ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 7 (1999) 85–91

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Influence of polymerization parameters and entrapment in poly(hydroxyethyl methacrylate) on activity and stability of GOD

Birgit Schulz [a,*], Anne Riedel [b], Peter U. Abel [a,c]

[a] *Sensor Research Center Greifswald, Brandteichstrasse 19, 17489 Greifswald, Germany*
[b] *Institute of Pharmacy, University of Greifswald, Greifswald, Germany*
[c] *Institute of Pathophysiology, University of Greifswald, Greifswald, Germany*

Abstract

The functional stability of biosensors used in vivo seems, most probably, to be limited by enzyme instability. Therefore, investigations have been carried out on enzyme stabilization by immobilization using poly(hydroxyethyl methacrylate) (pHEMA). On one hand, there are several factors connected to polymerization of the monomer hydroxyethyl methacrylate (HEMA) able to influence the activity of enzymes during their entrapment in the polymer. The factors we investigated are: the concentration of the monomer, different temperatures (50–65°C), ultraviolet radiation, and the concentration of radicals generated by different initiator concentrations. Glucose oxidase (GOD) was used as a model enzyme. It was found that the most important GOD inactivating factors are the monomer itself and temperatures higher than 50°C. On the other hand, despite of these influences a long term stable immobilization of GOD by entrapment in pHEMA could be realized.

Keywords: Immobilization; Glucose oxidase; Entrapment in pHEMA; Biosensor; Stability

1. Introduction

Since sensor alterations cannot be compensated by cleaning and recalibration during in vivo residence, one of the main prerequisites for the functional stability of biosensors is the stable immobilization of the biological component, i.e., the enzyme. This means that on the one hand, the activity of the immobilized enzyme is hardly changed over a certain period and is high enough to realize a diffusion controlled biochemical reaction. On the other hand, it means that the enzyme is tightly fixed at the electrode surface during the whole time of sensor application.

Poly(hydroxyethyl methacrylate) (pHEMA) is a hydrophilic polymer with good affinity to metallic surfaces like platinum. It is described as a well biocompatible material [1]. The properties of pHEMA can be modified in a wide range, e.g., by copolymerization or additives, to adapt it to the application conditions of the sensor [2,3]. The immobilization of an enzyme by means of pHEMA can be realized by physical entrapment during polymerization [4–6] or by covalent coupling to the chemically activated polymer surface [7–9]. The properties of

* Corresponding author. Tel.: +49-3834-550447; Fax: +49-3834-550449

1381-1177/99/$ - see front matter
PII: S1381-1177(99)00034-X

pHEMA, especially its hydrophilic character and biocompatibility, are also suitable prerequisites for the use of the polymer as a carrier for immobilized enzymes in biosensors for in vivo application.

In this study glucose oxidase (GOD) was used as a model enzyme. As a first step, the influence of some polymerization parameters on the activity of the enzyme was examined to differentiate the influence of the reagents in the polymerization mixture (monomer concentration, kind of solvent) from that of the process conditions (ultraviolet radiation, temperature, concentration of radicals generated from the initiator). In the second step, the long term stability of GOD activity immobilized on platinum sticks used as model electrodes and stored in buffer solution at room temperature has been determined.

2. Experimental

2.1. Reagents

Glucose oxidase (GOD, EC 3.1.3.4, type II, 346 U/mg at 37°C, from *Aspergillus niger*) was purchased from Boehringer Mannheim, Germany. Hydroxyethyl methacrylate (HEMA) and *N,N′*-azobisisobutyronitrile (AIBN) were obtained from Fluka Chemie, Buchs, Switzerland. Peroxidase (POD, EC 1.11.1.7, type II, 150–200 U/mg, from horseradish) and tablets of *o*-dianisidine dihydrochloride were supplied from Sigma–Aldrich, Deisenhofen, Germany. The mostly used imidazole buffer consisted of 3.41 g imidazole (Boehringer, Ingelheim, Germany), 3.42 g Titriplex® (Merck, Darmstadt, Germany), 1.0 g sodium benzoate (Sigma–Aldrich), 6.0 g sodium chloride (Fluka Chemie, Buchs, Switzerland) and 20.6 ml 1 N hydrochloric acid (Merck) in 1000 ml distilled water. A pH of 7.0 was adjusted potentiometrically. All substances used were of analytical purity.

