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FUNCTIONAL IMMOBILIZED BIOCATALYSTS

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CONTENTS

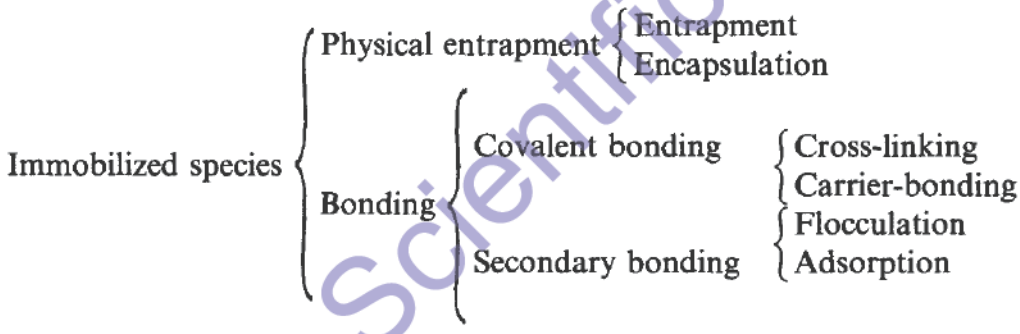
1. INTRODUCTION

As early as 1916, Nelson and Griffin[1] studied the absorption of invertase on charcoal and noticed that the adsorbed enzyme exhibits the same activity as the native enzyme. However, the purpose of their study was not to prepare a solid-supported, water-insoluble enzyme, i.e. an immobilized enzyme, now defined as "physically confined or localized in a certain defined region of space with retention of catalytic activity, and which can be used repeatedly and continuously". It is thus believed that the first attempt to prepare an immobilized enzyme was made in 1953, when Grubhofer and Schleith[2] coupled such enzymes as carboxypeptidase, diastase, pepsin, and ribonuclease with diazotized poly(*p*-aminostyrene). Prior to this, several immunologists[3–5] had succeeded in immobilizing antigens and antibodies by covalently binding these proteins to chemically well-defined, water-insoluble polymer supports such as

diazonium derivatives of cellulose and poly(styrene); this approach to protein immobilization seems to antedate studies on immobilized enzymes.

The direct immobilization of whole microbial cells, instead of enzymes, can eliminate the necessity for extracting enzymes from cells, and thereby enable the utilization of one or more enzymes existing in the cells. In 1960, Hattori and Furusaka[6] announced their study on the immobilization of *Escherichia coli* cells onto an ion exchange resin using an adsorption technique, apparently the first article on immobilized cells. Since then, many studies have been carried out which explore the possibilities for the use of immobilized cells as well as immobilized enzymes. At present, both immobilized enzymes and cells are known generically as "immobilized biocatalysts".

Immobilizing enzymes or microbial cells generally provides us with the following advantages: (i) continuous operation becomes practical; (ii) biocatalysts can be recovered and reused after reactions; (iii) biocatalysts can be formed into shapes, such as membranes or beads, required for fitting to specific reaction processes; (iv) in some cases, biocatalysts become stable with regard to changes in temperature, pH and inhibitor concentration. Immobilization methods can be classified according to the following scheme,



in which the type of enzyme or cell bonding in the immobilized biocatalyst is the dominant parameter. This scheme, however, is oriented more towards the state of the immobilized biocatalyst and less towards the route along (or process by) which the catalyst has been prepared. Both cross-linking and carrier-bonding methods cannot, in principle, be utilized in whole cell immobilization.

A glance at the outline of immobilized biocatalysts above shows that they involve research subjects which lie within interdisciplinary areas of biology, chemistry, physics, and engineering. In particular, the rapprochement between biochemistry and polymer chemistry seems to have played an important role in the methodological development of preparations for immobilized biocatalysts. A number of articles on the preparation and characterization of immobilized biocatalysts have been published, together with their applications in a variety of fields besides synthetic chemical reactions, for example, chemical and clinical analysis, medicine, and food processing, etc. These results have been reviewed by many of the pioneers in this and related fields.[7-26] The technology for immobilizing enzymes and cells is already believed to be relatively mature. In

addition, the nature of immobilized biocatalysts has become somewhat more transparent to us. The key now is to come up with new uses and new systems that can fulfil specific needs.[27]

A renewed interest in this research field may lead to the construction of "functional" immobilized biocatalysts that *surpass the conventional definition, or usually credited advantages, of immobilized biocatalysts with regard to their capabilities as catalysts*,[28,29] for example, immobilized enzyme systems in which an enzymatic process can be controlled by externally applied stimuli such as light, electric fields, pH, temperature, and mechanical force. In such cases, what is crucial in system construction is not to rely on a possible alteration in the property of the biocatalyst (e.g. an enhanced thermal stability) which has frequently been expected as a result of immobilization, but *to impose a new capability on a biocatalyst system as the result of a process of rational design*. As the principal means of reaching this goal, polymer chemists and scientists may consider developing supporting matrices; such functional polymers as pH-sensitive microcapsules, thermosensitive polymer gels, and reversibly soluble polyelectrolyte complexes have been employed for this purpose. This review aims to provide an up-to-date overview of the development of functional immobilized biocatalysts and to discuss how to apply the developing tools of the polymer and material sciences in the construction of such systems. In some cases the concept of biomimetic engineering — simulating natural biofunctions in the design and construction of artificial materials — has been taken into consideration.

