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Catalase Effects on Glucose-Sensitive Hydrogels

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ABSTRACT: Glucose-sensitive hydrogels (GSHs) responsive to both pH value and glucose concentration have been prepared by polymerizing solutions containing hydroxypropyl methacrylate, (*N*,*N*-dimethylamino)ethyl methacrylate, and tetraethylene glycol dimethacrylate in the mole ratio 70:30:2. Various concentrations of both glucose oxidase (GOx) and catalase were physically immobilized in the hydrogel matrix. The presence of GOx makes the hydrogels glucose-responsive, and catalase was added in order to explore its effects on swelling kinetics. In response to pH changes, hydrogels containing varying amounts of catalase show identical swelling kinetics and identical equilibrium degrees of swelling. However, in response to increases in glucose concentration, the rate of GSH swelling is dramatically increased by the presence of catalase when GOx activity is limited by the amount of oxygen available. In addition, catalase improves the enzymatic stability of the GOx by converting hydrogen peroxide to water.

Introduction

The current growth of hydrogel applications in drug delivery and biosensors is ascribed in part to the biocompatibility of hydrogels and in part to fast and reversible volume changes in response to external stimuli such as temperature, pH, electric and magnetic fields, or analyte concentration. Thus, these hydrogels are sometimes called "stimulus responsive polymers".[1] The swelling and shrinking of stimulus responsive polymers has been successfully used to control drug release in several systems.[2−17] A pH-sensitive hydrogel undergoes very large and reversible volume changes in response to pH changes within the hydrogel. Two main types of pH-sensitive hydrogels are acidic hydrogels and basic hydrogels. Acidic hydrogels by definition will be ionized and hence swollen at high pH and uncharged and unswollen at low pH.[8,16] Swelling behavior of a basic hydrogel has the opposite dependence on pH. The pH sensitivity is caused by pendant acidic and basic groups such as carboxylic acids, sulfonic acids, primary amines, and quaternary ammonium salts.[8,15,16] Carboxylic acid groups for example are charged at high pH and uncharged at low pH, whereas the reverse is true for primary amine groups and quaternary ammonium salts. The transition pH for a given pendant group is primarily determined by the pK_a value for that pendant group and by the hydrophobicity of nearby monomers in the polymer chain.[16] Hence, one can manipulate the critical pH value at which the pH-sensitive hydrogel undergoes a volume transition. pH-sensitive hydrogels can be derived by choosing pendant groups with the appropriate pK_a values and by adjusting the hydrophobicity by choosing from a number of monomers such as poly(alkyl acrylate), poly(alkyl methacrylate), poly(2-hydroxyethyl methacrylate) (p-HEMA), poly(2-hydroxypropyl methacrylate) (p-HPMA), poly(acrylamide), poly(*N*-vinylpyrrolidone), poly(vinyl alcohol) (PVA), poly(ethylene oxide) (PEO), and poly(etherurethane).[2−20] The monomers listed above can also be used in various combinations to form copolymers.

A pH-sensitive hydrogel containing glucose oxidase (GOx) enzyme is called a glucose-sensitive hydrogel (GSH) due to its responsiveness to environmental glucose concentrations. Thermally stable GOx is a flavin-containing glycoprotein which catalyzes a reaction that is very specific for glucose and which produces gluconic acid and hydrogen peroxide in the presence of glucose and oxygen as shown below. Therefore, increases in the environmental glucose concentration lower the pH value within the GSH.

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Several attempts have been made to utilize this catalytic reaction in glucose biosensors.[20−23] Glucose biosensors based on amperometric methods are the most highly developed. In the amperometric method, an electrode is used which produces a current proportional to the diffusional flux of hydrogen peroxide to the electrode surface or, alternatively, proportional to the diffusional flux of oxygen to the electrode surface.[20−23]

At steady state, the diffusional flux of hydrogen peroxide to the electrode surface equals the rate at which hydrogen peroxide is produced by the GOx reaction in the hydrogel adjacent to the electrode. However, unlike the hydrogels considered here, the hydrogels in amperometric glucose biosensors do not swell in response to pH changes.