2.2. Determination of GOD activity

The activity of GOD was determined photometrically using POD and *o*-dianisdine in a discontinuous mode at 37°C. The reaction was interrupted by 2 M or 9 M sulphuric acid (H_2SO_4), respectively, after exactly 5 min. The absorption of the coloured reaction product was measured at 436 or 530 nm (spectrophotometer DU 640, Beckman, München), respectively. 2 M H_2SO_4 and 436 nm were used for an enzyme activity of 0.5 to 10 U/ml, 9 M H_2SO_4 and 530 nm for 0.03 to 1.5 U/ml. A correlation of the GOD activities measured with both variants of the same method exists. For both methodical variants, a blank was determined containing all reagents except for GOD, respectively.

The activity of GOD in the immobilized state was determined by dipping the electrode with immobilized enzyme into the mixture of *o*-dianisidine and glucose for exactly 5 min. After removal of the electrode POD was added and allowed to react for 2 min with hydrogen peroxide generated in the former step. The reaction was interrupted by sulphuric acid.

2.3. Determination of the influence of HEMA concentration on GOD activity

Mixtures of 20, 40, 60, 80, and 90% HEMA with GOD dissolved in imidazole buffer or distilled water were prepared. After an incubation time of 30 min as well as 24 h at room temperature, samples for activity determination were taken. The blank contained all reagents except for GOD and the corresponding HEMA concentrations.

2.4. Determination of the influence of ultraviolet radiation on GOD activity

Solutions of GOD in imidazole buffer or water (0.04 mg/ml) were exposed to ultraviolet radiation at 365 nm (UVK 125, Narva, Berlin, Germany) in a distance of 4.5 cm from the radiator. The solutions were water cooled and

stirred. After irradiation times of 0.5, 1, 2, 3 and 4 h, samples for activity determination were taken.

2.5. Determination of the influence of temperature on GOD activity

To examine the polymerization heat dependent on the water content in the reaction mixture, various well defined volumes of water were added to a constant volume of HEMA with initiator (2 mg AIBN/ml HEMA) resulting in water contents of 0 to 70%. The polymerization was started at a temperature of 80°C. To test the stability of GOD in solutions of imidazole buffer or distilled water (0.04 mg/ml), they were heated to 65, 60 and 50°C. The periods of sampling were dependent on the enzyme stability expected and reached from a few minutes up to 4 h.

2.6. Determination of the influence of initiator / radical concentration on GOD activity

Mixtures of HEMA containing 2, 4, 6 or 9 mg AIBN/ml and GOD solutions (imidazole buffer) were exposed to ultraviolet radiation at 365 nm for 10 min. The mixtures were water cooled and stirred. The activity in the samples was measured compared to the analogous treated mixtures without AIBN.

2.7. Immobilization of GOD in pHEMA

Mixtures of HEMA containing AIBN (6.0 or 12.0 mg/ml HEMA) and solutions of GOD in imidazole buffer were made to realize a monomer concentration of 40% and an enzyme concentration of 1.5 mg/ml reaction mixture. The mixtures were polymerized at 365 nm over approximately 10 min. Model platinum sticks were dip-coated and the polymer was hardened completely. Platinum sticks loaded with GOD entrapped in pHEMA were stored in imidazole buffer at room temperature for stability tests.