2. CHARACTERISTICS OF IMMOBILIZED BIOCATALYSTS

Before discussing the present subject in detail, it seems necessary to outline the characteristics of immobilized biocatalysts in their catalytic reactions, especially the characteristics of immobilized enzymes. Figure 1 shows a schematic illustration of an immobilized enzyme system in which the enzyme molecules are bound to a porous matrix. When enzymes are immobilized with appropriate supports, the size of the biocatalyst apparently increases; for instance, when an enzyme molecule in the form of a globular protein with a diameter of 50 Å is bound to a polymer bead with a 100 μm diameter, the size of the biocatalyst becomes 20,000 times larger than that of the "original" enzyme. Such an immobilized biocatalyst is easier to handle than the original enzyme in many operations involving catalytic reactions. However, the step from homogeneous to heterogeneous catalysis results in differences in aspects of the kinetic behavior of the enzyme. The best starting point for characterizing immobilized enzymes might be an understanding of the differences in the kinetic behavior of their native and immobilized states.

The effects of immobilization on the kinetic behavior of enzymes can be classified as follows: (i) conformational and steric effects; (ii) partitioning effects;

FIG. 1. Schematic illustration of immobilized enzyme molecules bound to porous supporting matrix.

(iii) microenvironmental effects; and (iv) diffusional or mass-transfer effects (these effects have already been discussed in greater detail; see pp. 397–443 in Ref. 18 and pp. 127–220 in Ref. 19 for examples; the more typical articles have been cited here as references). The conformational and steric effects are due to changes in enzyme structure[30,31] and to steric hindrances in the immediate vicinity of the enzyme molecules,[32–36] respectively, both of which result from immobilization primarily when using covalent binding methods (see Fig. 2). The partitioning effects arise from the electrostatic[37–41] and hydrophobic[42,43] interactions of the supporting matrices with substrates, products, and other effectors such as inhibitors, accelerators, and small ions. As a result of the partition, the concentrations of these species in the immediate vicinity of the immobilized enzyme differ from those in the bulk phase. Such a difference influences the rate of immobilized enzyme reaction, and is called a microenvironmental effect.[38] The diffusional or mass-transfer effects are related to resistance to the translocation of substrates, products, and effectors from the bulk phase to the site of the enzyme reaction within the matrix (or vice versa).[44,45] All effects other than conformational changes and steric hindrances alter the rate of immobilized enzyme reaction by causing differences between the bulk and matrix phases in the concentrations of substrates, products, and effectors (see Fig. 3). In many cases, the rate of an immobilized enzyme reaction is lower than that of the same amount of soluble enzyme at a given concentration of substrate and effectors. This is called a "loss" in enzyme activity upon immobilization, and usually arises as the result of one or more of the effects described here.

Enzymic catalysis in homogeneous systems generally obeys the following

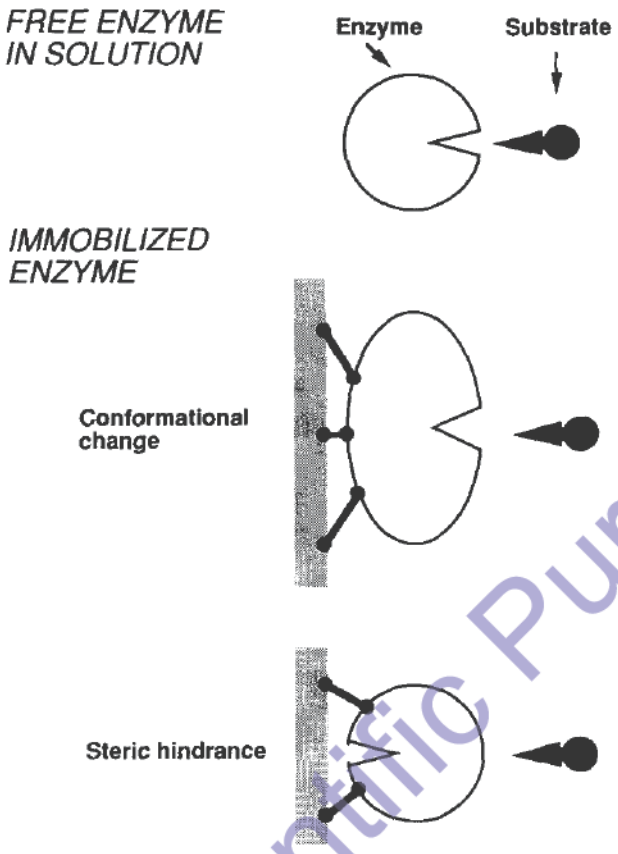


FIG. 2. Schematic illustrations of conformational change and steric hindrance of enzyme molecule bound to supporting matrix through covalent binding methods.

Michaelis–Menten scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \tag{1}$$

where E denotes the enzyme, S is the substrate, ES is the enzyme/substrate complex, P is the product from the substrate, and k is the first-order rate constant for the corresponding process. The rate of this enzymic reaction, V, can be given by:

$$V = \frac{k_{+2}[E]_f[S]_f}{K_m + [S]_f} = \frac{V_{max}[S]_f}{K_m + [S]_f} \tag{2}$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \tag{3}$$

$$V_{max} = k_{+2}[E]_f \tag{4}$$

where $[E]_f$ and $[S]_f$ represent the bulk concentrations of the enzyme and substrate, respectively, and V_{max} is the saturation rate (or maximum velocity) of the enzymic reaction. Also, K_m is the Michaelis constant and is equal to $[S]_f$ when $V = V_{max}/2$.

