Tr. J. of Chemistry
21 (1997) , 387 – 393.
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Immobilization of Glucose Oxidase and Urease in Hydrogel Matrices

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Received 12.6.1997

Immobilization of glucose oxidase and urease in hydrogels of 2-hydroxyethyl methacrylae, and N-vinyl pyrrolidone (NVP) was achieved by irradiation (using UV kand γ- rays). The effect of radiation on entrapment efficiencies, retention of activities and swelling rates was obtained. To optimize the system, duration of exposure, reaction temperature, co-monomer concentrations, initiator and cross linker compositions were varied. The repeated reusability of the generated products was also tested. It was found that γ-irradiation at very low temperatures did not have a detrimental effect on the enzyme activity. Incorporation of more hydrophilic monomer (NVP), influenced matrix morphology and entrapment efficiencies and retained enzyme activities positively. Repeated use studies revealed that there is a gradual loss (higher in the NVPL incorporated group) of activity following 10 runs. With the use of the present approach it is believed that the construction of biosensors using hydrogels of arious forms is possible.

Introduction

Immobilization technology is becoming an important field in the biomedical science and biotechnology. A large number of bioactive materials such as drugs, proteins, plant and animal cells and microorganisms of various classes were successfully immobilized with very high yields on appropriate supports [1–3]. These immobilized products were intented for use in the construction of artificial organ systems, bionsensors or bioreactors (for the production of the chemicals, stereospecific compounds, etc.)[4,5] Immobilization is advantageous because i) it extends the stability of the bioactive species by protecting the active material from deactivation, ii) enables repeated use, iii) facilitates easy separation and speeds up recovery of the bioactive agent and iv) it provides significant reduction in the operation costs[6,7].

The availability of a large number of support materials[5] and methods of immobilization (encapsulation, entrapment, covalent bonding, etc.)[1] leaves virtually no bioactive species without a feasible route

of immobilization. On the other hand, there are also certain disadvantages to immobilization such as the increased cost of the processed bioactive agent, and diffusional restrictions due to the support presence. It is thus important that the choice of immobilization over free bioactive agent should be well justified.

Urease converts urea to ammonium ion and carbon dioxide. The ammonium ions produced by enzyme-catalysed urea hydrolysis are toxic in humans at blood levels above 10^{-4} mol dm^{-3} [8-10]. Glucose oxidase (GOD) converts glucose and oxygen to gluconic acid and hydrogen peroxide removing oxygen and glucose from food stuff or producing gluconic acid for use in the tanning processes. They can also be used in biosensors (detection of glucose and urea) or removal of undesired chemicals (i.e. urea from blood)[2,8] after immobilizaton in/on supports of various forms. Development of an efficient immobilization approach for these enzymes is, therefore, quite important for the reasons stated above.

In the present study immobilization of these important enzymes, via entrapment within hydrogel matrices by gamma and UV irradiation was aimed. This approach was especially selected to avoid the high temperature immobilzation steps involving reactive chemicals. The preparation and properties of entrapped urease and glucose oxidase within hydrogels matrices were compared with respect to the immobilization efficiency, recovered activity yield and enzyme stability.

Materials and Methods

Materials

Glucose oxidase (GOD, EC.1.1.3.4; oxygen 1-oxido reductase, Type II from Aspergillus niger), peroxidase (POD, EC.1.11.1.7), o-dianisidine, bovine serum albulmin (BSA), Folin-Ciocalteu, urea kit (Catalog no:595 Bun color reagent, trichloroacetic acid solution (3.0 % w/v), diacetlyl monoxime solution (0.18% w/v), Bun acid reagent and urea nitrogen standard solution)were all obtained from Sigma Chem. Co. (USA).

2-Hydroxyethyl methacrylate (HEMA), N-vinyl pyrrolidone (NVP) and Ethyleneglycol dimethacrylate (EGDMA) were obtained from Fluka AG (Switzerland). HEMA and NVP were vacuum distilled in the presence of hydroquinone inhibitor and stored in the refrigerator until use. EGDMA inhibitors were removed by alkaline salt extraction (20% NaCl and 5% NaOH), washed twice with distilled water and dried with $CaCl_2$ stored in the refrigerator until further use. α,α'-Azo-isobutyronitrile (AIBN), ammonium persulfate (APS), N,N'-methylenebisacrylamide (BisAA) and D-glucose were obtained from Fluka AG. and used as received.

