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MYOGLOBIN ENTRAPMENT IN POLY(VINYL ALCOHOL) DENSE MEMBRANES

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Abstract - Our goal in this study was the immobilization of myoglobin in poly(vinyl alcohol) dense membranes. Glutaraldehyde was investigated both as the crosslinking agent, aiming to increase the membrane stability in aqueous medium, and as the vehicle to bind myoglobin and PVA. Reaction and membrane synthesis were carried simultaneously in mild operating conditions in order to maintain the native protein folding. Membrane characterization comprised the water swelling degree, DSC, TGA, UV-visible spectroscopy, FTIR analysis and oxygen transport in a dialysis cell. The incorporation of myoglobin in the film decreased the water swelling degree and improved the membrane thermal properties compared to unmodified PVA membrane. The reduction of ferric iron in the prosthetic group of the protein to the ferrous form was observed. The increased affinity between oxygen and the immobilized myoglobin did not favor the release of this solute from the biocarrier.
Keywords: Myoglobin; Oxygen/nitrogen separation; Poly(vinyl alcohol); Membranes; Immobilization.

INTRODUCTION

Oxygen is one of the most important commodities, being used in a broad range of applications such as the metallurgical industry. Moreover, ultra-pure oxygen is frequently needed in the medical field. The natural source of molecular oxygen is atmospheric air, where it corresponds to approximately 20% in volume. Air fractionation is attained by cryogenic technologies, such as distillation or pressure swing adsorption. These methods have none or very little expected advancement regarding process efficiency and energy consumption. The high energy demand of the traditional technologies has motivated the development of alternative routes to accomplish oxygen/nitrogen separation (Baker, 2002).

Gas separation through dense membranes emerged as an advantageous technique in 1980, when the synthesis of anisotropic membranes caused the hydrogen/nitrogen separation to be economical and conquered industrial acceptance (Baker, 2002). The high selectivity and low energy input of such technology decreased the operating costs. Since then, attempts to develop membranes with higher selectivity and flux are the main task to fulfill economical and technical requirements (Robeson, 2008).

Concerning air fractionation through membranes, the production of pure nitrogen is easier than that of

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oxygen-enriched streams, due to the fact that the feed is diluted in the latter and it usually permeates preferentially (Baker, 2004). Therefore, the development of membranes with better selectivity to molecular oxygen is pursued by means of altering the packing density of the polymer, inducing molecular sieving (Wang *et al*., 2005), or the modification of the polymer top layer by plasma (Ruaan *et al*., 1998). The literature reported an increase in the oxygen selectivity over nitrogen up to 10, followed by a reduction in permeate flux. Despite being the most studied gas pair, oxygen/nitrogen separation has shown only minor displacement of the upper bound limit, defined as the line combining materials with the highest separation factor and the highest permeability (Robeson, 2008).

Facilitated transport membranes comprise an alternative to the mechanism of simple diffusion. The facilitation is attained with the addition of a specific carrier to the desired component. The carrier-mediated transport can be described in three steps: a) the reaction between the carrier and the solute at the feed/membrane interface, b) the diffusion of the complex through the membrane and c) the decoupling reaction at the membrane/permeate interface. Transport due to simple diffusion is also likely to occur. However, the facilitated mechanism is favored for low partial pressures of the solute, like oxygen in air. The increase of the partial pressure of the component may cause the saturation of the carrier sites (Baker, 2004, Ferraz *et al*., 2007).

Natural oxygen carriers, like hemoglobin, were evaluated in *ex-vivo* tests by means of supported liquid membranes in the early 1960s (Scholander, 1960). Hemoglobin-containing membranes exhibited a facilitation factor of 8, compared to pure water, and a selectivity oxygen/nitrogen of 14, but the autoxidation of the carrier revealed a lack of stability. The knowledge of the bioinorganic chemistry of the bonding of molecular oxygen and the central iron atom in the heme group led to the development of synthetic carriers, mimicking the prosthetic environment (Baker, 2002). The use of such carriers in supported liquid membranes reached the apex in 1985, when Roman and Baker (1985) patented a system with O_2/N_2 selectivity of 30 and oxygen permeability of 1,500 Barrer, operating at 25 °C for up to three months. However, the evaporation losses of solvent and carrier oxidation brought about the investigation of more stable configurations. For instance, Nishide and co-authors proposed the use of cobalt-based synthetic carriers immobilized in a polymeric matrix (Shoji *et al.*, 2008). Although high selectivity was achieved, up to 118, the flux of 2.6 Barrer and the membrane lifetime of 3 months were low (Nishide *et al*., 1998). The minimum ideal conditions presented by Figoli and co-workers for a competitive membrane-based process are O_2/N_2 selectivity of 20, permeate flux of 0.015 $m^3m^{-2}h^{-1}bar^{-1}$, with 1 stage operation, feed pressure lower than 10 bar and temperatures between 0 and 40 °C. The minimum lifetime expected for the carrier is 1 year (Figoli *et al*., 2001).