The most important physical property of a GSH is its ability to change volume in response to changes in environmental glucose concentrations, due to changes in pH within the hydrogel. This physical phenomenon has been applied in insulin delivery devices to control insulin permeability through GSHs.[4−6,17] Also, in our lab, GSHs have been studied in devices for measuring glucose concentrations in various solutions using a pressure transducer. For such applications, two major problems with the GOx enzymatic process have been identified: insufficient oxygen supply for the reaction and the decay of the GOx activity with time due to peroxide-induced degradation.[21−25] In recent years, numerous attempts have been made to solve the first

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problem by using oxygen substitutes or external oxygen reservoirs.[22−24,26] Both problems mentioned above can be partially alleviated using the following enzymatic reaction of catalase:

$$H_2O_2 \xrightarrow{\text{catalase}} {}^1/_2O_2 + H_2O$$

The catalase reaction produces oxygen which helps meet the oxygen requirement of the GOx enzymatic reaction. Furthermore, the removal of hydrogen peroxide has been shown to reduce peroxide-induced degradation of the GOx enzyme.[27−33] On the basis of the catalase reaction, we hypothesize that catalase may increase swelling kinetics and enzyme stability in GSHs due to the removal of hydrogen peroxide and the production of oxygen and water in situ. In both insulin delivery devices and glucose biosensors, GOx stability is essential for long-term use in vivo. For insulin delivery devices and the pressure-based glucose biosensor being studied in our lab, rapid swelling kinetics is also important. To our knowledge, there have been no previous experimental studies on the effects of the amount of catalase on the swelling kinetics of GSHs. Amperometric glucose biosensors with hydrogen peroxide electrodes cannot employ catalase because the catalase reaction given above influences the electrode current.[34−41] Amperometric glucose biosensors with oxygen electrodes and an excess of catalase have been studied by Lucisano and Gough.[22,23] As mentioned previously, the hydrogels containing the immobilized enzymes in amperometric glucose biosensors do not swell or deswell in response to glucose concentration changes. Albin et al. studied experimentally the kinetics of pH changes within a GSH in response to changes in glucose concentration.[42] However, the GSHs used did not contain catalase. The swelling kinetics of GSHs for insulin delivery with an excess of immobilized catalase have been studied theoretically by Albin et al.[42] and Klumb and Horbett.[24] In these theoretical studies, a flowing glucose solution was assumed to replenish the glucose consumed by reaction, and the hydrogel was assumed to contain macropores. The presence of macropores allowed the authors to assume that the diffusion coefficients and concentrations at equilibrium of the various species are the same inside and outside of the hydrogel.[24] These theoretical results do not directly apply to our experimental system, because the GSHs studied do not contain macropores.

Experimental Section

Materials. Hydroxypropyl methacrylate (HPMA, Polysciences, Inc.) and (*N*,*N*-dimethylamino)ethyl methacrylate (DMA, Polysciences, Inc.) were vacuum-distilled in the presence of polymerization inhibitor prior to use in copolymerization. Tetraethylene glycol dimethacrylate (TEGDMA, Polysciences, Inc.), ethylene glycol (EG, Aldrich), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, Aldrich), ammonium persulfate (APS, Aldrich), glucose oxidase (GOx, Sigma), and catalase (Sigma) were used as obtained. Sodium dihydrogen phosphate (ICN), potassium hydrogen phosphate (ICN), TRIS HCl (ICN), TRIS Base (ICN), KCl (Sigma), and NaCl (Sigma) were used as received. Buffers were prepared from citric acid, Tris HCl, Tris Base, and PBS with pH adjusted with NaOH or HCl to the desired range. Measurement of pH was performed using a Corning pH meter with G-P Combo w/RJ pH probe. Calculated amounts of NaCl were added to the buffer solutions in order to adjust the ionic strength to 0.15 M, mimicking physiological conditions.