Urease (EC.3.5.15.3), urea and all the other analytical grade chemicals were purchased from Merck AG. (Germany).

Immobilization of GOD and ureas in HEMA and NVP via Gamma irradiation

Immobilization of GOD and urease in HEMA and in a HEMA:NVP(1:1) mixture were carried out according to a method described previously[2].

A solution consisting of monomer(s) (10 cm^3) and citrate buffer (7.0 cm^3, 0.1 M,pH 5.4) containing 20 mg GOD or phosphate buffer (7.0 cm^3, 0.1 M,pH 8.0) containing 20 mg urease was placed in a polymerizaition tube and mixed by vortexing. It was then purged for 2 min with nitrogen gas before introduction to the γ-irradiaton chamber (dose rate: 630 Gy/h). Polymerization reactions were carried out at three different temperatures (25°C, 0°C and -196°C) for a duration of 4h. The resultant gels were washed with the same fresh buffer (50 cm^3) and stored at 4°C until use.

Immobilization of glucose oxidase in HEMA via UV irradiation

HEMA, EGDMA and Biss AA (cross linker), AIBN (initiator), GOD and phosphate buffer (0.1 M, pH 7.0) were introduced into a test tube according to Table 1. This was then poured into a glass mold (ϕ=4.5cm) and exposed to UV (12 W lamp, P.W. Allen Co) for 30 min. at 25°C while a nitrogen atmosphere was maintained in the mold during the exposure. The resultant hydrogel was washed with phosphate buffer (50 cm^3, 0.1 M, pH 7.0) and stored at 4°C until use.

Table 1. Preparation conditions for GOD entrapment in pHEMA hydrogels under UV irradiation

Sample Type	Monomer (M)		Crosslinker (mM)	Initiator (mM×10^4)
	HEMA	BisAA	EGDMA	AIBN
HEMA-30	2.34	-	-	6.1
HEMA-40$_1$	3.11	-	-	6.1
HEMA-40$_2$	3.11	-	-	12.2
HEMA-50	3.92	-	-	6.1
HEMA-30$_{x1}$	2.34	64	-	6.1
HEMA-40$_{x2}$	3.11	-	110	6.1
HEMA-40$_{x3}$	3.11	-	220	6.1
HEMA-50$_{x4}$	3.92	64	-	6.1

Each preparation mixture (5 cm^3) contained glucose oxidase (5mg) and it was carried out 25°C in phosphate bufer (0.1 M, pH 7.0)

Urease activity determination

The determination of the activites of free and immobilized ureas were carried out according to the procedure given in Sigma Blood Urea Nitrogen Kit (Sigma Catalog No: 535).

For the construction of a calibration curve Bun acid reagent (3.0 cm^3), Bun color reagent (2.0 cm^3) and urea nitrogen solution (0.02 cm^3, 3.3 - 16.6 mM urea) were transferred to a series of test tubes and were maintained for exactly 10 min in boiling water. After cooling, the absorbances at 525 nm were determined with a double beam spectrophotometer (Shimadzu Model 2100 S, Japan).

100 cm^3 urea nitrogen solution (6.6 mM) was preincubated at 35°C for 10 min and the reaction was started by adding 10 mg urease. A 0.02 cm^3 aliquot was transferred to a solution consisting of Bun color (2.0 cm^3) and Bun acid reagent (3.0 cm^3) at specific time intervals (15 min). The decrease in urea concentration was measured as described above.

For the determination of immobilized urease activity, hydrogel pieces (ca 0.1 g) were introduced to the urea nitrogen solution (6.6 mM, 10cm^3) to initiate the reaction and the decay of urea was followed as above. The activity of the immobilized urease was presented as a percentage of the activity of free enzyme of same quantity. One unit of activity represent the decomposition of 1 mmole urea min^{-1}, at 35°C at pH 7.0.

