. On the surface, the pore number is much more important than for both membranes 6 and 7.

The water permeability coefficient K of membrane 7 and membrane 8 are, respectively, 2 times and 137 times greater than that calculated for membrane 6. The water permeability performances are improved when these membranes are precipitated from KS611 polyamides 4, 6 solutions containing 5% by weight of PVP1 or of PVP2. This improvement is more significant for membrane 8 (Table II).

Membranes 7 and 8 exhibit a higher total water content than membrane 6. Membrane 7 presents the highest total water content of the three membranes 6, 7 and 8 but this amount is still lower than that obtained for the membrane prepared without solvent evaporation (Table III).

Both membranes 7 and 8 contain more non-freezing water and freezing water than membrane 6. Membrane 7 is characterized by the highest weight of non-freezing and freezing water which are, respectively, 48 g and 161 g for 100 g of dry membrane (Fig. 3).

When the KS611 polyamide 4, 6 membranes were prepared from a solution containing 5% by weight of PVP1 (membrane 7) or PVP2 (membrane 8), the protein desorption was not significantly different between the two membranes: 28.39 ± 3.32 mg/ml versus 25.84 ± 3.77 mg/ml. However, these values were significantly higher than those observed in the membrane prepared without PVP (membrane 6: 17.56 ± 1.65 mg/ml) (Fig. 4).

The addition of PVP leads to a notable evolution of the membrane morphology: the volume occupied by the macropores is significantly reduced and the structural spheres become porous with the addition of

PVP, or disappear when PVP2 is added. These morphological changes lead to an improvement of the water permeability performances of the membranes. The increase of the hydraulic permeability coefficient is higher when PVP2 is added.

For the KS611 membranes, the addition of PVP increases the amount of desorbed proteins. For the KS200 membranes the PVP addition decreases this amount.

3.2. Biological evaluation

Membrane 3 was the only polyamide 4,6 membrane submitted to biological evaluation.

3.2.1. Glucose and insulin diffusion

Glucose passed through the KS200 polyamide 4,6 membrane after the first few minutes of the diffusion test (Fig. 5a). Approximately, 46% of the initial amount of glucose passed through the membrane after 1 h of the diffusion test (Fig. 5a). This result indicates that after 1 h, 63% of the theoretical equilibrium concentration of glucose was reached with the KS200 polyamide 4,6 membrane.

After only 5 minutes of the diffusion test, ^{125}I-labelled insulin passed through the KS200 polyamide 4,6 membrane. The diffusion of ^{125}I-labelled insulin increased progressively to reach 25% of the initial amount of radioactivity after 1 h (Fig. 5b). Expressed in percentage of theoretical equilibrium concentration of insulin, this rate corresponded to 34% for the KS200 polyamide 4,6 membrane. Furthermore, the total remaining radioactivity linked to the membrane was low: about 0.5% of the initial radioactivity.

3.2.2. Biocompatibility studies

In vitro: *culture of pancreatic islets.* Using phase contrast microscopy, pancreatic islets appeared as round structures bounded by an intact outline after one week of culture. Similar morphological aspects were observed when the islets were cultured in the presence of membrane 3 or on dishes (Fig. 6).

In vivo. After one month of implantation, the implants made of KS200 polyamide 4,6 membrane (membrane 3) were found intact in the right ileal fossa and only covered with omentum. Furthermore, the implantation area was unaffected. Neither tissue necrosis, nor cellular inflammation was observed. Over a one month period, the structure and surface membrane remained unaltered (Fig. 7a). Some areas of cellular adhesion composed of fibroblasts and macrophages were observed on the surface of the membranes (Fig. 7b).