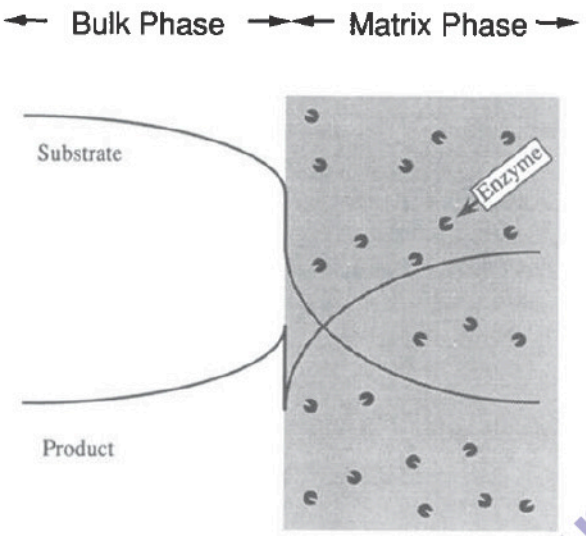


FIG. 3. Schematic illustration of the concentration profiles of substrate and product in an immobilized enzyme-catalyzed reaction system. The illustration shows a system in which both diffusional resistance and partition occur; the concentrations of the species distributed between the two phases due to partition gradually decrease or increase from the interface towards the interior of the matrix phase because of diffusional resistance.

Assuming that heterogeneous catalysis by immobilized enzymes obeys the Michaelis–Menten scheme and also that all of the effects described above are negligible, the rate of the immobilized enzyme reaction, V', which is actually determined by measuring changes in the bulk concentrations of the substrate and the product, should be equal to V in eq. (2). However, this is not the case in general; for example, in the presence of concentration gradients, as shown in Fig. 3, enzymes at different local positions within the support exhibit different activities, even if they have the same "intrinsic" kinetic parameters (these values are not necessarily the same as those estimated from homogeneous catalysis by the native enzyme, but both are equal under conditions where the conformational and steric effects are negligible; see pp. 139–144 in Ref. 19). As a rule, the observed V', which is an "overall" rate taken to be the sum of all "local" rates, involves one or more of the effects caused by immobilization. A topic of great interest in previous studies[38–59] was how to estimate immobilization effects from the dependence of V' on the bulk concentrations of substrate, inhibitor, and other effectors. "Apparent" (or "effective") values for the saturation rate (V_{max}^{app}) and Michaelis constant (K_m^{app}) were then estimated from the dependence of V' on bulk substrate concentrations under conditions in which the other factors remained fixed, as well as in studies of the effects of inhibitors in homogeneous enzymatic catalysis.

According to most previous studies,[38,40,43,46–54] variations in K_m^{app} are due to the effects of the partitioning, microenvironment, and diffusion or mass transfer, all

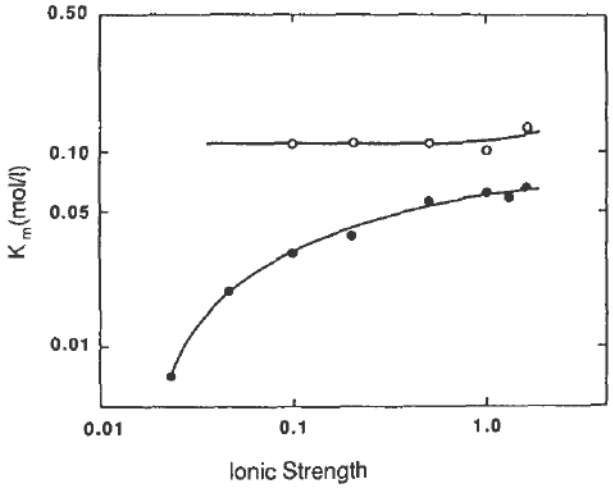


FIG. 4. Effect of ionic strength on the K_m values of native bromelain (○) and of bromelain immobilized on carboxymethyl cellulose (●) for the hydrolysis of *N* α-benzoyl-L-arginine ethyl ester at pH 7.0. The ionic strength was adjusted with KCl (from Wharton *et al.*[40]).

of which yield differences between the matrix and bulk phases in the concentrations of substrates and products. In particular, changes in K_m^{app} caused by the electrostatic partitioning effect have been widely investigated,[38,40,46–49] and it is known that K_m^{app} increases when the charges of the matrix and the substrate are the same in sign,[46,48] but decreases when they carry opposite charges.[38,40,47] In both cases, K_m^{app} approaches the intrinsic K_m when such electrostatic effects are eliminated by increasing the ionic strength. A typical example[40] of the change in K_m^{app} with ionic strength is shown in Fig. 4 (in this case the intrinsic K_m (K_m^{app} at very high ionic strength) is lower than the value of K_m for the native enzyme, suggesting that some interaction (possibly several) between the supporting matrix and the substrate, other than the electrostatic interaction, might be of significance in determining the magnitude of K_m^{app}).

Several early studies reported that changes in V_{max}^{app} upon immobilization generally fall into two types: the value remains unchanged[55–58] or decreases[42,48,49,59] upon immobilization. In contrast to K_m^{app}, however, the meaning of this phenomenon has not yet been discussed in sufficient detail in connection with the effects of immobilization. As can be seen from eq. (3), V_{max} is defined as a function of both k_{+2} and the enzyme concentration ($[E]_f$). Consequently, it seems to be true that V_{max}^{app} decreases as a result of steric hindrances and/or conformational changes when the amount of the enzymes immobilized without any change in k_{+2} is less than that of the enzymes initially applied. However, V_{max}^{app} remains unchanged in cases where such loss is negligible, even if the reaction is governed by diffusional and partitioning effects. This is because V_{max} is independent of substrate and product concentrations even when the concentrations of these species within the supporting matrices are, as mentioned

above, altered by diffusional and partitioning effects (this is not true in cases where the product acts as an inhibitor or accelerator or where other inhibitors or accelerators are present in the system[42]). Taking these results into account, a few studies have utilized the observed changes in V_{max}^{app} to characterize immobilized enzymes. For example, Kitano *et al.*[48] estimated the amount of alkaline phosphatase effectively bound onto anionic lattices (i.e. terpolymer of acrylic acid, styrene, and divinyl benzene) by comparing the V_{max}^{app} values of the native and immobilized enzymes, while Kokufuta and Takahashi[49] discussed a change in trypsin conformation due to complexation with a strong polyelectrolyte, potassium poly(vinyl alcohol), by studying changes in V_{max}^{app}.