The market size of oxygen and the spectacular selectivity of facilitated transport membranes are responsible for maintaining the interest of researchers in this area, especially because of the impact that competitive cost oxygen-enriched air could cause in many niches (Baker, 2002). For instance, the development of the artificial gill, an apparatus to remove oxygen from water to air, is closely related to facilitated transport membranes (Nagase *et al*., 2003).

The investigation of the role of the globin on the stability of hemeproteins (Sugawara *et al*., 1995) and the development of Molecular Biology tools, which make feasible changes in the amino acid sequence (Hargrove *et al*., 1996), justify the regained interest in the use of these biocarriers in facilitated transport membranes. The main breakthrough in this area is the stabilization of the biomolecules inside the polymeric matrix, preserving their physiological function. Considering the importance and applicability of the hemeproteins, the goal of this work comprises the understanding of the behavior and stability of the stroma-free biocarriers, which can be useful in other areas, such as in biosensors (Vidal *et al*., 1999) and artificial blood (Eike and Palmer, 2004).

In this paper, we investigated the immobilization of myoglobin in poly(vinyl alcohol), PVA, aiming at the synthesis of facilitated transport membranes for oxygen permeation. Myoglobin, Mb, was selected because it has only one oxygen-binding site, whereas the choice of PVA was based on its hydrophilicity, biocompatibility and film-forming ability. Oxygen transport tests were performed in the liquid phase, by using a dialysis cell equipped with an oxygen sensor. Tests were performed in the liquid phase because the oxygen permeability through the semi-crystalline PVA is about 0.0019 Barrer (Mulder, 1996).

The choice of a hydrophilic polymer to evaluate the immobilization of myoglobin was based on the fact that the biological activity of the protein was evolved in an aqueous milieu. However, the high water swelling degree damages membrane integrity. Therefore, the simultaneous crosslinking of PVA in mild conditions was investigated in order to maintain the physiological role of the immobilized protein by using glutaraldehyde, GA.

EXPERIMENTAL

Materials

Poly(vinyl alcohol), PVA, (85-146 kDa, 99%), glutaraldehyde, GA, sol. 50 wt% and horse heart myoglobin (minimum 90%), Mb, were purchased from Aldrich® (Milwaukee, WI). All solutions were prepared using distilled water.

Poly(Vinyl Alcohol) Membranes

PVA was dissolved in distilled water at 100 °C. The solution was cooled to room temperature. Metmyoglobin (Fe^{3+}) was dissolved in distilled water at 25 °C. The solution was transferred to a flask containing PVA aqueous solution and stirred with a magnetic stirring bar at 50 rpm, for 3 minutes. GA solution was transferred to the system by using an automatic pipette. The casting solution was stirred for 1 minute, at 50 rpm. PVA content was fixed at 4 wt% and the final pH was 6.2 $\pm$ 0.1. The casting solution was poured into a Petri dish in order to prepare flat dense membranes by solvent evaporation. The system was dried at 40 °C for 15 hours. Reaction and membrane formation were conducted simultaneously.

The effects of myoglobin and glutaraldehyde contents on membrane properties were investigated by means of altering the protein and the aldehyde mass ratio to PVA, respectively. The GA/PVA mass ratio was set at 0.2 g/g, whereas the Mb/PVA ratio was varied from 0.2 to 1.9 g/g.

Membrane Characterization

Water swelling, UV-visible spectroscopy, TGA, DSC and FTIR were performed for the characterization of membrane properties. They were used to infer the crosslink density, myoglobin physiological form, thermal stability, glass transition temperature, melting enthalpy and the chemical structure of the films.

Water Swelling

Films were cut into strips of (2 x 2) cm^2 and immersed in distilled water for 48 hours, at 40 °C (Yeom and Lee, 1996). Then, the swollen size, l_w, was recorded and the strips were placed in a desiccator, at 25 °C, in vacuo, until constant weight, when the dried membrane size, l_d, was measured. The water swelling degree, R, was calculated by Equation (1):

$$R = \frac{(l_w - l_d) \times 100}{l_d} \quad (1)$$

UV-Visible Spectroscopy

Strips of (0.4 x 2) cm^2 of dense membranes were placed in the cuvette of an UV-visible spectrometer (UV mini 1240, Shimadzu, Tokyo, Japan) and the absorbance spectra were compared in order to determine the physiological form of myoglobin in the polymeric film. PVA films with no added-myoglobin were used as the reference.