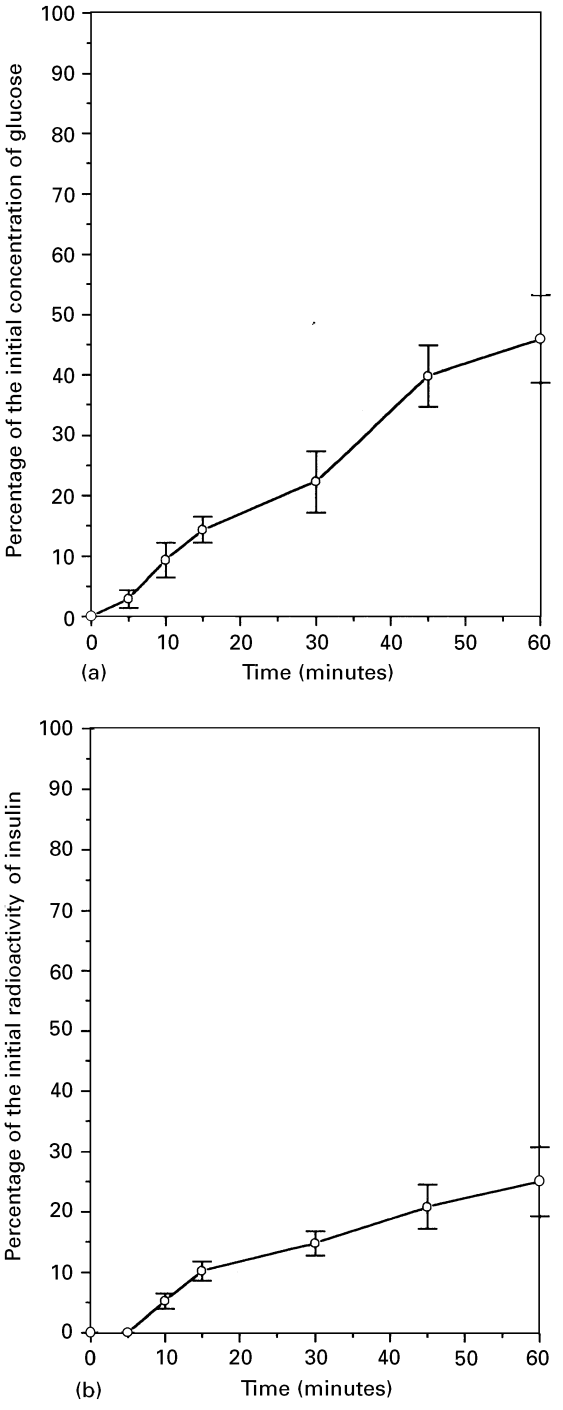

Figure 5 Glucose (curve a) and insulin (curve b) diffusion through the KS200 polyamide 4,6 membrane called membrane 3. Results are expressed as means ± SEM of six experiments.

4. Discussion

Membrane 3 was prepared by immersion in water of a polyamide 4,6 solution after 5 minutes of solvent evaporation. The polymer solution was composed of 15% by weight of KS200 polyamide 4,6 and 1% by weight of PVP1 in formic acid. Its asymmetric structure is composed of a dense top layer on porous spheres. Such a structure assures high hydraulic permeability and good glucose and insulin diffusion. The low adsorbed mass of proteins on its surface, its non-toxicity towards the islets and the limited cell adhesion observed when it was implanted prove the biocompatibility of membrane 3.

The main reason for which the polyamide 4,6 material was chosen for the preparation of porous flat membranes for islet encapsulation is its high potential hydrophilicity. Polyamides 4,6 are structurally regular