Glucose oxidase activity assay

The activities of both the soluble and immobilized GOD preparation were determined by measuring the concentration of hydrogen peroxide, a side product of glucose conversion in the medium. The assay mixture

($100\ cm^3$) consisted of POD (3 mg, 60 U), o-dianisidine (0.16 mM) in phosphate buffer (0.1 M, pH 7.0). A 2.3 cm^3 of assay mixture and glucose solution (0.1 cm^3, 100 mM) are mixed and preincubated at 25°C for 10 min. The reaction was initiated by the addition of 0.1 cm^3 GOD solution (containing 1 mg GOD cm^3) to the assay mixture and terminated by the addition of sulfuric acid solution (1.5 cm^3, 30 % v/w). The absorbance at 520 nm was determined by a double beam UV/Vis spectrophotometer (Shimadzu, Model 2100 S). One unit of GOD is defined as the amount of enzyme which oxidizes 1μmole of β-D-glucose to gluconic acid and H_2O_2 per minute at 25° C at pH 7.0.

Protein determination

The amount of protein in the crystalline enzymes and in the hydrogel wash solutions after immmobilization was determined according to Lowry et al[11]. Crystalline bovine serum albumin was used as a standard in the construction of the calibration curve.

Determination of water content of the hydrogels

Water content of hydrogels were determined by drying fully hydrated hydrogel samples of known weight in a vacuum oven until they reached constant dry weight (ca for 24 h).
The water content was calculated according to the following equation:

$$\text{Water content (w/w)}=(W_w\text{-}W_d)/W_d, \quad (1)$$

where W_w and W_d are the wet and dry weights of the hydrogel, respectively.

Results and Discussion

Entrapment of glucose oxidase and urease in HEMA and HEMA/NVP via Gamma-irradiation

One of the most important parameters that influence the specific activity of the immobilized enzyme is the reaction temperature during entrapment process. In this study three different temperatures (-196°C, 0°C and 25°C) were used during the polymerization stage.

Immobilizatin efficiency of GOD in γ-irradiated pHEMA gels at 25°C, 0°C, and at -196°C were 92.9, 89.9, and 66.4 %, and the relative specifiç activity of the GOD entrapped in pHEMA gels were 30.2, 45.0 and 49.6 % at the same temperatures, respectively. It was observed (Table 2) that immobilization yields in pHEMA gels decreased as the temperature of the reaction decreased. The relative activities, however, were increased upon decrease of the temperature.

For urease immobilization, on the other hand, it was observed that decreasing immobilization temperature did not appreciably influence the immmobilization efficiency as much as the relative activity. At -196°C the immobilization efficiency was 83.1 % (with 48.5 % relative activity) but this declined to 77.0 % at 25°C retaining only 39.1 % of the activity of the free enzyme. This proves that the damage caused by the highly energetic gamma radiation could be reduced by lowering the temperature and possibly restricting the mobility of the free radicals formed on the enzyme.

In all the samples the incorporation of the hydrophilic comonomer, NVP, although did not influence the immobilizaiton efficiency, but its presence augmented the relative activities regardless of the enzyme used

(Table 2). This positive influence could be due to the enhanced water content levels of the gels (Table 2). As there would be better diffusion through the water filled matrices, accumulation of the reaction products, which could either have a detrimental effect on the enzyme or decrease the rate, would be less in the vicinity of the active site.

Table 2. Properties of the GOD and urease containing pHEMA and pHEMA/NVP hydrogels prepared by exposure to gamma irradiation

Sample Type	Immobilization Efficiency (%)		Recovered activity (%)		Water Content (g/g dry gel)	
	GOD	Urease	GOD	Urease	GOD	Urease
HEMA (RT)	92.9	77.0	30.2	39.1	1.60	1.85
HEMA (0°C)	89.9	82.5	45.0	2.05	2.34	
HEMA (-196°C)	66.4	83.1	49.6	48.5	1.96	2.08
NVP/HEMA (RT)	84.9	77.7	63.9	52.2	3.04	3.21
NVP/HEMA(0°C)	81.9	69.9	83.4	56.4	5.47	5.17

Entrapment of glucose oxidase in pHEMA via UV radiation

A series of hydrogels were produced for the optimization of polymer systems with using HEMA, EGDMA (crosslinker) and initiator (AIBN and ABS) and enzyme (glucose oxidase). The enzyme-hydrogel system were characterized with respect to activity yield, immobilization efficiency and water content. (Table 3).