3. Results

3.1. The effect of HEMA concentration on GOD activity

A 50% higher activity of GOD dissolved in imidazole buffer was found in contrast to GOD dissolved in distilled water. Nevertheless, the values were used as a 100% basis to be able to compare the effects of both solvents on the stability of GOD under polymerization conditions. While HEMA concentrations increased from 20 to 90%, the GOD activity decreased significantly. Up to a HEMA concentration of 60%, the loss of activity was 40 to 50% but a further increase of the monomer concentration up to 90% resulted in an additional loss of activity of approximately 10% only. The incubation time of GOD and HEMA had a low influence on the enzyme activity (Fig. 1). It was shown that the main loss of activity occurs during the first 5 min.

3.2. The effect of ultraviolet radiation on GOD activity

The exposure to ultraviolet radiation at 365 nm led to a decrease of GOD activity dependent on the irradiation time. The activity loss occurred in both solvents in a linear manner but at

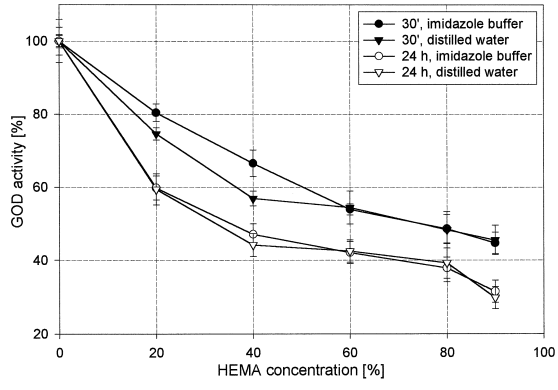

Fig. 1. The effect of the HEMA concentration on the activity of GOD dissolved in different solvents and contacted with the monomer for different times.

higher intensity in water. The correlation between activity y (%) and irradiation time x (min) is expressed by the following equations: $y = -0.1127^{*}x + 100.267$ $(r = 0.999)$ for GOD dissolved in imidazole buffer and $y = -0.2023^{*}x + 98.876$ $(r = 0.967)$ for GOD dissolved in distilled water. Consequently, after an irradiation time of 4 h the activity in water decreased by 50% but in buffer by 25% only.

3.3. The effect of temperature on GOD activity

As shown in Fig. 2, an increasing water content in the reaction mixture resulted in a lower temperature rise during the exothermic process of polymerization and also in a prolonged process duration.

Since a maximum temperature rise of 30 K was detected during the polymerization of HEMA without water on one side (Fig. 2) and GOD becomes unstable at temperatures of more than 40°C on the other [10], we examined the stability of GOD at temperatures of 50, 60 and 65°C. At 65 and 60°C, GOD was inactivated rapidly (Fig. 3). Nevertheless, a higher thermal stability of GOD dissolved in distilled water was to be seen at 65°C as well as 60°C already. At 50°C, the effect of the solvent on the GOD stability was very obvious. The activity of GOD dissolved in water was reduced by 10% only, over a 3 h period. However, the activity of GOD dissolved in imidazole buffer was decreased by 70% during the same time (Fig. 3). At 65°C, the enzyme inactivation followed a first order kinetic. The inactivation rate constants of 0.218 min^{-1} for buffer dissolved GOD and 0.140 min^{-1} for water dissolved GOD were conformed with the Arrhenius equation. At 60 and 50°C, the inactivation kinetic could not be determined.

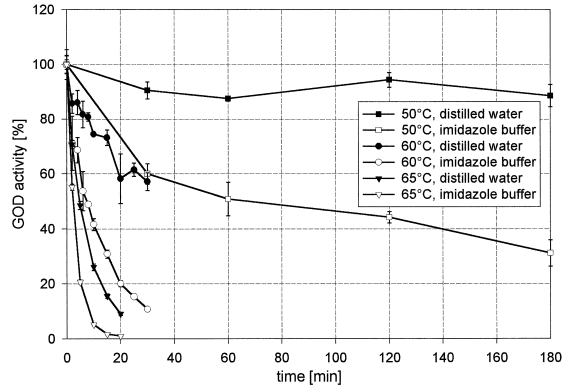

Fig. 3. The effect of the solvent of GOD on the thermal stability of the enzyme at 50, 60 and 65°C.

