POLYVINYLALCOHOL–COLLAGEN MEMBRANES FOR ENZYME IMMobilIZATION *

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SUMMARY

PVA–collagen membranes were prepared by co-crosslinking a mixture of PVA–collagen with epichlorohydrin, to obtain membranes 35 μm thick (dried). Best results were recorded with a composition of 6% PVA and 6% collagen.

CM and AE derivatives were obtained by treating the PVA–collagen membranes with monochloroacetic acid and chloroethylamine, respectively. The CM–PVA–collagen membranes were used for enzyme immobilization by carbodiimide, while PVA–collagen and AE–PVA–collagen were used for the carbodiimide or glutaraldehyde procedures. The immobilizations were performed under identical conditions using the GOD enzyme. The different immobilized GOD membranes were applied on amperometric oxygen electrodes and the decrease of the oxygen concentration in various glucose samples was measured. Best results — a relatively short response time (2–3 min) with a response rate of 80% of the saturation value per minute (80%/min) for a 0.1 M glucose solution — were obtained in the case of AE–PVA–collagen membranes activated with glutaraldehyde. A linear calibration curve was recorded with different standard solutions by multiple additions and flow injection techniques. The procedure for preparing this new type of membrane is easy to perform and the preliminary results indicate some attractive perspectives for its use in various fields including clinical analysis.

ADDITIONAL ACRONYMS USED IN THIS PAPER

AE– aminoethyl–
CL– epichlorohydrin–
CM– carboxymethyl–
EDC 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide
GOD glucoseoxidase

INTRODUCTION

Among other polymeric materials (e.g. agarose [1], cellulose [2], nylon ([3,4], polyacrylamide [5], etc.) both collagen [6,7] and polyvinylalcohol [8,9], were largely used as supports (bead, granular or membrane forms) for enzyme immobilizations.

In this paper, a new type of polymeric material, based on a PVA–collagen compound, is proposed as a macromolecular support for enzyme immobilization.

In a previous note [10], some physical and chemical properties of PVA cross-linked with epichlorohydrin (CL–PVA) were described. The excellent mechanical properties found [11] for CL–PVA beads (osmotic shock ranging between 86 and 91%) were ascribed to high interchain hydrogen associations. The intensities of these associations are higher in case of reduced reticulation degrees and lower in case of an increased cross-linking, because the interchain glycerine bridges (8.6 Å) are longer than the interchain hydrogen bonds (5.7 Å).

These suppositions are strongly supported by I.R. and X-ray data [11].

The reason for introducing the new type of material (PVA–collagen compound) for enzyme immobilization is to join the high reactivity of collagen with the mechanical properties of PVA.

It was possible to obtain this type of compound because the two materials, PVA and collagen, when mixed, are highly compatible in a large concentration range up to their maximal solubility limits [12]. The immobilized enzyme realized with this new procedure can be exploited in different biotechnological applications: in this paper the preparation of some glucose sensors by coupling the membrane with an oxygen or hydrogen peroxide probe is described. This coupling gives very useful applications in clinical analysis and in biochemical studies.

EXPERIMENTAL

Materials

Both low (20000) molecular weight (LMW-PVA) and high (100000) molecular weight (HMW-PVA) soluble collagen (Serva, MW 20000) and A. niger crystalline GOD (Sigma, 125 units/mg) were used.

Collagen membranes were obtained from Yellow Spring Instrument Co., Inc., OH, USA.