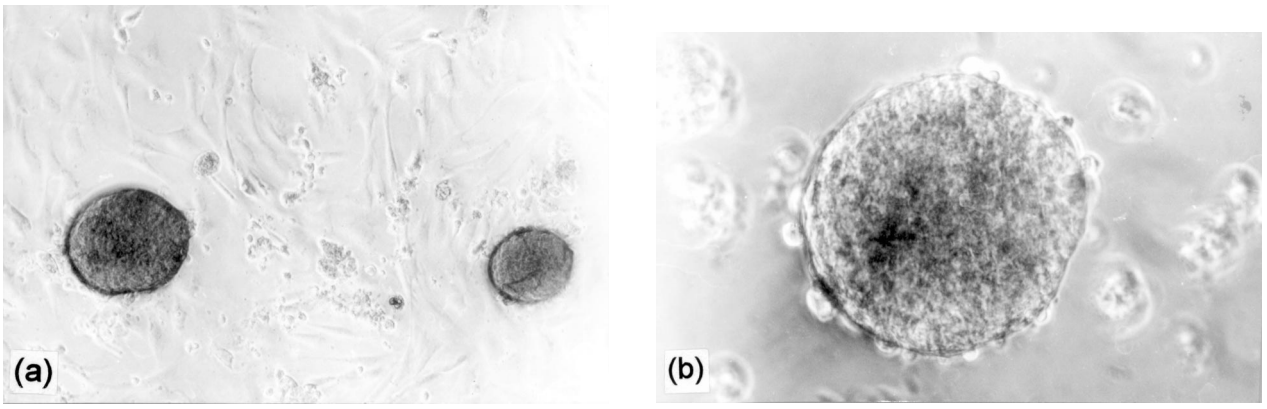

Figure 6 Phase contrast micrograph of a rat islet of Langerhans after 7 days of culture (a: ×80) on a dish and (b: ×200) on the surface of membrane 3. Note the similar morphological aspect of the islets in both cases.

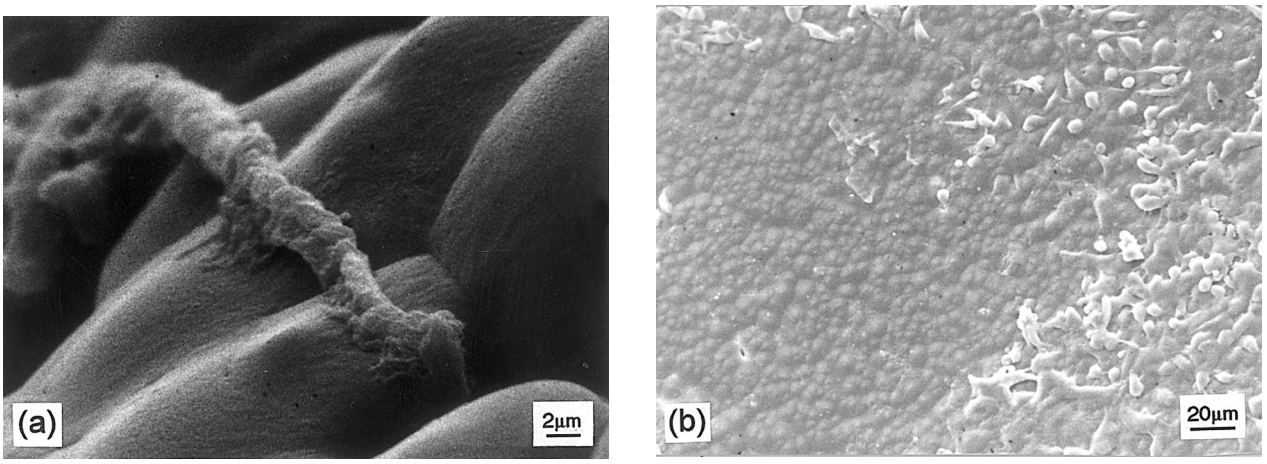

Figure 7 SEM micrographs showing the surface of membrane 3 one month after implantation in the peritoneal cavity of mice. (a) Note the preservation of the surface structure after implantation. On this micrograph, a fibroblast is entering one of the few pores on the surface. (b) Note that cellular adhesion is limited on the membrane surface.

chains consisting of a series of methylene groups linked together by amide groups. Their hydrophilicity is due to the presence of these amide groups in amorphous regions which, to the extent that they are not involved in intermolecular hydrogen bonding, are available for interaction with water. This should lead to good water permeability and thus good diffusion properties for glucose and insulin dissolved in water. The high value of the calculated hydraulic permeability coefficient reflects the high water flux through membrane 3. The results of the glucose and insulin diffusion tests show the relatively good diffusion of these products through membrane 3.

Moreover, according to many important observations [25–27] on protein surface activity which have been explained in terms of contact surface hydrophobicity, the high potential hydrophilicity of polyamide 4,6, especially on the membrane surface, would induce a low adsorption of proteins. The decreasing of the level of surface membrane fouling would then imply an improvement of its biocompatibility.