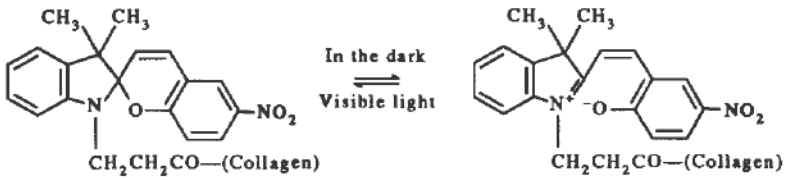
As a result, we can obtain information about the effects of the supporting matrices on immobilized enzyme reactions by monitoring changes in K_m^{app} and V_{max}^{app}. This approach is readily available for application in the design, construction and characterization of functional immobilized biocatalysts.

3. CONTROL OF ENZYMATIC ACTIVITY BY STIMULUS-SENSITIVE POLYMER SUPPORTS

In the nervous system, electrical stimulus triggers the release of synaptic transmitters which cause a sharp initiation and then termination of a specific enzyme reaction. This is a typical example of the signal-responsive control of biocatalytic activity. Immobilized biocatalysts, provided with such biological functions, have attracted considerable interest because of their potential applications in a variety of fields, for example, as biochemical switches, biosensors, drug delivery devices, controllers for bioreactors, etc. Several attempts have been made to regulate enzymatic processes using external stimuli such as light,[60] electric fields,[61] mechanical stress,[62–65] pH,[66,67] and temperature.[68,69] Polymeric fibers and membranes, liposomes, pH-sensitive microcapsules, and hydrogels have been employed as supports in the construction of signal-responsive immobilized enzymes.

3.1. *Photocontrol*

Several photosensitive molecules, such as compounds of spiropyran,[70] exist in either an ionized or non-ionized state under irradiation by light, the transition to which is usually accompanied by a color change known as a photochromic phenomenon. Karube *et al.*[60] attempted to immobilize urease within a photosensitive membrane prepared from collagen fibrils which had been modified with a spiropyran compound, β-1-(3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyran)):



(5)

The immobilization was carried out by casting a mixed suspension composed of the enzyme and the modified collagen fibrils onto a Teflon plate, followed by treatment with an aqueous glutaraldehyde solution. The membrane with the immobilized enzyme showed a photochromic color change; that is, it was colored red in the dark and bleached under irradiation with visible light. The enzymic activity of the membrane was assayed by measuring the concentration of ammonia formed via the hydrolysis of urea (eq. (6)), which was used as the substrate:

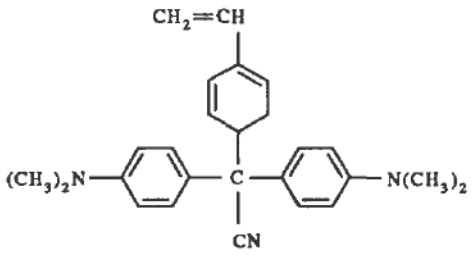
$$(NH_2)_2C{=}O + 3H_2O \rightarrow CO_2 + 2NH_4OH \qquad (6)$$

It was observed that activity under irradiation was reduced from that in the dark over the range of pH 5–8, with a shift of the optimum pH towards lower pH values. The value of V_{max}^{app} decreased with irradiation, but the K_m^{app} value (0.030 $\pm$ 0.002 M) remained unchanged: V_{max}^{app} (in μmol NH_3/min per mg of membrane) was 7.6 $\pm$ 0.3 in the dark and 4.9 $\pm$ 0.2 under visible light. On the other hand, the apparent diffusion coefficient (D) through the membrane for urea under visible light was found to be smaller than that in the dark, whereas the diffusivity of ammonium chloride under visible light was greater than in the dark: D in cm^2/sec = 1.4 $\pm$ 0.2 $\times$ 10^{-6} (urea) and 2.5 $\pm$ 0.1 $\times$ 10^{-6} (ammonium chloride) under visible light; 2.0 $\pm$ 0.1 $\times$ 10^{-6} (urea) and 1.9 $\pm$ 0.1 $\times$ 10^{-6} (ammonium chloride) in the dark. However, the reduced diffusivity of the substrate under visible light was not responsible for the decrease in activity because there was no change in K_m^{app}. In order to explain this contradiction, Karube *et al.* speculated that under visible light, the microenvironment around the immobilized enzyme loses hydrophilicity because of the deionization of the spiropyran molecules (see eq. (5)), which may reduce the rate of the decomposition of the enzyme/substrate complex into the products (ammonium and biocarbonate ions) (the present author notes that if this is the case, K_m^{app} may also vary, since K_m^{app} is a function of the decomposition rate (k_{+2}) of the ES complex; see eqs (1) and (3)).