Table 3. Properties of the GOD containing pHEMA prepared by exposure to UV irradiation

Sample Type	Immobilization Efficiency (%)	Recovered Activity (%)	Water Content (g/g dry gel)
HEMA-30	97.2	78.3	0.69
HEMA-40_1	97.3	71.1	0.65
HEMA-40_2	97.7	66.0	0.69
HEMA-50	97.6	69.3	0.60
HEMA-30_{x1}	98.5	70.8	0.63
HEMA-40_{x2}	97.9	67.3	0.59
HEMA-40_{x3}	98.4	64.1	0.52
HEMA-50_{x4}	98.3	58.7	0.51

(30, 40 and 50): percent of HEMA in polymerization mixture
(x): the presence of crosslinker

The immobilization efficiency for HEMA based hydrogels was not changed significantly (varied beteen 97.2 to 98.5) and was higher than observed with gamma irradiation. This suggest that the highly penetrating gamma rays are more detrimental for activity of the enzyme. The highest GOD activity was obtained with (HEMA 30) composition (Table 3).

Since HEMA 30 has the highest water content, the observation mode with pHEMA-NVP gels was confirmed. When AIBN concentrations was doubled (in HEMA-40_2 composition with respect to HEMA-40_1) the polymerization time decreased from 2 to 1 h and activity was decreased from 71.1 to 6.0 %. The dependence of glucose oxidase activity on the concentration of monomer and crosslinking agents (BisAA and EGDMA) was also tested (Table 1). The recovered activity of enzyme decreased by increasing monomer and crosslinker concentration.

The recovered glucose oxidase activity in the crosslinked hydrogels was always less than their uncrosslinked counterpart. This is due to the increase in resistanceed to diffusion of the substrates (glucose and oxygen) into, and products (gluconic acid and hydrogen peroxide) out of the hydrogel micropores upon crosslinkage.

Activity during repeated use

The reusability of glucose oxidase and urease entrapped in hydrogels were studied by using the same enzyme-gel pieces in upto 10 successive batch test. (Figures 1-3).

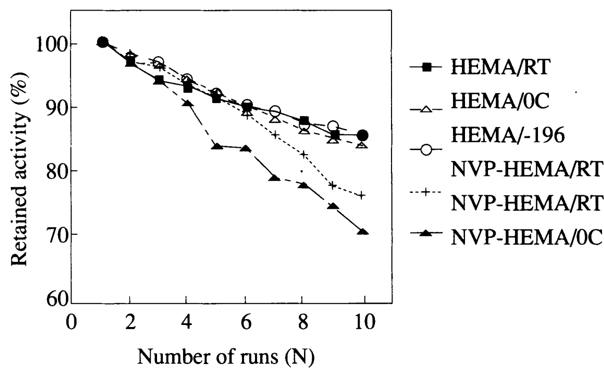

Figure 1. Repeated use studies of GOD incorporated into pHEMA and pHEMA/NVP hydrogels via gamma irradiation (reaction conditions: phosphate buffer; 0.1 M, pH: T = 25° C)

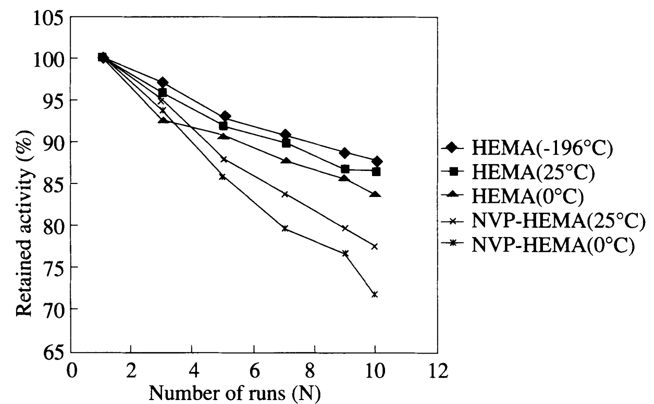

Figure 2. Repeated use studies of urease immobilized pHEMA and pHEMA/NVP hydrogels via gamma irradiation (reaction conditions: urea nitrogen solution; 6.6 mM, pH: 7.0; T=35° C)