Glucose-Sensitive Hydrogel (GSH) Preparation. Each glucose-sensitive hydrogel was prepared by redox polymerization between two glass plates (10 cm × 10 cm) with the gap set using a Teflon spacer (0.40 mm). These slide molds were held together by metal clamps to provide a uniform internal cavity for the pregel solutions. Each pregel solution contained HPMA, DMA, TEGDMA, EG, TEMED, and APS in the mole ratio 70:30:2:10:0.03:0.001. The concentration of these species in the solution was adjusted to ensure complete monomer conversion in the final product. Calculated amounts of glucose oxidase and catalase solutions were prepared separately and added into the pregel solution. Pregel solutions were degassed by bubbling nitrogen for 10 min and/or by stirring under a rough vacuum for 5−10 min and then injected into the slide mold. The molds were kept at 4 °C for 12−16 h to facilitate complete polymerization. The hydrogel slab was separated from the two glass plates with a razor blade and cut into a 9.0 mm by 9.0 mm square disk using a long-blade cutter. All gel disks were washed in 0.5 X and 1.0 X PBS at least 3 days (2−3 times a day) and then stored in 1.0 X PBS buffer at 4 °C overnight or until use.

pH Swelling Studies. Hydrogel disks were immersed in 50 mM Tris buffer (pH 10) overnight or until they reached a constant weight value at room temperature (22−23 °C). The total ionic strength of each buffer at each pH was adjusted to the same value (0.15 M) with a calculated amount of NaCl. Periodically, disks were withdrawn from the buffer solution and weighed after removal of excess surface solution by light blotting with a laboratory tissue. The disk weights were individually monitored in this way until they reached a constant value. This typically required from 6 to 24 h depending on the value of the pH and the composition of the sample, and the buffer was replaced several times to maintain a constant pH during the experiment. The relative swelling ratio (RSR) for a gel sample was calculated as RSR = [Ww − Wd]/Wd where Ww and Wd are swollen and dry disk weights, respectively. Dry weights were determined by weighing gel samples after at least 10 days of drying in an oven at 60 °C. The weighing error was estimated as follows. One particular hydrogel was swollen to an equilibrium amount, weighed, and then replaced into the same equilibrium bath. This cycle was repeated five times, and a standard deviation was calculated from the weight measurements.

Glucose Swelling Studies. Hydrogel disks were immersed in PBS (pH 7.2) overnight or until they reached a constant weight value. A series of glucose solutions (75, 150, and 300 mg/dL) were prepared in a 100 mL bottle prior to use. The hydrogel disks were placed in the glucose solution, and the temperature was controlled using a Precision Scientific Inc. dual chamber water bath and a Thermolyne 42000 incubator. Oxygen saturation was accomplished by controlled bubbling of oxygen (2−3 bubbles per second) through the glucose solution with constant stirring during the experimental period. In certain other experiments, no oxygen bubbling was performed, and the solution was exposed to oxygen only at the air/solution interface at the top of the solution container. Periodically the sample gels were taken from the glucose bath, weighed after removing the excess buffer solution on the surface, and then replaced in the bath. The relative swelling ratio for a gel sample was calculated as RSR = [W(t) − Wd]/Wd where W(t) and Wd are sample weights at time t and dry weights, respectively.

Results and Discussion

Equilibrium Swelling of GSHs. Figure 1 shows the equilibrium degree of swelling as a function of pH at fixed ionic strength. The HPMA/DMA hydrogels used here were selected because these gels have been used for insulin delivery in response to the concentration of glucose.[17] The basic pendant group, a tertiary amine on DMA, becomes charged at low pH, thereby swelling the hydrogel (Figure 1). It has been demonstrated that the swelling response is most sensitive at a pH close to the pK_a value of the ionizable group of the hydrogel.[16] However, this effect depends on the relative hydrophi-