Membrane preparation

Taking into account the limited solubility of both PVA and collagen, some preliminary experiments were performed using different concentrations of HMW-
PVA (up to 12%), LMW-PVA (up to 24%), collagen (up to 12%) and sodium hydroxide (up to 5 \text{ M}), in various ratios PVA/collagen/NaOH, in order to establish the optimal composition of the PVA–collagen compound for our aims. Best results were obtained with a composition of 6% HMW-PVA, 6% collagen and 3 \text{ M} NaOH solution. PVA was dissolved by stirring in ethanol (a volume of ca. 50% of the total volume was calculated for the mixture), then an equal volume of water and the calculated amount of collagen were added. Thereafter the ethanol was eliminated by moderate heating. The proper amount of solid NaOH (to obtain a 3 \text{ M} solution) and water to obtain the required volume) should be added just before the reticulation step (prolonged contact of the collagen with sodium hydroxide should be avoided in order to prevent hydrolysis). In preliminary experiments, various ratios PVA–collagen/crosslinking reagent, were assayed. The Cl. concentration in the reaction medium was in the range 2.5%–10% (V/V). We also tried to reticulate the wet membranes by tanning, first with the NaOH solution and then with CL, but no satisfactory results were obtained for HMW-PVA–collagen (from the point of view of mechanical properties); better results were obtained when LMW–PVA was used. However, with the tanning system, some difficulties occur concerning the non-uniform structure of products and due to the fact that it is difficult to appreciate exactly the ratio PVA–collagen/CL. Best results were obtained in the case of reticulation in solution when the epichlorohydrin was added to the PVA–collagen–NaOH solution, under stirring and moderate heating, so as to obtain a concentration of 5% epichlorohydrin (V/V), in solution. The reaction medium (still liquid) should be poured on delimited horizontal glass (e.g. Petri dishes) surfaces to obtain a 0.9 mm thick liquid layer, which should be kept immediately overnight (18 h) for cross-linking.

After washing with large volumes of water, the membranes were dried (for another 10–18 h) on glass surfaces, to obtain dried membranes of PVA–collagen–CL (cross-linked) 35 \text{ \mu m} thick. As a proof for cross-linking, the membranes should remain insoluble in water, at 100^\circ\text{C}.

\textit{Derivation of PVA–collagen–CL membranes}

Wet membranes, obtained by previously described procedures (10 cm diameter), were treated (two membranes in a Petri dish) with 10 cm\textsuperscript{3} of 10 \text{ M} NaOH solution and with various amounts (ranging between 2.5 and 10 g) of 1-chloro-2-ethylamine (chlorhydrate) or monochloroacetic acid, respectively (dissolved in minimal volumes of water), for 18 h, adding occasionally some drops of 10 \text{ M} NaOH to maintain a pH > 9. The obtained AE– and CM–PVA–collagen–CL membranes were thoroughly washed with water and dried.

\textit{Enzyme immobilization}

The carbodiimide procedure was used in the case of both CM– and AE–PVA–collagen–CL; each membrane (2 cm diameter) was treated for 24 h with
2.5 cm³ of a solution of 0.1 M EDC (chlorhydrate) (Merck) in 0.1 M monosodium phosphate and 4 mg of GOD, under moderate stirring. Otherwise, membranes of the CM- and AE-type were treated for 1–3 h with the 0.1 M EDC solution, washed with 0.1 M phosphate buffer, pH 7, and then each wet membrane was treated for 24 h with 4 mg of liophilized GOD (uniformly distributed on the central area of these membranes).

The glutaraldehyde procedure was used with both PVA–collagen–CL (non-derivatized) and AE–PVA–collagen–CL derivative. The membranes, washed with 0.1 M borate buffer, pH 8.5, were each treated for 1 h with 10 cm³ of a 12.5% solution of glutaraldehyde (in the same borate buffer solution), at room temperature and then washed with 0.1 M phosphate buffer, pH 7.5; each wet membrane was treated for 24 h with 4 mg of liophilized GOD as in the case of the reaction with EDC, for immobilization.

In all cases, after the immobilization, the membranes were washed with water, and then with a solution of 1 M NaCl in 0.1 M phosphate buffer, pH 7, until no absorbance was observed at 280 nm in the supernatant washing solutions. Thus, the non-covalently bound enzyme (generating eventual artifacts) was eliminated from the membrane surfaces.