Considering the low measured amounts of desorbed proteins on the polyamide 4,6 membranes, the non-toxicity of the material towards the islets and the limited cell adhesion on the membrane surface, our assumptions on the good biocompatibility of the polyamide 4,6 material were proven. The amounts of desorbed proteins from the KS200 polyamide 4,6 membranes were notably low. The values are in the same range as those obtained for the AN 69 membrane (Hospal, Meysieu, France). In Kessler's work [28], the AN 69 membrane composed of 69% polyacrylonitrile and 31% sodium methallyl sulphonate, was described as biocompatible. Moreover, its hemocompatibility has already been demonstrated in renal dialysis. The higher amount of desorbed proteins measured on membrane 5 cannot be attributed to a notable difference in membrane hydrophilicity. In fact, the large open surface structure of membrane 5 induces an important increase of the active area for protein adsorption. This is the only explanation for the high quantity of desorbed proteins noted for membrane 5. A partial desorption can explain the low amounts of desorbed proteins. It would be necessary to use another substance more active for protein desorption and to compare the results.

Most of the commercially available membranes are obtained by the immersion precipitation process. The main interest of this process is to achieve the preparation of asymmetric membranes by controlling the initial stage of phase transition which occurs by the exchange of solvent and non-solvent resulting in

a very dense top layer or skin supported by a porous sublayer. Such a membrane combines the high selectivity of the dense layer with the high permeability rate of the porous layer. Such asymmetric membranes would represent a good compromise for a semi-permeable membrane that should be permeable to glucose and insulin and act as an immunological barrier. To optimize the polyamide 4,6 membrane structure taking into account the interest of an asymmetric structure, three parameters were varied in this study: the type of polyamide 4,6, the introduction of a 5 minutes solvent evaporation step, and the addition of PVP in the initial polymer solution.

Two polyamides 4,6 of different molecular weight were used: KS200 and KS611 polyamide 4,6. They exhibit different intrinsic viscosity. It has been reported [29] that the intrinsic viscosity of the polymer has a great effect on the membrane properties: increasing the intrinsic viscosity of the polymer enables the production of membranes with a higher permeability. The observed influence of the intrinsic viscosity of the polymer was not attributed to the increase in initial solution viscosity directly connected to the polymer molecular weight. It was due to an improvement of the quality of the skin of the membrane. The number of imperfections in the skin should decrease when the intrinsic viscosity of the polymer augments.

SEM observation does not exhibit distinct differences in the surface structure between membrane 2 and membrane 6 which might emphasize the influence of the type of polyamide 4,6. Moreover these membranes exhibit about the same hydraulic permeability coefficient. In fact the molecular weight difference between both polyamides 4,6 might be too small to notice large differences in membrane performance.

Several authors [16–20] consider the volume fraction of water in polymer membranes as one of the important factors controlling permeability of water through membranes.

According to Uragami *et al.* [16], the non-freezing water amount in the membrane has a determinant influence on permeation mechanisms through the polymer membranes. The amount of non-freezing water depending on membrane morphology is inversely proportional to the packing density of the polymer. For the cellulose membranes studied by Uragami *et al.* [16], the amount of bound water decreased with increasing evaporation time. This is because the dense surface layer of the membrane increased with the evaporation period. This decrease in the amount of non-freezing water observed for the KS611 polyamide 4,6 membranes means that the density of polymer packing for membrane 5, with 5 minutes of solvent evaporation, is higher than for the membrane prepared without solvent evaporation. But this evolution was not checked for the KS200 polyamide 4,6 membranes. As the membranes obtained without solvent evaporation time exhibit an open cell structure and contain a lot of water, the method of using a paper to wipe the membranes to remove the water excess is too drastic. Part of the water contained in the cells can be extracted with the paper and this can induce distortion of the calculated values of the different water weights.

According to Taniguchi and Horigome's work [17], the ratio of water content of each stage closely depends on the structure of the membrane. In this work, as the membranes are porous, it was more interesting to study the correlation of total water or freezing water content with the membrane structure in order to better understand the water permeability performances.

The introduction of a 5 min solvent evaporation step is an important parameter influencing both the structure and the final membrane properties. Solvent evaporation and gelation of the film induce an increase of polymer concentration in the film and especially at its surface. Such an evolution promotes the formation of a dense top layer and thus a more selective membrane regarding the glucose and insulin exchange. On the other hand, it prevents macropore formation in the membrane [30, 31]. The reduction of pore size and the number of pores explains the strong reduction of the total water amount and the weight of freezing water. In this manner pure water flux is strongly reduced and mechanical properties largely improved. This was observed in membrane 2 and membrane 6 precipitated in water after 5 minutes of solvent evaporation. Membrane 6 is the best example showing the lowest total water content, weight of freezing water and non-freezing water, a small hydraulic permeability coefficient and the densest structure.