There are three physiological forms of myoglobin: metmyoglobin, metmb (iron III), which is biologically inactive, deoxymyoglobin, deoxymb (iron II not bound to oxygen), and oxymyoglobin, oxymb (iron II bond to molecular oxygen). The color of each myoglobin form is different. For instance, the typical absorbance pattern of metmb (409 and 630 nm) differs significantly from oxymb (418, 543 and 581 nm) and this property was used to infer possible changes in the protein form during membrane synthesis.

Infrared Spectroscopy

The functional groups of the modified PVA were determined by FTIR analysis (1720X, PerkinElmer) of the membranes. Each sample was scanned 20 times with resolution of 2 cm^{-1}. The incorporation of myoglobin into the film was evaluated by means of the absorbance pattern typical of alpha-helical proteins.

Thermal Analysis

Differential scanning calorimetry, DSC, and thermogravimetric analysis, TGA, were performed to characterize the modification of the thermal properties of PVA due to crosslinking and bonding to myoglobin.

For TGA tests (TGA 7, PerkinElmer, Norwalk, CT), 8 mg of the sample were heated from 50 to 600 °C at 10 °C/min. The weight loss as a function of temperature was used to infer the chemical modifications in the polymer structure.

DSC (DSC7, PerkinElmer) curves were obtained by heating 15 mg of the films from 30 to 250 °C, at 10 °C /min, under nitrogen flow of 22.5 mL/min. Samples were cooled to 30 °C, at 200 °C /min, followed by a second heating step, from 30 to 250 °C, at 10 °C/min. The results were compared in order to infer the spatial organization of the PVA polymeric chains.

Membrane Performance

The permeability of oxygen in myoglobin-containing membranes was measured in liquid phase, by using a dialysis cell. The apparatus is presented in Figure 1. The membrane was placed between the feed and permeate, using two cellulose acetate microfiltration membranes (Millipore®, with pore diameter of 0.45 μm and 30% porosity) to increase the mechanical resistance.

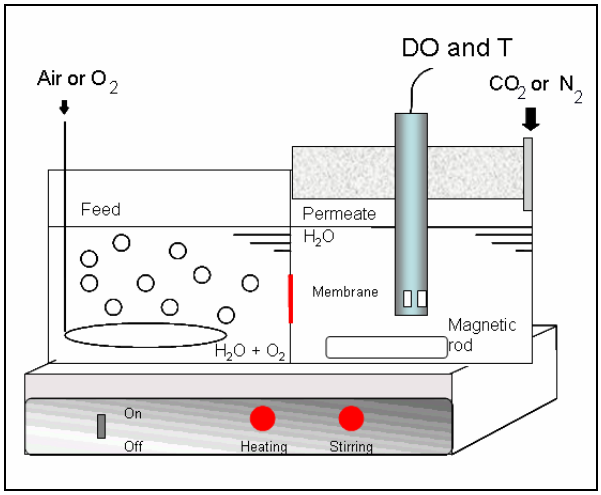


Figure 1: Scheme of the dialysis cell used in oxygen permeation tests. DO and T refers to the dissolved oxygen and temperature sensor.

Oxygen was supplied by bubbling compressed air or pure oxygen on the feed side. Permeate was deoxygenated using nitrogen or carbon dioxide prior to the experiment. A dissolved oxygen and temperature sensor (5739 DO, model 58, YSI, Yellow Springs, OH), DO and T in Figure 1, was used to quantify oxygen content on the permeate side. Measurements were taken for 30 minutes, in time intervals of 30 seconds. Tests were performed in triplicate, at 25 and 40 °C.

Oxygen Affinity

The affinity between the myoglobin–immobilized PVA membranes and oxygen was investigated by means of UV-visible spectroscopy. The method was developed in order to characterize any changes in the oxygen dissociation curve, compared to the non-immobilized myoglobin.

Strips of (2 x 0.4) cm^2 of the samples were placed in the cuvette of a UV-visible spectrometer. Distilled water was added to the system, which was closed to the atmosphere. The solution was bubbled with gas streams with different compositions (pure N_2, pure O_2 and O_2/N_2 mixtures of 5:95, 10:90 e 15:85 and 21:79 in weight) for at least 2 hours, until the equilibrium was reached. The system was sealed and the visible spectra of the strips were recorded. Absorbance at 543, 556 and 581 nm was used to determine the physiological form of the immobilized myoglobin.

RESULTS AND DISCUSSION

The immobilization of myoglobin in the PVA matrix was investigated by using GA, a bifunctional aldehyde, which is able to bind the protein and the polymer together. The mechanism of the reaction of aldehydes and hydroxyl groups is well known. However, the reaction between proteins and glutaraldehyde is non-specific (Migneault *et al*., 2004).