**Analytical procedures**

The immobilized GOD on the new membranes was used in various systems for the measurement of glucose concentration in standard solutions by recording the oxygen decrease or the hydrogen peroxide formation according to the following scheme:

\[
\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic} + H_2O_2
\]

The oxygen consumption was recorded with an oxygen probe (Orion Res. 97-08), while the formation of hydrogen peroxide was measured with a platinum electrode polarized at +600 mV versus Ag|AgCl (hydrogen peroxide probe).

The functional parameters of the new membranes were determined, through a calibration curve or using a multiple-addition technique of the same sample, with both oxygen and hydrogen peroxide sensors.

As the response of the Orion oxygen probe is expressed in conventional pH units, we report the results as a percentage of the oxygen saturation value. Accordingly, the response rate is expressed as the variation of the percentage of oxygen saturation per min.

As a flow-analysis system, an Instrumentation Laboratory oxygen probe and a flow cell were used (IL 213). The results are expressed in the same units (% of saturation value).
In all cases, the membranes with immobilized GOD were fixed with O-rings on the external electrode surface, with or without another dialysis (cellulose acetate) or nylon-net membrane.

The efficiency of enzyme immobilization on the obtained membranes was evaluated by comparing the apparent retained activity with the activity of the same amount of enzyme in solution; i.e. by comparing the oxygen decrease recorded with the glucose sensor (GOD membrane fixed on the oxygen probe) with the oxygen decrease recorded with an oxygen probe and the same amount of enzyme (4 mg) dissolved in 10 cm³ of 0.1 M glucose solution. The measured retained activities are considered apparent because diffusional phenomena through the membranes, affect the assays. However, it can be considered a relative measurement useful for comparison of the several kinds of immobilized enzyme membranes obtained.

RESULTS AND DISCUSSIONS

In this paper, aspects concerning enzyme immobilization and electrochemical behaviour of our membranes are described, while aspects concerning the physical and chemical properties, will be dealt with in a following paper [12]. However, it is worth mentioning that the CL concentration is an important factor for the mechanical properties of PVA-collagen membranes. When concentrations lower than 5% were used, no cross-linking was found, while for higher concentrations the membranes, even cross-linked ones, exhibit poor mechanical properties (fragile, non-elastic).

This fact was ascribed to different intensities of the PVA interchain hydrogen associations and is in good agreement with our previous data [11]; when a higher concentration of epichlorohydrin is used, the density of interchain glycerine bridges (8.6 Å) is higher and the arrangement of interchain hydrogen associations (5.7 Å) is probably hindered. The same fact was observed when higher concentrations of collagen were used in the PVA-collagen composition. Probably, in this case the PVA interchain hydrogen associations are hindered by the excess of collagen in the gel network (experiments concerning these aspects are under way).

Some preliminary experiments were carried out in order to establish if, and to what extent, the access of the oxygen to the electrode is affected by our PVA-collagen membranes (without immobilized enzyme) and by other additional protective (dialysis or nylon) membranes. As shown in Fig. 1, no major modifications in permeability were observed in the case of PVA-collagen or both PVA-collagen and dialysis membranes. The same results were obtained with the 0.2 M sodium sulphite solutions, giving a similar oxygen signal decrease when recorded with and without additional membranes of PVA-collagen.

Screening the activities of immobilized GOD by different techniques on various membranes indicates that the best results (fig. 2) were obtained with AE-PVA-collagen-CL (apparent retained activity 14.1%) and PVA-collagen-CL (retained activity 10.3%) when glutaraldehyde was used for immobilization. The higher activity obtained with AE-derivative is due to the higher number of amino groups available.
Fig. 1. Permeability of oxygen through different membranes not loaded with GOD. The solution was 10 cm$^3$ of 0.1 $M$ glucose and at the time indicated by the arrow 2 mg of GOD were introduced into the stirred solution. (1) Oxygen probe without membranes; (2) oxygen probe with an AE–PVA–collagen–CL; (3) oxygen probe with an AE–PVA–collagen–CL and a dialysis membrane.

However, it is worth mentioning that in both these cases the retained activities are higher than when commercial collagen membranes are used in similar immobilization and assay procedures (retained activity 8.9%).