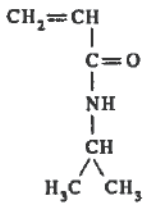
Prior to this study, the same research group reported the preparation of photosensitive enzymes by modifying α-amylase, α-chymotrypsin and urease with spiropyran.[71] Also, a spiropyran-bound enzyme was entrapped in a collagen membrane and attempts made to regulate its enzyme activity photochemically.[72]

As a result, it has become apparent that photosensitive polymers are available for the regulation of immobilized enzyme activities. To our knowledge, however, the sharp initiation–termination (on/off) control of immobilized enzyme

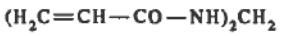
activity is still not possible using photochemical methods. A very recent study[73] has reported the synthesis of a photosensitive hydrogel consisting of bis(4-dimethylamino)phenyl-(4-vinylphenyl)methyl leucocyanide (BDVMLC; as photosensitive monomer), *N*-isopropylacrylamide (NIPA), and *N,N′*-methylenebis(acrylamide) (MBA; as cross-linker):



(BDVMLC)



(NIPA)

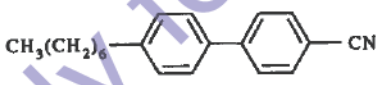


(MBA)

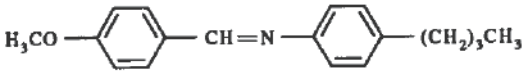
Under ultraviolet irradiation, the gel synthesized was found to undergo a discontinuous volume change at a specific temperature (this phenomenon has been known as the volume–phase transition of polymer gels, and will be discussed in detail in Section 7.1). Thus, such photosensitive polymers would seemingly be more useful as supports in the on/off control of immobilized enzyme activity.

3.2. *Control using electric fields*

The activity of immobilized enzymes can also be controlled by means of electric fields. A typical example of such a system is a collagen membrane into which lipase has been immobilized with a liquid crystal using the entrapping method.[61] To form the liquid crystal within the membrane phase, one of the following chemicals is used: 4-cyano-4′-heptylbiphenyl (CHBP) or 4′-methoxybenzylidene-4-*n*-butylaniline (MBBA).



(CHBP)



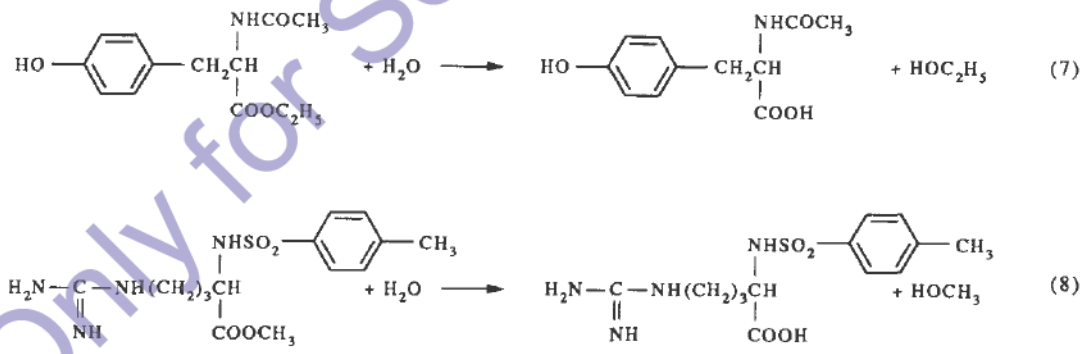
(MBBA)

The membrane with immobilized lipase exhibits only 3% of the activity of free lipase when using olive oil emulsions as the substrate. However, the immobilized enzyme activity is increased by a factor of 1.7 when the membrane is used for the cathode on a platinum electrode. This effect of the electric field is not found in a membrane which contains the enzyme but not the liquid crystal. In addition, the activities of the enzyme immobilized both with and without the liquid crystal are nearly independent of pH over the range of 4–8. Therefore, the observed increase under the electric field was interpreted in consideration of the following possible sequence of events: alkaline species are produced on the cathode during

electrolysis and dissociate the acidic groups in the collagen molecules, in turn swelling the membrane through their electrostatic interaction, thereby promoting the "lubrication" of the liquid crystal and consequently eliminating the diffusional resistance to the transport of the substrate from the outside to the inside of the membrane. When the on/off control of activity is tested by changing the terminal voltage (3, 0, and again 3 V), the initial activity at 3 V falls to about 60% at 0 V, then returns to the initial value. In this system, however, further activity control could not be performed because the membrane peeled from the surface of the cathode.

3.3. *Mechanical control*

A very interesting study based on a simple but clever idea has been reported by Klibanov *et al.*,[62] who attempted to control the activity of α-chymotrypsin or trypsin bound to a fine elastic polymer fiber, such as Nylon, using mechanical changes, i.e. the stretching and relaxation of the fiber support (see Fig. 5(A)). They assumed that the conformation of enzyme molecules rigidly attached to the fiber by several covalent bonds would be changed from a "catalytically active" to an "inactive" state by application of tension to the fiber support. The validity of this assumption was demonstrated by the results in Fig. 5(B), from which it can be seen that the activity of the bound α-chymotrypsin or trypsin decreased with an increase in the degree of fiber stretching when the enzymatic hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (see eq. (7)) or *Nα-p*-tosyl-L-arginine methyl ester (see eq. (8)) was studied using the stretching device shown in Fig. 5(C).



It should be noted that since the observed drop in activity was due to a decrease in V_{max}^{app} but not to a variation in K_m^{app}, diffusional or mass-transfer effects were not dominant factors in this system. This idea, proposed by Klibanov *et al.*, was then applied to the activity control of glucose oxidase adsorbed onto a porous poly(vinyl chloride) membrane,[65] although in this case changes in both V_{max}^{app} and K_m^{app} were observed with stretching.