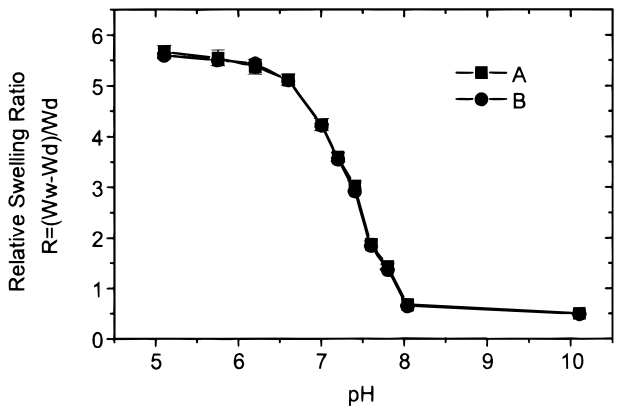

Figure 1. Equilibrium swelling profiles of the HPMA/DMA/TEGDMA (70:30:2 mole ratio) hydrogels with thickness 0.4 mm containing a fixed concentration of GOx (1000 units/mL of pregel solution), with (A) and without (B) catalase (600 units/mL of pregel solution) at various pH values of 50 mM Tris buffer at fixed ionic strength of 0.15 M and at room temperature. The hydrogel was incubated for 48 h at each pH (three independent measurements at each data point).

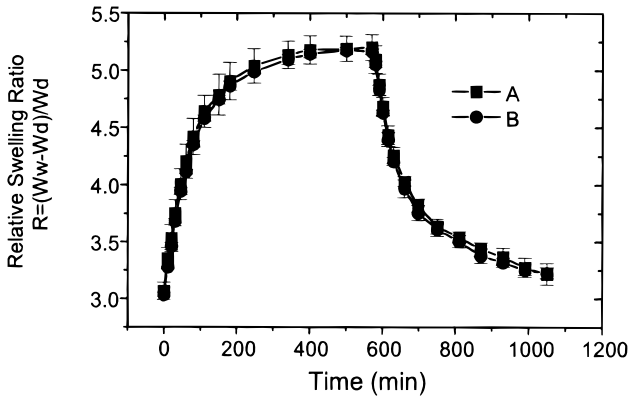

Figure 2. Kinetic swelling profiles of the HPMA/DMA/TEGDMA (70:30:2 mole ratio) hydrogels with 0.4 mm thickness containing a fixed concentration of GOx (1000 units/mL of pregel solution), with (A) and without (B) catalase (600 units/mL of pregel solution). The hydrogel was cycled at room temperature between solutions at pH 7.4 and pH 6.2, both solutions containing 50 mM Tris buffer at a fixed ionic strength of 0.15 M. Hydrogels equilibrated with pH 7.4 were suddenly exposed to pH 6.2 at time equal zero; the pH was switched back to pH 7.4 at 570 min (three independent measurements at each data point).

licity of the ionizable monomer compared to the neutral comonomer. The transition pH can be changed by increasing the hydrophobicity of the gel. Figure 1 shows that the hydrogel begins to swell at pH 8, which is below the pK_a value of the pendant amine (approximately 10). When the pH inside the gel varies during swelling experiments, the hydrophobicity changes due to a conformational change of the hydrophilic moiety. Cross-linking density is important for pH-sensitive swelling, with an increase in cross-linking density reducing the equilibrium degree of swelling. Glucose-sensitive hydrogels containing a fixed amount of GOx (1000 units/mL), and five different catalase concentrations (0, 100, 300, 600, 900 units/mL) have been examined and show identical equilibrium swelling behavior in response to pH changes. The equilibrium data demonstrate that different concentrations of catalase do not influence equilibrium swelling in the HPMA/DMA-based hydrogels with approximately 2 mol % TEGDMA cross-linker.