The enzymes are immobilized asymmetrically on all membranes obtained (the immobilization procedure is carried out only at one single face) and it was inter-

Fig. 2. Response time of a glucose sensor assembled with different membranes with immobilized GOD in 10 cm$^3$ of 0.1 $M$ glucose standard solution. (1) AE–PVA–collagen–CL; (2) PVA–collagen–CL; (3) collagen (YSI).
Fig. 3. Response time of a glucose sensor in 10 mM standard solution in the range 1–100 mM. Figures give the concentration in mM. GOD immobilized on an AE–PVA–collagen–CL membrane.

esting to evaluate the activity when the membranes were fixed with the side with immobilized enzyme facing the electrode surface: for commercial collagen membrane, the activity measured decreased by 53%, while for AE–PVA–collagen–CL, the decrease was only 35%. This advantageous behaviour is probably due to a higher permeability of substrate through the AE–PVA–collagen–CL membranes.

The retained activity values for GOD immobilized on the various membranes mentioned via the carbodiimide procedure were lower (mainly for CM–PVA–collagen–CL membranes), an intramembrane reaction between the introduced carboxymethyl group and the amino groups of collagen probably being the reason for the reduced activities. Therefore, all measurements reported were carried out with the GOD immobilized on the AE–PVA–collagen–CL membranes. A good dependence of the electrode response at various glucose standard solutions was observed either when the sensor was immersed in different buffer standard solutions (Fig. 3) or when multiple additions were performed with the sensor dipped in a buffer solution (Fig. 4). This dependence is linear up to 10 mM glucose (Fig. 5). The detection limit of the oxygen electrode with GOD immobilized on an AE–PVA–collagen–CL membrane is 0.2 mM; it is comparable with the values obtained with other enzyme sensors. This limit depends mainly on the oxygen probe and on the thickness of the membrane with immobilized enzyme.

The short response time (2 min and 80%/min for the 0.1 M glucose solution) and the time needed to recover the base line permits its use in estimating clinical samples.

The new membranes can also be used in connection with an amperometric hydrogen peroxide sensor; this system also shows a linear dependence of measured current (pA) on the concentration of glucose (in a wide range of concentrations), the membrane being permeable to hydrogen peroxide.
Fig. 4. Multiple additions of a 0.2 \textit{M} standard solution in 10 cm$^3$ phosphate buffer, pH 7.0, recorded with a glucose sensor with a GOD-loaded AE–PVA–collagen–CL membrane.

Fig. 5. Calibration curve of a glucose probe with an AE–PVA–collagen–CL membrane.

Fig. 6. Flow injection analysis of 0.3 cm$^3$ of 0.1 \textit{M} glucose standard solution. Flow rate 2 cm$^3$/min. Buffer phosphate 0.1 \textit{M}, pH 7.0. GOD immobilized on an AE–PVA–collagen–CL membrane.
The immobilized GOD on AE–PVA–collagen–Cl membranes was also used in a flow-injection analysis system (with an oxygen electrode IL-213), and a high reproducibility was established when identical amounts of glucose were assayed (30 μmoles, i.e. 0.3 cm³ of 0.1 M) (Fig. 6).

The analytical results (especially in terms of analysis time) can be improved substantially by decreasing the thickness of the membrane; the flexibility of the composition and of the procedure permit in fact to reduce the thickness to a few micrometers without affecting the mechanical properties; experiments on this line are under way.

Finally, the lifetime of the immobilized enzyme on AE–PVA–collagen–CL is quite appealing. After two months of operation the decrease of activity was only 20%.

CONCLUSIONS

The proposed membrane type has a mechanical stability higher than that of a collagen membrane; it maintains the necessary chemical reactivity for binding enzymes through a derivation procedure. The thickness of the membrane can be varied easily and with it the response rate of the system.

Enzyme electrodes can easily be assembled by fixing the membrane on an oxygen or hydrogen peroxide probe.

From the results obtained, it is possible to conclude that this new type of membrane exhibits some features that justify further interest in and development of GOD probes for clinical analysis and related fields.

REFERENCES