The main challenge was to perform the immobilization of the biomolecule in mild operating conditions so that the structure and biological activity of the carrier were maintained. This task was investigated by promoting the reaction in acid free medium and in the absence of organic solvent. The crosslinking of PVA in such conditions was accomplished in a former work (Figueiredo *et al*., 2009). The existence of an optimum GA/PVA ratio was shown for the reaction conducted at 40 °C. An excess of GA molecules can promote PVA branching and consequently increase the spacing among the polymeric chains. In the absence of metmyoglobin, the lowest water swelling degree of GA-modified PVA membranes was reached for a GA/PVA mass ratio of 0.01 g/g. Membrane swelling was (44 $\pm$ 5)% (Figueiredo *et al*., 2009).

In this work, membrane synthesis was conducted with metmyoglobin, the inert form of the protein, since the primary objective at this stage was the evaluation of the protein entrapment in PVA. The experimental procedure commonly used to accomplish iron reduction is based on the reaction of metmyoglobin and sodium dithionite, followed by gel filtration of the oxymyoglobin and a concentration step, using an ultracentrifuge. This protocol was avoided considering that the chemical behavior of the different physiological forms of myoglobin in the reaction with PVA and GA would be the same, regardless of the oxidation state of iron.

The addition of metmb to the membrane with GA/PVA mass ratio of 0.01 g/g did not bring about a significant change in the water swelling behavior. Water swelling degree was 48%. Moreover, the extraction of some protein to the aqueous phase indicated that the GA content was very low to promote

the entrapment of metmb into the polymeric chain. Therefore, the GA/PVA mass ratio was set at 0.2 g/g, while the myoglobin content related to PVA was investigated from 0.2 to 1.9 g/g. The results for water swelling tests of myoglobin–containing PVA membranes are presented in Table 1. Tests were made in triplicate in some experimental conditions and the deviation was calculated. Membrane average thickness was 30 μm.

Table 1: Water swelling degree (R) of the membranes at 25 °C. GA/PVA mass ratio was 0.2 g/g.

Mb/PVA mass ratio (g/g)	R (%)
0.2	Infinite
0.4	19
0.9	23 ± 1
1.4	15 ± 5
1.9	20 ± 2

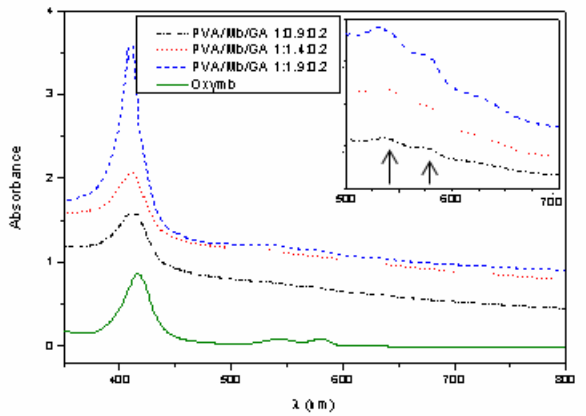
It was noted that the increase in GA content caused the major reduction in the water swelling, compared to the increase in Mb content. Membranes with a GA/PVA ratio of 0.2 g/g and Mb/PVA of 1.4 g/g showed the best result, with an average water swelling degree of 15%. For PVA/Mb/GA of 1:1.9:0.2, the water swelling degree was around 20%. This result can be explained in terms of the reaction between the hydroxyl groups of PVA and Mb with GA, which leads to crosslinking and reduces chain mobility. As a consequence, membrane swelling in water was decreased.

It was noted that the increase in Mb/PVA mass ratio from 0.2 to 0.4 g/g caused significant reductions in the water swelling degree of the membranes. In addition, no protein was extracted to the aqueous phase. Samples with Mb/PVA mass ratio higher than 1.9 showed phase separation and poor flexibility.

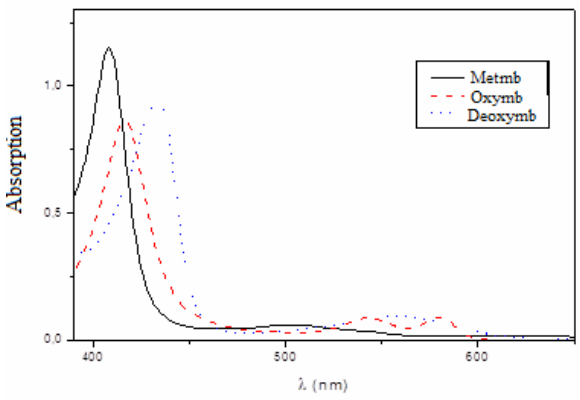
The change in water swelling degree and absence of protein extraction evidenced the reaction between Mb, GA and PVA. As a consequence, the tridimensional network formed by Mb-containing GA-crosslinked PVA membranes was changed. It probably decreased PVA free volume. The unmodified PVA membrane dissolves in water while Mb/GA/PVA samples showed an average water swelling degree, R, of 19%. It can be used to infer the crosslinking density of PVA, which changed because Mb and GA changed the spatial organization of the polymeric chains.