Prior to the publication of their results, Klibanov *et al.*[18,63] succeeded in regulating the activity of fiber-bound α-chymotrypsin using the stretch–relax

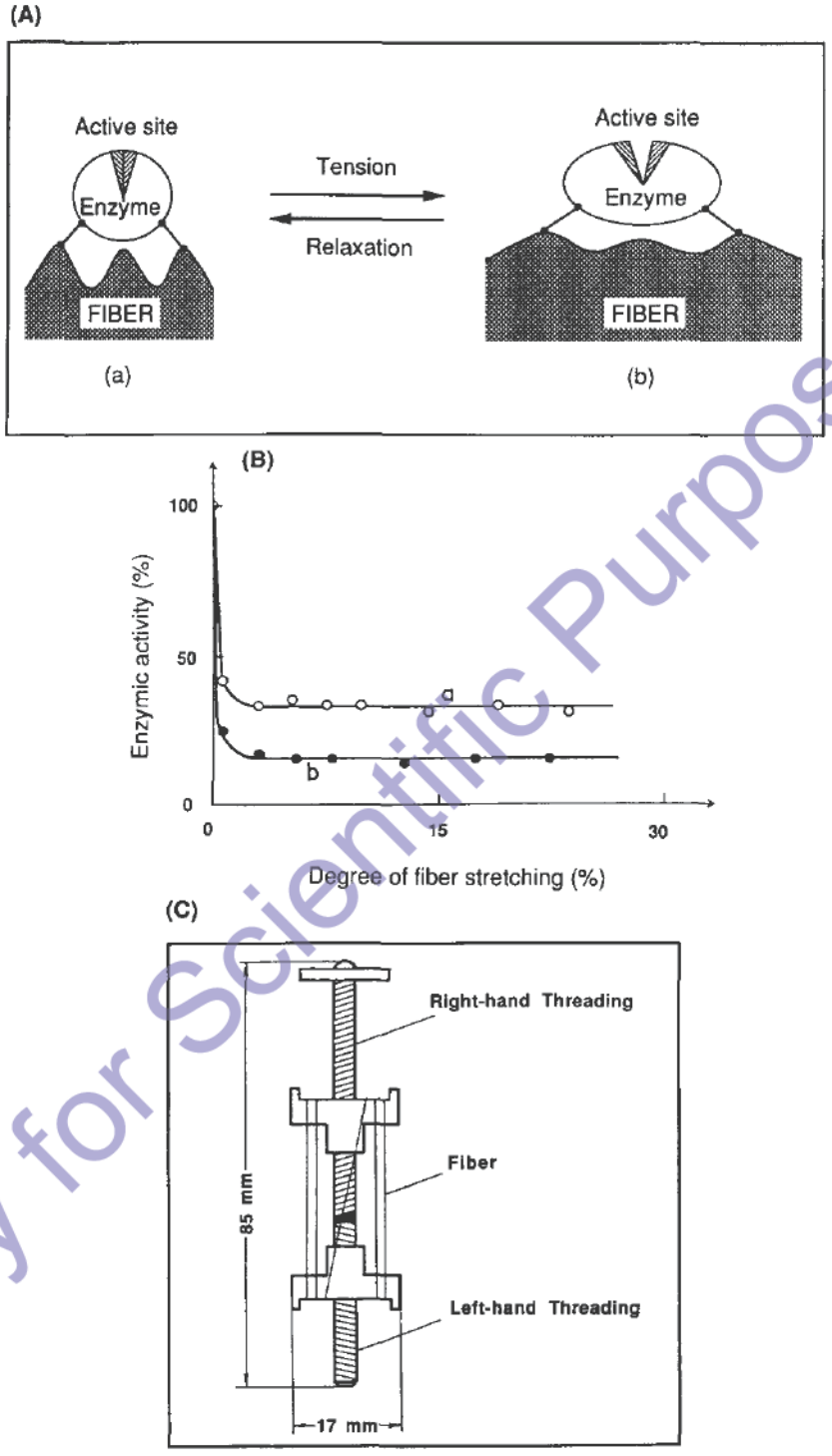


FIG. 5. Mechanical control of immobilized enzyme activity: (A) Schematic illustration of reversible deformation of enzyme molecule bound to fiber induced by stretching: (a) normal fiber and (b) stretched fiber. (B) Changes in activities of Nylon fiber-bound α-chymotrypsin towards *N*-acetyl-L-tyrosine ethyl ester (6 mM; ○) and trypsin towards *N* α-*p*-tosyl-L-arginine methyl ester (3 mM; ●) with the degree of stretching of the fiber (pH 8.0; ionic strength 0.1 adjusted by KCl; 25°C). (C) Stretching device for enzyme-carrying fibers (from Klibanov *et al.*[62]).

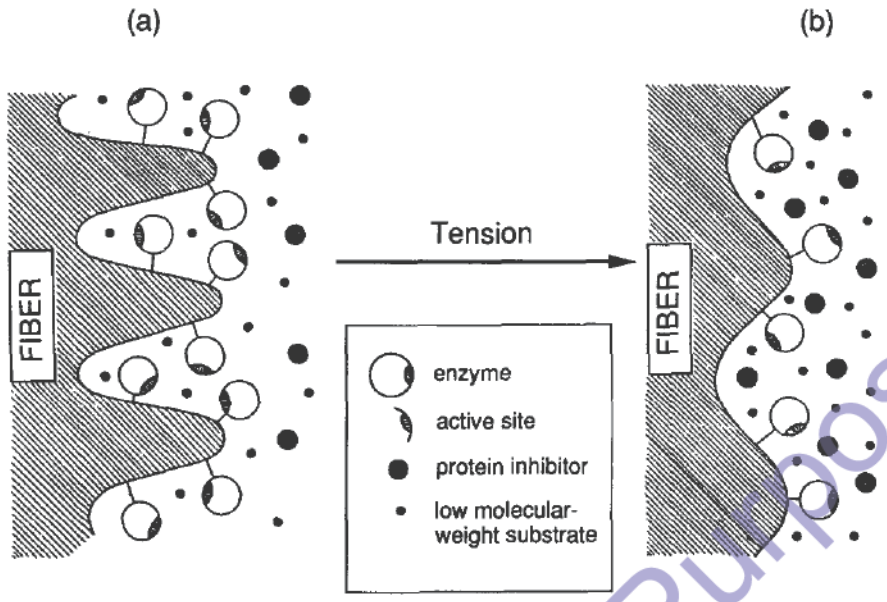


FIG. 6. Schematic illustration of interaction of Nylon fiber-bound α-chymotrypsin with high molecular-weight inhibitor (protein) and low molecular-weight substrate: (a) unstretched fiber and (b) stretched fiber (from Klibanov *et al.*[63] this figure is taken from p. 570 in Ref. 18).