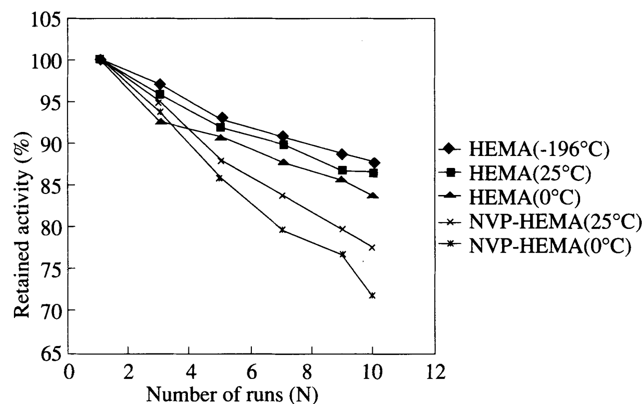

Figure 3. Retained activities for GOD entrapped in pHEMA hydrogels prepared by UV-irradiation (reaction conditions: phosphate buffer; 0.1 M, pH: 7.0; T=25° C)

It is observed that the activities of both γ-irradiated enzymes entrapped in pHEMA were relatively stable upto 10 repeated uses regardless of the gel density, enzyme type, and irradiation temperature (Figures 1 and 2). Activity retained was between 86% for GOD and 88% to 84% for urease, with respect to their initial activities, at the end of this period. The lowest activities retention in whole pHEMA set (84% for both enzymes) was obtained with the 0° C preparation.

The activites of GOD and urease entrapped in γ-irradiated pHEMA/NVP hydrogels prepared under two different temperatures (0° C and RT) decreased more rapidly, regardless of the type of the enzyme and the preparation temperatures. For GOD at the end 10 repeated uses the retained activities were about 77%

and 71% for RT and 0°C, respectively, and for urease under the same conditions they were 84% and 72% (Figures 1 and 2).

The reuseability of UV-irradiated glucose oxidase immobilized in pHEMA-30 and pHEMA-50 were tested as described above and the results are presented in Figure 3. The activity loss behaviour could best be described as biphasic. The activity of the samples were decreased with a very shallow slope up to the end of 8th run. The activities of pHEMA-30 and pHEMA-50 were then more rapidly decreased and only 50% and 47% of their initial activity was retained after 10 repeats, respectively.

The reason for decrease in activity with repeated use could be due to gradual inactivation of enzyme as a result of the poisonning effect of the products, H_2O_2 for GOD and ammonia for urease.

When the influence of the type of radiation is compared it apears that UV led to higher water content gel type, with more initiall activity and better retention in the first 8 runs. Thus the type radiation has a significant effect. The harmful influence of reactive chemicals was not as great as it was expected. The more rapid decline in activity with UV hydrogels is worth further investigation.

Conclusions

In the present study, immobilization of GOD and urease within hydrogel membranes were successfully achieved via UV and γ-irradiation. Entrapment efficiencies were considerably better than those reported in the literature. The entrapment efficiency and relative activity of the enzymes following was closely related to the temperature and the initiating radiation type in polymerization medium. As temperature increased the entrapment values increased but the relative activity decreased due to higher damage. Incorporation of more hydrophilic comonomer NVP enhanced the relative activity in all tested formulations. The finding in general, indicated that the use of radiation may not be as detrimental to the final product performance if the reaction temperature can be controlled.

It can be concluded that by using electromagnetic radiation as intiators preparation of stable immobilized enzyme system is possible. This would, therefore, enable researchers to construct durable biosensors which can be applied in the pharmaceutical, chemical and food industries.

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