These results indicated that Mb can be attached to the PVA matrix by means of the reaction with GA in mild conditions such as pH of 6.2, 40 °C and solvent-free medium up to a Mb/PVA mass ratio of 1.9. The evidence of the reaction between PVA, GA and Mb is that the extraction of the protein from the membrane was not noted during water swelling tests.

UV-visible spectra of myoglobin-containing membranes were recorded for the investigation of the physiological form of the protein. The original color of metmyoglobin is brown. The red color of the samples suggested the reduction of iron during membrane synthesis. This color change is another evidence of the reaction between Mb, GA and PVA. The spectra are presented in Figure 2a, while the typical spectra of the physiological form of the protein are presented in Figure 2b. The sample without myoglobin was used as a reference (blank) as well as the aqueous solution of oxymyoglobin. The baseline displacement can be attributed to local fluctuations in membrane thickness.



(a)



(b)

Figure 2: (a)Visible spectra of Mb–immobilized GA-crosslinked PVA membranes and (b) Typical spectra of metmyoglobin, oxymyoglobin and deoxymyoglobin.

Upon analyzing Figure 2(a), one can easily recognize the absorbance pattern typical of myoglobin in the Soret region. Moreover, the peaks at 543 and 581 nm, highlighted in the detail of Figure 2, indi-

cated that the physiological form of myoglobin immobilized in the film was oxymb (Fe^{2+} — O_2), which is biologically active.

This surprising result led to the conclusion that the reaction mixture caused the reduction of iron (III) to iron (II), which simplified the procedure of membrane synthesis. The procedure to reduce metmyoglobin to oxymyoglobin comprises the protein reaction with sodium dithionite, followed by gel filtration and concentration by means of an ultracentrifuge. The advance of membrane preparation technique allowed the use of metmyoglobin to obtain oxymyoglobin in a quick and labor-saving way.

It should be pointed out that myoglobin showed the same UV-visible spectrum as the protein in aqueous solution (continuous line in Figure 2a), which evidenced that the microenvironment regarding to the electron configuration in the heme prosthetic group was maintained. In another words, the reaction between PVA, GA and Mb changed the iron oxidation state and the resulting UV-vis profile was typical of oxymyoglobin.

The chemical characterization of modified PVA membranes was performed by Fourier Transform Infrared analysis. The spectra for the commercial myoglobin (lyophilized sample in KBr) and PVA films are shown in Figure 3.

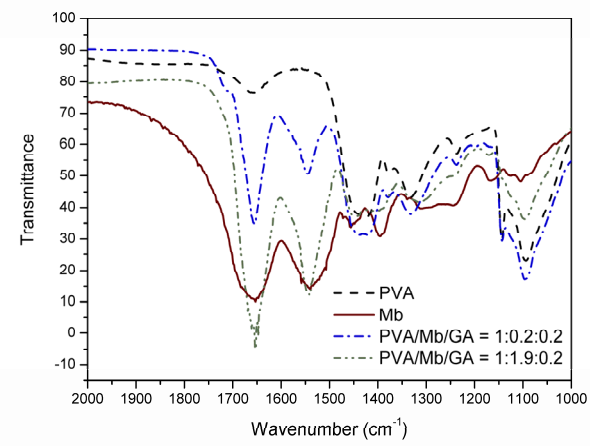


Figure 3: Infrared spectra of myoglobin–immobilized PVA membranes compared to lyophilized Mb and unmodified PVA sample.

For lyophilized metmyoglobin, the infrared spectrum showed two main peaks, which were attributed to amide I (from 1615 to 1700 cm^{-1}) and amide II (from 1650 to 1515 cm^{-1}) bands. In our case, the highest absorbance occurred at 1652 and 1544 cm^{-1}. Amide I is related to the C = O stretching vibration of the peptide linkage in the protein backbone. Amide II is due to the combination of N – H bending and C – N stretching (Wang *et al*., 2004). Regarding the unmodified PVA sample, a small band was noted around 1650 cm^{-1}, probably related to the C = O stretching of residual acetate groups in the 99% hydrolyzed PVA.

Both PVA films containing Mb and GA showed peaks at 1652 and 1544 cm^{-1}, typical of amide I and amide II bands. Altogether, these results suggested that the secondary structure of the protein was highly maintained in the film. According to the literature, alpha-helical proteins, like myoglobin, show the highest band around 1655 cm^{-1}, while beta-sheets shows two bands in the amide I region, at 1630 and 1690 cm^{-1} (Arkin, 2006). Moreover, if Mb was denaturated during the reaction and film synthesis, amide I and II bands would have diminished considerably (Wang *et al*., 2004).