Kinetics of GSH Response to pH. For insulin delivery and glucose biosensor applications, it is of great practical importance that the GSH responds to glucose concentration changes in the body almost immediately, within 5 min. A pH-sensitive hydrogel cannot react to changes in glucose concentration until the pH value inside the hydrogel changes. Once the pH value inside the gel changes, mass transfer of water into or out of the gel will occur. The mass transfer of water is driven by osmotic pressure forces, which in turn are generated by ion-exchange processes and polymer conformational changes within the gel. pH-sensitive hydrogels have ionizable groups such as carboxylic acids, tertiary amine groups, and sulfanilamide groups. Ionization of these groups generates strong osmotic pressure forces and swells the gel; deionization of these groups deswells the gel. One can drastically decrease the time it takes a species to diffuse into the interior of gel by decreasing the gel thickness and/or increasing the gel porosity. Since cross-linking density also affects the response time, thin and lightly cross-linked hydrogels without macropores have been prepared, and their behavior is shown in Figure 2. The HPMA/DMA-based hydrogels containing GOx and catalase reversibly contract and dilate when then external pH value is cycled as shown Figure 2. Initially the hydrogel was equilibrated with a pH value of 7.4. At time equal to zero in Figure 2, the hydrogel was suddenly subjected to a pH value of 6.2, and the swelling was followed as a function of time. After the degree of swelling leveled off, the external pH was switched back to 7.4, and the shrinking (deswelling) kinetics was observed. Several important points are apparent from Figure 2. First, the degree of swelling returns to its initial value after the deswelling is complete; i.e., the swelling process is reversible. Second, the swelling and shrinking process is reproducible and identical for various concentrations of catalase (0, 100, 300, and 600 units/mL) in the GSH. Thus, there is no influence of catalase and GOx on the swelling kinetics of the basic hydrogel with 2 mol % cross-linking ratio, at least in the enzyme concentration range studied.

Catalase Effects on Swelling Kinetics and GOx Stability. Now consider the combined diffusion and reaction process that occurs when a thin (0.4 mm) hydrogel, equilibrated with PBS buffer at pH 7.2, is suddenly placed in 100 mL of a glucose solution at concentration 150 mg/dL (Figure 3). Glucose diffuses into the GSH, and the enzymatic GOx reaction produces gluconic acid, which ionizes the basic pendant group. This ionization is the driving force for the swelling behavior. Since the reaction is irreversible, it will proceed until all of the glucose in the surrounding solution is converted, which accounts for the long time required to reach equilibrium. Of course in the body, the surrounding glucose is continuously replenished, and a glucose biosensor containing a GSH operates in a steady-state or dynamic equilibrium condition. Under the solution conditions of Figure 3, the glucose concentration far exceeds that of the dissolved oxygen, which is also true under physiological conditions. Thus, oxygen is the limiting reactant in the GOx enzymatic reaction. To achieve oxygen saturation conditions (curves D and E in Figure 3), pure oxygen was continuously bubbled through the solution. Even under these conditions, the GSHs with coimmobilized catalase show somewhat faster swelling kinetics at earlier times than the GSHs without catalase in several independent experiments. This is probably due to higher local oxygen concentrations inside the hydrogel via the catalase enzymatic

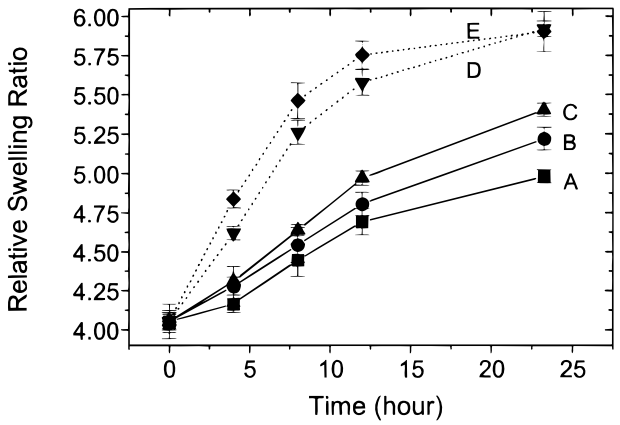

Figure 3. Kinetic swelling profiles at room temperature for HPMA/DMA/TEGDMA (70:30:2 mole ratio) hydrogels of thickness 0.4 mm and containing GOx (1000 units/mL of pregel solution) and various concentrations of coimmobilized catalase. At time equal zero, the hydrogels were placed in a PBS solution initially at pH 7.2 with an initial glucose concentration of 150 mg/dL and exposed to air without bubbling (A, 0 units of catalase/mL; B, 300 units of catalase/mL; C, 600 units of catalase/mL in pregel solution) or exposed to oxygen saturation with bubbling (D, 0 units of catalase/mL; E, 600 units of catalase/mL in pregel solution) (three independent measurements at each data point).