cycle (Fig. 6) in the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester in the presence of pancreatic trypsin inhibitor (a high molecular-weight inhibitor). The principle utilized is related to the control of the support-induced steric hindrance effect on the association of the inhibitor with the immobilized enzyme; that is, activity is retained under unstretched conditions of the fiber because the inhibitor is not easily accessible to the enzyme, whereas the stretching of the fiber favors access by the inhibitor and thus causes a fall in activity. In constructing a biocatalyst system according to this principle, the primary requirement for reversible control of its catalytic activity is that the inhibitor should be of a competitive type, along with the impossibility of contact with the active site under unstretched conditions of the support. Needless to say, an inhibitor cannot be used which will become concentrated within the microenvironment as the result of its interaction with the supporting matrix. In fact, one inhibitor from soybeans (soybean inhibitor) may not have been effective in the reversible regulation of the fiber-bound, α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester because it may not have satisfied these requirements.

The regulation of enzymic activity by mechanical means is also possible in a gel consisting of acrylamide (AAm).[18,64] For example, when the trypsin-catalyzed conversion of chymotrypsinogen to chymotrypsin, i.e. the tryptic activation of chymotrypsinogen, was studied in a gel block with a high polymer concentration (33.5%, w/w), the rate of the reaction increased to about 20 times that in the initial state as the result of a 35% vertical compression of the block; following

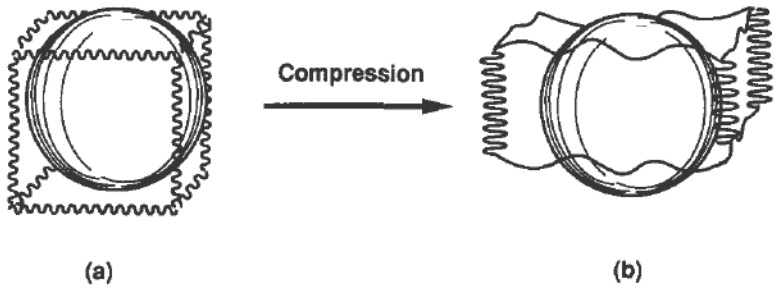


FIG. 7. Schematic illustration of an elementary unit of polymeric gel with entrapped enzyme molecule (a) before compression and (b) after compression (from Berezin *et al.*[64] this figure is taken from p. 576 in Ref. 18).

decompression, this increased activity fell back to its initial low level.[64] In order to explain such mechanical changes in activity, the model shown in Fig. 7 has been proposed. Protein molecules, such as trypsin (as the enzyme) and chymotrypsinogen (as the substrate), are assumed to be entrapped in a certain elementary unit of the three-dimensional lattice of the gel; the protein globule cannot emerge from this lattice unit if the polymer concentration of the gel is sufficiently high, i.e. there is almost no diffusion through the gel porosity. When pressure is applied perpendicular to the upper facet, the unit will strain into a parallelepiped. The volumes of both units before and after compression are equal, because the gel, like water, is practically incompressible. Thus, the compression may simultaneously induce a decrease in the areas of the side facets and an increase in the area of the upper and lower facets of the lattice unit. This would drastically change the rate of diffusion, and hence the diffusion-controlled reaction rate. As a result, the conclusion drawn from the model in Fig. 7 is that mechanical compression of the gel may, in principle, facilitate diffusion, thereby increasing the rate of the reaction between the protein molecules (the enzyme and the substrate) within the gel phase.

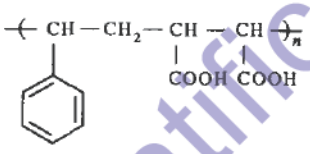
3.4. *Control using minor pH differences*

In several of the immobilized biocatalyst systems described above, catalytic activity could be reversibly controlled by the application and removal of external stimuli. However, a complete shutdown of activity has not yet been achieved. One goal of studies on functional immobilized biocatalysts in this field is the complete on/off control of enzyme activity. Along this line, Kokufuta *et al.*[66,67] studied a new type of microencapsulated enzyme in which capsules with a surface-coating of polyelectrolyte were able to shut off the permeation of substrates through the capsule membrane in response to small differences in pH (± 0.5 units).

Since the preparation of enzyme-loaded microcapsules by Chang,[74] many attempts have been made to encapsulate aqueous enzyme solutions or cell

suspensions within semipermeable polymeric membranes. Parallel to, but independent of, the research in this field, Okahata *et al.*[75,76] studied functional microcapsules which allow the control of the permeation of water-soluble substances through the capsule membranes using external stimuli. With respect to the signal-responsive on/off control of biocatalytic activity, the most useful preparation which they studied[76] may be pH-sensitive microcapsules with surface-grafted polyelectrolytes, the permeability of which can thus be varied according to the pH level of the outer medium. However, since the polyelectrolytes used in the grafting were poly(acrylic acid) and poly(vinyl pyridine), changes in the permeability were observed over a wide pH range (3–12), at which enzyme activities are also changed. Therefore, the microcapsules prepared by Okahata *et al.*[76] cannot be directly adapted for the present purpose. In addition, there is worry concerning a loss in enzyme activity during the grafting procedure, which is usually made under severe chemical conditions.