It should be pointed out that the quantitative information about protein secondary structure from FTIR spectra has been studied (Goormaghtigh *et al.*, 2006). It could reveal how much of the protein native folding remained intact in the film. The native folding of a protein is the most stable thermodynamic state of the structure. Although it is a consensus that the native folding is greatly responsible to maintain the biological function of proteins, the idea that it should be 100% intact to allow its function is controversial (Guo and Clark, 2001).

The thermal properties of the membranes were analyzed by DSC and TGA. TG curves are presented in Figure 4. The unmodified PVA film showed three main stages of weight loss: the first one, up to 200 °C, devoted to the evaporation of volatile compounds, mainly water; the second stage, from 250 to 320 °C, usually associated with the polymer chain, as a result of the reaction between hydroxyl side groups, and the last one, higher than 420 °C, due to the breakage of the main chain. Sample PVA/GA (1:0.2) showed a slight increase in membrane thermal stability and the three stages of weight loss were less defined. This result suggested that the reaction between PVA and GA decreased the amount of hydroxyl groups involved in the polyene formation, leading to a decrease in weight loss.

Concerning the PVA/Mb/GA samples, an increase in membrane thermal stability was noted. The amount of volatile compounds was decreased and the other two weight loss stages were consecutive and smoother compared to unmodified PVA. This suggested that the addition of myoglobin to the system caused additional changes in PVA structure, which can be ascribed to the reaction between PVA, GA and Mb. Additionally, the increase in Mb content did

not modify the TGA profile, except by a decrease in content of volatile substances.

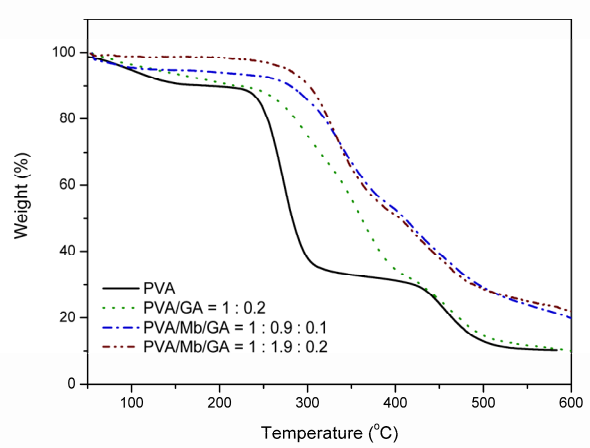


Figure 4: TG curves of myoglobin–immobilized PVA membranes.

The DSC second heating step curves are presented in Figure 5. The unmodified PVA membrane showed a glass transition temperature, T_g, of 60.5 °C, and a broad endothermic peak regarded to the polymer melting at 222 °C. These temperatures were lower than PVA grains probably due to the plasticization effect of water. For GA-crosslinked PVA membrane (PVA/GA = 1:0.2 g/g), T_g could not be determined and the melting event due to the crystalline domain was lower than the unmodified PVA membrane. It can be explained in terms of the change in spatial organization of PVA chains due to the reaction with GA.

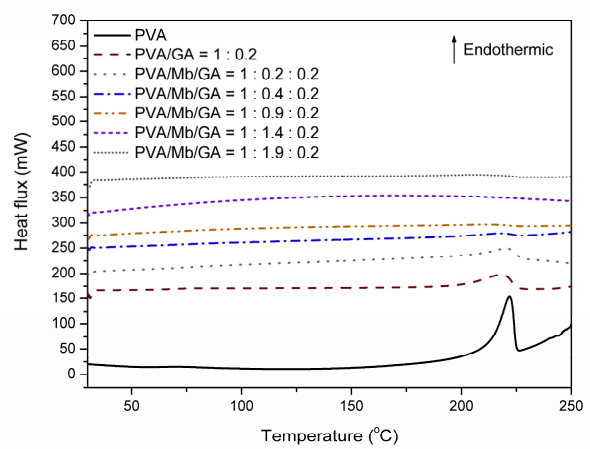


Figure 5: DSC curves of myoglobin–immobilized PVA membranes.

T_g values could not be determined for Mb-containing samples, probably due to the morphological changes caused by the reaction between PVA, GA and Mb. It was noted that the increase in myoglobin content decreased the melting enthalpy from unmodified PVA to Mb/PVA of 1.9, in which the crystallinity is not detectable anymore. This result can be reasoned in terms of the crosslinking reaction between Mb, PVA and GA. The reduction in chain mobility prevented the formation of the crystalline domain typical of unmodified PVA. As the result, the crystalline lattice of PVA could not be formed and the enthalpy for melting the film was diminished.

The thermal properties of myoglobin-containing PVA membranes showed different behavior compared to unmodified PVA. An increase could be noted in the thermal resistance and a decrease in crystallinity. The consumption of PVA hydroxyl groups increased the amorphous domain of the membranes, as mentioned before. These morphological and structural changes represented an increase in the effective area for permeation, which can lead to higher fluxes.