3.4. The effect of initiator / radical concentration on GOD activity

Since the initiator AIBN is not soluble in aqueous solvents, it was dissolved in HEMA

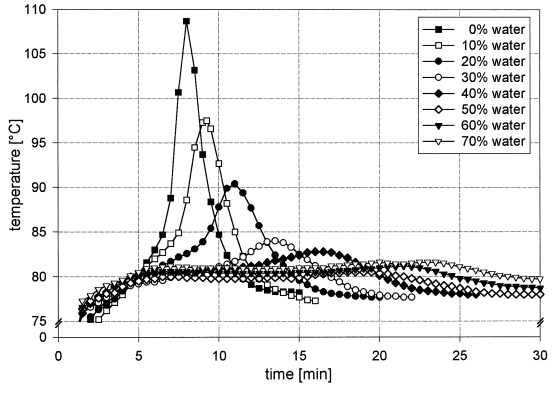

Fig. 2. The course of temperature during the polymerization of HEMA dependent on the water content in the mixture (initiation: 2 mg AIBN/ml HEMA, 80°C).

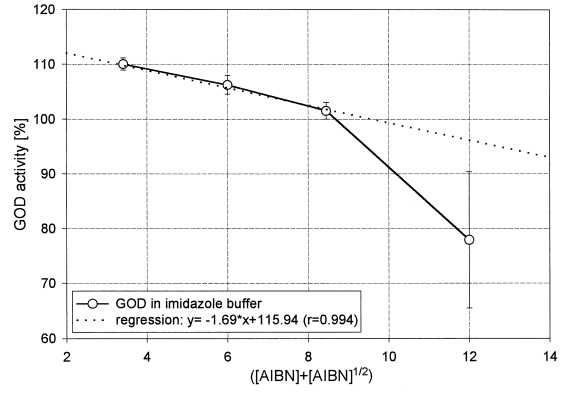

Fig. 4. The effect of the radical concentration expressed as $([AIBN]+[AIBN^{1/2}])$ in the first phase of polymerization process on the GOD activity.

and then mixed with GOD dissolved in imidazole buffer. During exposure to 365 nm, radicals were generated from AIBN. The total radical concentration during the first phase of the polymerization process is proportional to the sum of AIBN concentration and its square root ([AIBN] + $[AIBN]^{1/2}$). The expression was arithmetically derived from the polymerization kinetics [11,12]. In the presence of radical concentrations up to 8.45 mg/ml (corresponds to 6 mg/ml AIBN), a higher GOD activity was observed compared to that of a HEMA-GOD mixture without AIBN and a linear correlation between the increasing total radical concentration and the decreasing enzyme activity was determined in the first phase of the polymerization process (Fig. 4).

3.5. Long term stability of GOD entrapped in pHEMA

As concluded from Fig. 1, the highest GOD activity entrapped in pHEMA was expected using low monomer concentrations. This was to be observed also, only, the effect was weakly pronounced [12]. Investigating the long term stability, GOD was entrapped in a polymer composed of 40% HEMA and 60% of a solution of GOD in imidazole buffer. In the solved state, an increasing initiator concentration led to a decreasing GOD activity but an opposite effect was found in the immobilized state. Therefore, high AIBN concentrations of 6.0 and 12.0 mg/ml HEMA were applied in long term stability tests. Except for the first days, a stable activity of GOD was determined over more than 4 months (Fig. 5).