reaction. Also, the water production inside GSHs may partially help the fast initial swelling of the GSHs even though oxygen is the major controlling factor in swelling kinetics in our experiments. After 24 h at oxygen saturation, the pH value of the solution surrounding the GSH had dropped from 7.2 to 6.2. By comparing the swelling ratio of curve E in Figure 3 with Figure 1, one infers that the pH value inside the hydrogel was also about 6.2. Curves A, B, and C in Figure 3 correspond to experiments in which no oxygen was bubbled through the solution, and the only exposure to oxygen was at the air/solution interface. For these curves, the effect of catalase is significant. When the catalase concentration increases from 0, 300, to 600 units/mL in the GSHs, the relative swelling ratio after 24 h of glucose incubation increases from 4.96 ± 0.042, 5.21 ± 0.072, to 5.44 ± 0.043, at 25 °C. The latter value is increased by about 11% when the experiment is performed at 37 °C. However, the swelling ratio decreases when the catalase concentration in the GSH is increased from 600 to 900 units/mL, possibly by protein aggregation which denatures the enzyme or by limiting substrate diffusion. Thus, a catalase loading of 600 units/mL appears to be optimum.

The results in Figure 3 show that the oxygen and the water produced by the catalase reaction increases GOx activity, resulting in acceleration of the swelling kinetics. Theoretically, the oxygen requirement of the GOx reaction may be halved by the presence of catalase, provided that both enzymatic reactions are 100% efficient.[22−24] Catalase may also increase the rate of swelling by reducing peroxide-induced degradation of the GOx enzyme. Our experiments indicate that the activity of free GOx decreases by almost 60% after only 4 h of incubation in glucose solutions and that this decay in activity can be retarded by adding free catalase to the solution. Since the GOx reaction is irreversible, the final equilibrium degree of swelling should be the same for all of the curves in Figure 3, provided that oxygen is replenished by dissolution at the air interface. Curves A, B, and C never reach this plateau value. However, if the gel samples corresponding to curves A, B, and C are withdrawn from the glucose solution and placed in pH buffer at 7.2, all three samples take about 10 h to return to the slightly swollen equilibrium state at pH 7.2.

Several groups have tried to help solve the oxygen-deficit problem in glucose sensors and insulin delivery systems with a theoretical model that evaluates different possible device designs.[22−24] Klumb and Horbett have developed a theoretical model to evaluate possible designs for an insulin delivery system which is responsive to glucose.[24] They designed several insulin delivery devices with an oxygen-supplying system such as an oxygen diffusion tank to improve oxygen limitation in macroporous GSHs with an assumed excess of catalase at physiological conditions.[24] Our results further demonstrate the effect of the amount of catalase on swelling kinetics by the production of oxygen and water in the thin and lightly cross-linked nonporous GSHs. Alternatively, the oxygen depletion problem can be alleviated by using catalase and a semipermeable membrane that permits sufficient diffusion of oxygen and limited diffusion of glucose to the GSHs from exterior fluids such as blood.

Conclusions

Our data demonstrate that (1) the GSHs swell at low pH and at physiological glucose concentrations, (2) the GSHs with coimmobilized catalase show faster swelling kinetics in response to glucose changes than the GSHs without catalase, (3) the effect of catalase is most apparent when insufficient oxygen is present for GOx activity, as is likely to be the case under physiological conditions, (4) catalase has no effect on the swelling or deswelling of GSHs in response to pH changes, and (5) the swelling kinetics of GSHs may slowly decrease with time due to inactivation of GOx, and catalase helps prevents GOx inactivation by removing hydrogen peroxide.

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