The oxygen transport through the membranes was investigated by means of a dialysis cell, equipped with a dissolved oxygen sensor. The results for the tests conducted at 25 °C are presented in Figure 6. Tests were conducted in triplicate. The deviation was lower than 10%.

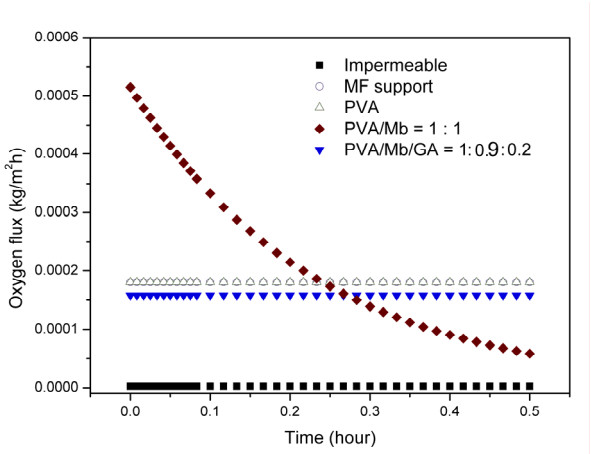


Figure 6: Oxygen transport through different membranes in the aqueous phase at 25 °C.

The "impermeable" membrane refers to an aluminum plate placed between the feed and the permeate compartments. This test was performed for the evaluation of the method, provided that no oxygen from the permeate atmosphere could influence the result. The low oxygen content during the tests confirmed the insulation of permeate.

The lack of mechanical resistance of the 30 μm-thick membrane required the use of two microfiltra-

tion membranes (Millipore, average pore diameter of 0.45 μm). The "MF support" test corresponds to two membranes without any dense film between them.

For the unmodified PVA membrane, the oxygen flux through the membrane was the same as the MF support, 3 x 10^{-7} kg/hm^2, denoting that the high water swelling degree of the polymer caused no additional resistance to mass transfer, as expected.

The oxygen flux through the myoglobin-immobilized membrane was lower than the unmodified PVA, showing no facilitation. It could be inferred that the permeation of oxygen occurred by simple diffusion through the crosslinked polymeric chains. The crosslinking of PVA increased the corresponding diffusion path, and, consequently, decreased the total flux.

Also shown in Figure 6 is the oxygen flux through an oxymb-containing PVA membrane prepared without the addition of GA. The initial flux is about three times higher than the unmodified PVA membrane, showing the facilitated transport of O_2. However, as the biocarrier was not chemically bound to the polymeric matrix, it was extracted to the aqueous medium, evidenced by the color change of the solution. As a consequence, the oxygen flux decreased with time. This result showed the ability of the myoglobin to transport oxygen in the PVA membrane, although the poor stability of such system did not allow the commercial use of the membrane. Moreover, the increase in the facilitation factor for oxygen also confirmed the validity of the dialysis method to evaluate the oxygen transport. It was assumed that the mass ratio Mb/PVA of 0.9 g/g is higher than the percolation threshold of the system and did not affect the transport rate.

The absence of facilitated transport for the myoglobin-immobilized PVA membrane can be explained in terms of the reaction of the protein with the GA molecule. Although the alpha-helical structure seemed to be maintained, the oxygen affinity was increased, which did not allow the release of oxygen on the permeate side. This result can be reasoned in terms of the fact that the reaction of the macromolecule is transmitted along the atoms and could affect the heme microenvironment. It is known that even a small displacement in the atoms at the heme pocket can cause significant changes in Mb-O_2 affinity.

It is worth noting that the increase in the affinity of oxygen to the biocarriers has been reported on the literature. For instance, Buehler *et al*. (2005) also observed the displacement of the oxygen dissociation curve to the left as the result of hemoglobin oligomerization with glutaraldehyde in the synthesis of the artificial blood, HBOC, hemoglobin-based oxygen carriers. It is quite reasonable to extend the same behavior to the reaction of myoglobin and PVA crosslinked by GA. Besides that, the immobilization of the biocarriers in a polymeric (solid) matrix can also avoid the nanometric displacement of the iron atom to the porphyrin plane of the heme environment during the oxygenation of the biocarrier. This could be the cause of the loss on the reversibility between the carrier and the solute.

In addition, Bonaventura and Bonaventura (1982) reported the synthesis of an artificial gill based on the immobilization of hemoglobin in hydrophilized polyurethane membranes. The unloading step was quite difficult, due to the high affinity between the immobilized hemoglobin and oxygen.