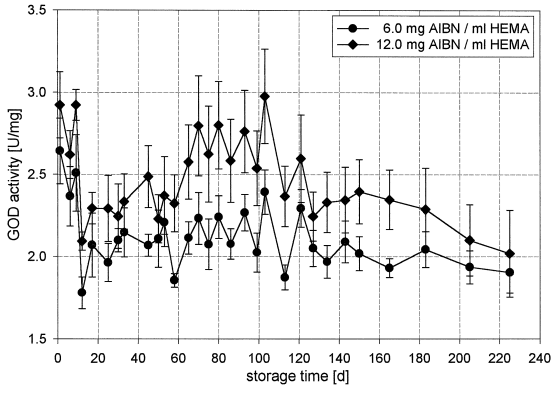

Fig. 5. Long term stability of GOD entrapped in 40% pHEMA made from different AIBN concentrations (storage conditions: imidazole buffer, room temperature; ranges = standard deviation of the mean).

4. Discussion

4.1. The effect of the HEMA concentration on GOD activity

To keep a high GOD activity, low monomer concentrations should be used for enzyme immobilization. But the HEMA concentration should not be reduced below 40% to prevent the polymer from becoming inhomogeneous and brittle [2,13] and lowering its adhesiveness to platinum. The decreased GOD activity with an increased HEMA concentration is caused by a reduced solubility of the enzyme in the monomer of organic nature, most probably. This was underlined by a visible protein precipitation at higher enzyme concentrations, a loss of activity independent on the time of contact with the monomer, and the possibility to reconstitute the activity after dilution with imidazole buffer if the enzyme was dissolved in imidazole buffer before contacting HEMA.

4.2. The effect of ultraviolet radiation on GOD activity

The wavelength of 365 nm used is able to induce the photochemical splitting of AIBN to produce reactive radicals as starting elements for the polymerization of HEMA. The GOD molecule contains structure elements which are able to absorb ultraviolet radiation. In agreement with the ultraviolet spectra which are showing a high absorption value of AIBN and a low value of GOD at 365 nm, the enzyme activity is hardly influenced by the radiation at this wavelength.

4.3. The effect of temperature on GOD activity

Derived from the low thermal stability of GOD above 50°C as expressed in Fig. 4, it is essential to minimize the temperature rise during the polymerization process of HEMA, e.g., by an increase of the water content in the polymerization mixture. In contrast to all other factors examined, the thermal stability of GOD showed a strong dependence on the solvent used. The amount of GOD activity and its thermal stability were influenced by the solvent in an opposite manner. If GOD is dissolved in an aqueous medium, the hydrophilic groups of the enzyme are directed to the surrounding water molecules, buffer ions interact with charged groups and stabilize the protein conformation of the solved state [14–16]. The substrate can reach the active center easily resulting in a high enzyme activity. But in the ion stabilized solved state, a larger protein area is exposed to the solvent than in an ion free solvent like water where stronger hydrophobic inter- and intramolecular interactions resulting in a more compact protein conformation. Since the heat quantity passing the boundary surface is proportional to the size of the passed area, it should be clear that GOD is more sensitive to thermal stress if it is dissolved in buffer than in water.

4.4. The effect of initiator / radical concentration on GOD activity

The process of polymerization via radicals consists of three phases [17]. In the first phase, there is an equilibrium of radical generation and termination. The radical concentration is dependent on the initiator concentration only. Using the described experimental arrangement, this first phase seemed to exist after an irradiation time of 10 min, but only if the initiator concentration is not higher than 6 mg/ml. At an AIBN concentration of 9 mg/ml, the second phase of the polymerization process should be already reached at the same irradiation time. In this phase, the grown polymer chains are forming a physical network hindering the diffusion of radical chains to react with others and to terminate the chains by this way. Therefore, the radical concentration is increasing and the enzyme inactivation no longer depends on the energy of those radicals generated from the initiator only.