In summary, the membranes obtained without solvent evaporation are characterized by a large open cell morphology with an important surface porosity which explains the high total water and freezing water contents calculated. Therefore very high water flux is measured through these membranes. On the other hand, such a structure with large pores cannot efficiently ensure the immunoprotection of the encapsulated cells.

When preparation of the membrane included a solvent evaporation step, the resulting membranes were denser and presented the lowest hydraulic permeability coefficients. In order to find an optimal structure between the large open pore membranes and the dense membranes, PVP was added to the casting solution.

The most important effects of the addition of PVP are suppression of macropores, improvement of the interconnection of pores and higher porosities in the top layer and sublayer [32, 33]. The importance of PVP effects depends on the strength of the interactions between PVP and polymer, the concentration of the additive, and the molecular weight of the additive. In the case of the KS200 polyamide 4,6 solution, only 1% by weight of PVP1 was added. The molecular weight difference between the two polyamide 4,6 justifies this choice. By adding PVP1 to the initial polyamide 4,6 solution, a notable increase of the weight of freezing water, the total water content and the hydraulic permeability coefficient K is observed due to the increase of porosity of the membrane throughout its entire thickness. The strong increase in water flux through the membranes obtained with a solution containing PVP2 could be attributed to the higher surface porosity. The differences in structure observed between membranes 7 and 8 illustrate the influence of the molecular weight of the PVP on membrane morphology.

It would be very interesting to study how the PVP can influence the membrane precipitation process in order to better understand its action on the formation of membrane pores and on their interconnection. Studies are in progress to develop these aspects of membrane preparation.

From these observations it appears that membrane 3 represents the best compromise between specific morphology and water permeability performances, and that is why membrane 3 was the only membrane tested for glucose and insulin diffusion.

An interesting result is that there is no delay in glucose diffusion through membrane 3 while glucose passed through the AN69 membrane only after 10 minutes [27]. Glucose diffusion through membrane 3 is slower than through the AN69 membrane, as 65% of the initial amount passed through the AN69 membrane after 1 h of diffusion. Nevertheless it should be noted that the difference in thickness between the two membranes is important: membrane 3 is about twice as thick as the AN 69 membrane.

Not only was the insulin diffusion through membrane 3 improved as compared to AN69, but the radioactivity bound to membrane 3 was very low. The insulin diffusion properties of membrane 3 are better than those of the AN 69 membrane. Soldani *et al.* [34] fabricated porous tubular membranes made with polyurethane-polydimethylsiloxane (PU-PDMS) material. These membranes exhibit excellent glucose and insulin diffusion properties, as after a few minutes, 100% of the glucose had been released from the capsule and after 25 minutes, 80% of the insulin. Nevertheless their biocompatibility was not evaluated by *in vivo* experiments. The same remark can be made about Zekorn and co-worker's study [35] in which cellulose, nylon and polysulfone commercial membranes were tested to determine the insulin diffusion rate. The greatest advantage of membrane 3 over most of the commercial membranes tested to immunoisolate islets of Langerhans, is that it combines satisfactory glucose and insulin permeability with good biocompatibility.

In conclusion, the immersion precipitation process allows the preparation of asymmetric membranes made of polyamide 4,6 suitable for islet encapsulation. To obtain the optimal membrane 3, the influence of three parameters on the precipitation process was studied. It was shown that the type of polyamide 4,6 does not seem to greatly influence the membrane structure. The introduction of a solvent evaporation step before the immersion in water leads to less brittle membranes characterized by a denser structure and that the addition of a second polymer in the initial solution of the polyamide 4,6 helps to obtain a more porous and more water permeable membrane.

In order to use membrane 3 for pancreatic islets encapsulation its glucose and insulin diffusion properties should be improved. Control of the membrane thickness should lead to a notable increase of diffusion rates. Good biocompatibility, tested by *in vitro* and *in vivo* experiments, is the greatest advantage of membrane 3 and confirms the accuracy of the material selected.

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