In order to evaluate the oxygen dissociation curve from immobilized myoglobin, UV-visible spectra were recorded after the exposition of the PVA/Mb/GA membrane 1:0.9:0.2 to oxygen/nitrogen mixtures with varying contents. The results are presented in Figure 7. The appearance of deoxymyoglobin (peak at 556 nm) and the decrease of the absorbance typical of oxymb (543 and 581 nm) with the reduction of oxygen partial pressure in the feed gas was expected. The expected behavior is shown by the arrows in the figure.

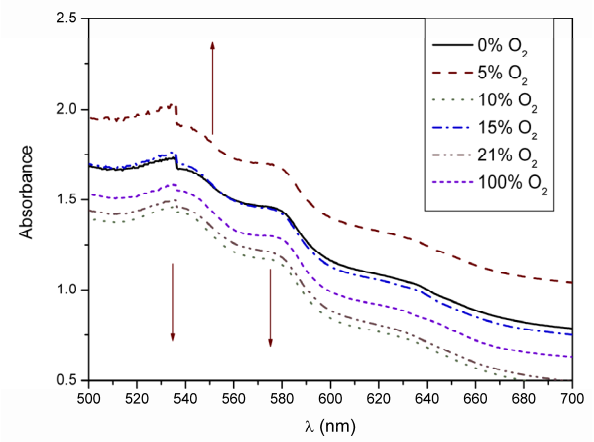


Figure 7: Visible spectra of a PVA/Mb/GA 1:0.9: 0.2 membrane submitted to oxygen-depleted air.

The spectra showed no change in myoglobin physiological form with the decrease in the oxygen content. This is the evidence that the reversibility of the reaction was lost during the chemical bonding of the biomolecule on PVA.

The effects of a pH difference between feed (pH 6) and permeate (pH 5) by bubbling carbon dioxide in the latter, as well as the increase in the operating temperature of permeation tests to 40 °C, were inves-

tigated for the release of oxygen from oxymyoglobin. However, these variables did not cause an increase in the oxygen transport, confirming the loss on the reversibility of the myoglobin and oxygen reaction. New membranes were prepared by using previously reduced myoglobin but no facilitation was observed.

Hemoglobin (minimum 95%, Fluka) was investigated as an alternative to myoglobin in the membranes due to the lower affinity to oxygen. The optimized mass ratio between PVA, hemoglobin and glutaraldehyde was 1:0.5:0.04, due to the lower solubility of hemoglobin in water compared to Mb. The reaction between the protein and GA was faster than myoglobin and GA. The formation of clusters of the hemoglobin in the Petri dish required the filtration of the casting solution before it was poured into the plate. No iron reduction was observed during membrane synthesis. Homogeneous films were produced at the optimum condition, with no extraction of the protein to the aqueous medium in water swelling tests. However, no facilitation of the oxygen transport occurred. The effects of pH and operating temperature did not bring about changes in the oxygen content profile. Altogether, these results showed that the immobilization of oxygen biocarriers in a PVA matrix can damage irreversibly the prosthetic environment, which decreased the oxygen flux through the membrane.

CONCLUSION

Myoglobin was successfully immobilized in poly(vinyl alcohol) by means of the reaction with glutaraldehyde, in the absence of organic solvents and acid catalysts. The water swelling degree of the films was decreased, which indicated that the crosslinking reaction between PVA and Mb with glutaraldehyde was effective. Regarding the thermal properties, the reaction caused a decrease in the crystalline domains and increased the thermal resistance.

Metmyoglobin was reduced during the reaction. The characterization of the chemical groups in the films showed that the amide I and II bands typical of the protein were kept, denoting high maintenance of the helical conformation of the protein inside the membrane. The increased affinity between the immobilized myoglobin and oxygen caused no decoupling of the solute at the membrane/permeate interface.

A facilitation factor of 3 was observed for non-immobilized myoglobin in PVA, confirming the oxygen transport ability of the protein. The use of hemoglobin as the biocarrier also revealed the lack of reversibility of the reaction of the prosthetic group and oxygen.

Even though there are evidences that the reaction between the biocarriers and GA is far away from the hydrophobic pocket of the heme group, the information seems to be transmitted along the macromolecule and the mechanism must be studied to allow the development of biocarrier-based facilitated transport membranes for oxygen separation.

NOMENCLATURE

PVA	poly(vinyl alcohol)
GA	glutaraldehyde
Mb	myoglobin
DSC	differential scanning calorimetry
TGA	thermogravimetric analysis
FTIR	Fourier Transform Infrared spectroscopy
Metmb	metmyoglobin, Fe^{3+}, physiologically inactive
Oxymb	oxymyoglobin, Fe^{2+} bound to oxygen
Deoxymb	deoxymyoglobin, Fe^{2+} not bound to oxygen
l_w	swollen membrane size
l_d	dried membrane size
R	Water swelling degree

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