4.5. Long term stability of GOD entrapped in pHEMA

Regarding long term stability of immobilized GOD, the entrapment in 40% pHEMA initiated with 6.0 and 12.0 mg AIBN/ml HEMA was the most successful method. The effect of a higher residual enzyme activity in polymers made with higher AIBN concentrations might be caused by the formation of more but shorter polymer chains. This could result in enhanced diffusion conditions in the polymer [12]. The use of 40% monomer is connected with several advantages. The temperature rise during the polymerization is reduced by the high water content. A duration of the polymerization process of 30 min is acceptable. A polymer made from 40% HEMA shows a sufficient adhesiveness to platinum. Low monomer concentrations of about 40% increase the permeability of pHEMA for small molecules and oxygen [18,19]. The conditions for enzyme reaction are improved because of the more hydrophilic microenvironment of the enzyme in the polymer matrix [20,21].

5. Conclusions

The investigations have shown that a certain loss of GOD activity caused by the composition of the polymerization mixture and process parameters cannot be avoided but it can be reduced by a critical selection of the parameters. As a compromise of essential polymer properties and the loss of activity, a HEMA concentration of 40% should be used. The concentration of the initiator and the composition of the solvent for the enzyme have to be optimized.

Nevertheless, the results demonstrate that the entrapment of GOD in pHEMA is a suitable method to realize long term stability of the enzyme as a main prerequisite for the functional stability of biosensors for in vivo application.

References

[1] J.-P. Monthéard, J. Kahovec, D. Chappard, in: R. Arshady (Ed.), Desk Ref. Funct. Polym., Am. Chem. Soc., Washington, DC, 1997, p. 699.
[2] J.J. Gooding, E.A.H. Hall, Biosens. Bioelectron. 11 (1996) 1031.
[3] H. Yasuda, M. Gochin, W. Stone Jr., J. Polym. Sci. A 1 (4) (1966) 2913.
[4] D.J. Strike, A. van den Berg, N.F. de Rooij, M. Koudelka-Hep, ACS Symp. Ser. 556 (1994) 298.
[5] G. Jobst, I. Moser, M. Varahram, P. Svasek, E. Aschauer, Z. Trajanoski, P. Wach, P. Kotanko, F. Skrabal, G. Urban, Anal. Chem. 68 (1996) 3173.
[6] I. Gürsel, V.N. Hasirci, Biomaterials 13 (1992) 150.
[7] C.E. Hall, E.A.H. Hall, Anal. Chim. Acta 281 (1993) 645.
[8] L. Doretti, D. Ferrara, P. Gattolin, S. Lora, Biosens. Bioelectron. 11 (1996) 365.
[9] M.Y. Arica, V. Hasirci, Biomaterials 14 (1993) 803.
[10] R. Wilson, A.P.F. Turner, Biosens. Bioelectron. 7 (1992) 165.
[11] J. Ulbricht, Grundlagen der Synthese von Polymeren, 2nd edn., Hüthig and Wepf, Basel, 1992, p. 53.
[12] B. Schulz, P. Abel, A. Riedel, in preparation.
[13] M. Yoshida, M. Kumakura, I. Kaetsu, Polymer 20 (1979) 3.
[14] H.J. Hecht, D. Schomburg, H. Kalisz, R.D. Schmid, Biosens. Bioelectron. 8 (1993) 197.
[15] S. Yoshioka, Y. Aso, K.-I. Izutsu, T. Terao, Pharm. Res. 10 (1993) 1484.
[16] T. Arakawa, S.N. Timasheff, Biochemistry 21 (1982) 6545.
[17] H.-G. Elias, Makromoleküle, Vol. 2, 5th edn., Hüthig & Wepf, Basel, 1992, p. 81.
[18] L. Doretti, D. Ferrara, G. Barison, S. Lora, Appl. Biochem. Biotechnol. 49 (1994) 191.
[19] M.Y. Arica, V. Hasirci, Biomaterials 8 (1987) 489.
[20] M. Cantarella, F. Alfani, L. Cantarella, A. Gallifuoco, in: G.F. Bickerstaff (Ed.), Methods in Biotechnology, Vol. 1, Humana Press, Totowa, 1997, p. 67.
[21] M.Y. Arica, V. Hasirci, J. Chem. Tech. Biotechnol. 58 (1993) 287.