Several polyelectrolytes involving polypeptides are known to undergo a conformational transition in a specific and very narrow pH range. For example, the following alternative copolymer of styrene and maleic acid,



shows a rapid increase in solution viscosity at pH = 4.5 when polyion charges increase, as demonstrated by the pH dependence of electrophoretic mobility (see Fig. 8(A)).[77] This polyelectrolyte property is distinct from those of other poly (dicarboxylic acids), such as an alternative copolymer of methyl vinyl ether and maleic acid[77,78] and poly(itaconic acid).[77] These results suggest that the copolymer of styrene and maleic acid undergoes a conformational transition from a tighly coiled chain to an extended one (see Fig. 8(B)) when the hydrophobic interaction between the styrene units is overcome by the electrical force arising from the dissociation of the maleic acid units. Thus, the problems described above seem to be solved if this kind of polyelectrolyte is adsorbed onto the outer surface of a microcapsule without covalent bonding.

The pH dependence of the permeability constant for the copolymer-coated microcapsules is shown in Fig. 9(A),[67] from which we can see a rapid change in the permeability at pH 5.5 $\pm$ 0.5, close to the pH range at which the conformational transition has been observed. Therefore, the role of the copolymer in the permeation process can be explained using the schematic illustration in Fig. 9(B): in the range of pH $<$ 5, the capsule surface is covered with a flat layer of contracted and entangled copolymer chains, which reduces its permeability; and at pH $>$ 6, the expansion of the copolymer chains on the capsule surface possibly forms a looped layer which enhances permeability. This permeation characteristic of the prepared pH-sensitive microcapsule made possible the initiation–termination control of the invertase-catalyzed hydrolysis of sucrose

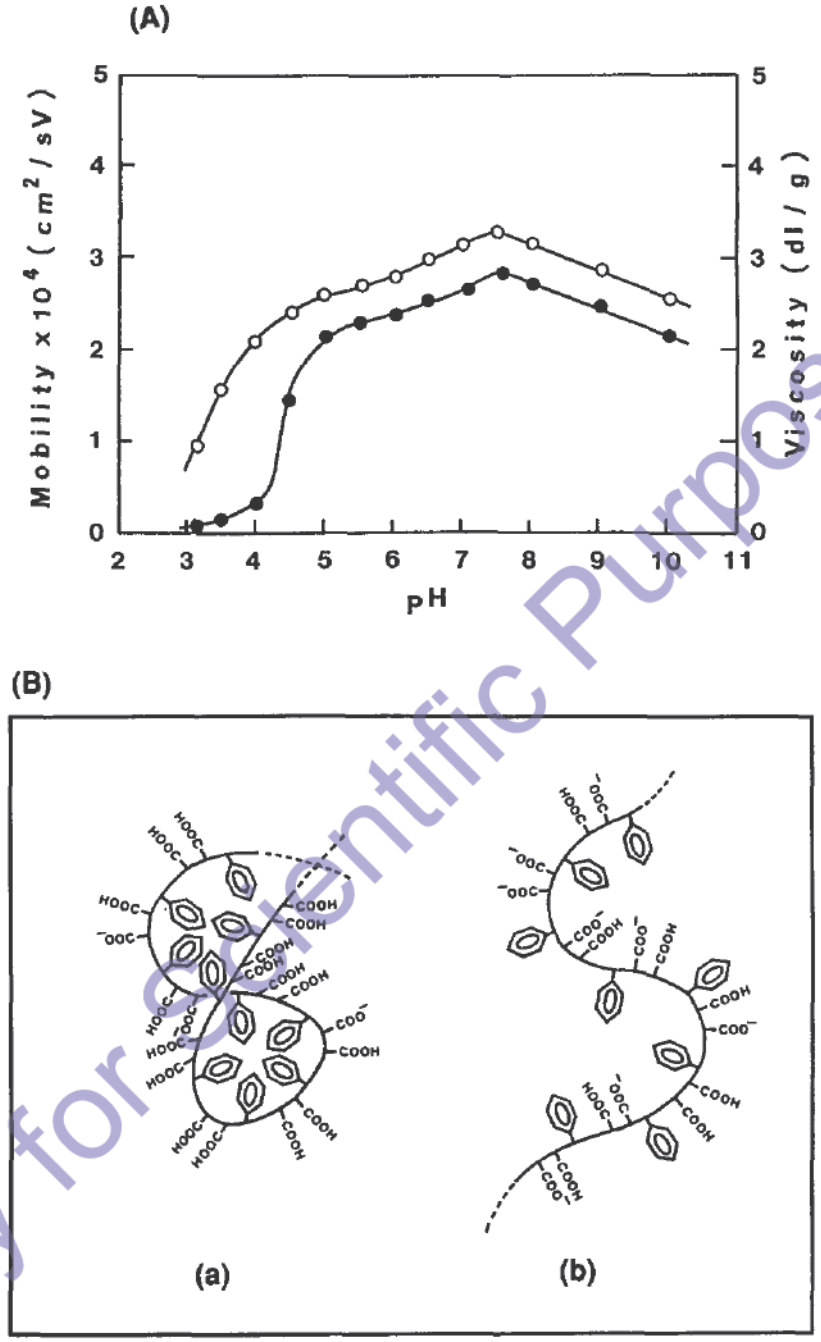


FIG. 8. Conformational transition of alternative copolymer consisting of styrene and maleic acid: (A) pH dependence of mobility (○) and viscosity (●), both of which represent intrinsic values obtained by extrapolation to zero polymer concentration of linear plots of mobility or viscosity against a polymer concentration in the range of 0.005–0.2 g/dl. (B) Schematic illustration of conformational transition of (a) tightly coiled chain due to hydrophobic interaction between the phenyl groups to (b) extended chain due to electrical repulsion between the charged carboxylic groups (from Kokufuta *et al.*[67,77]).

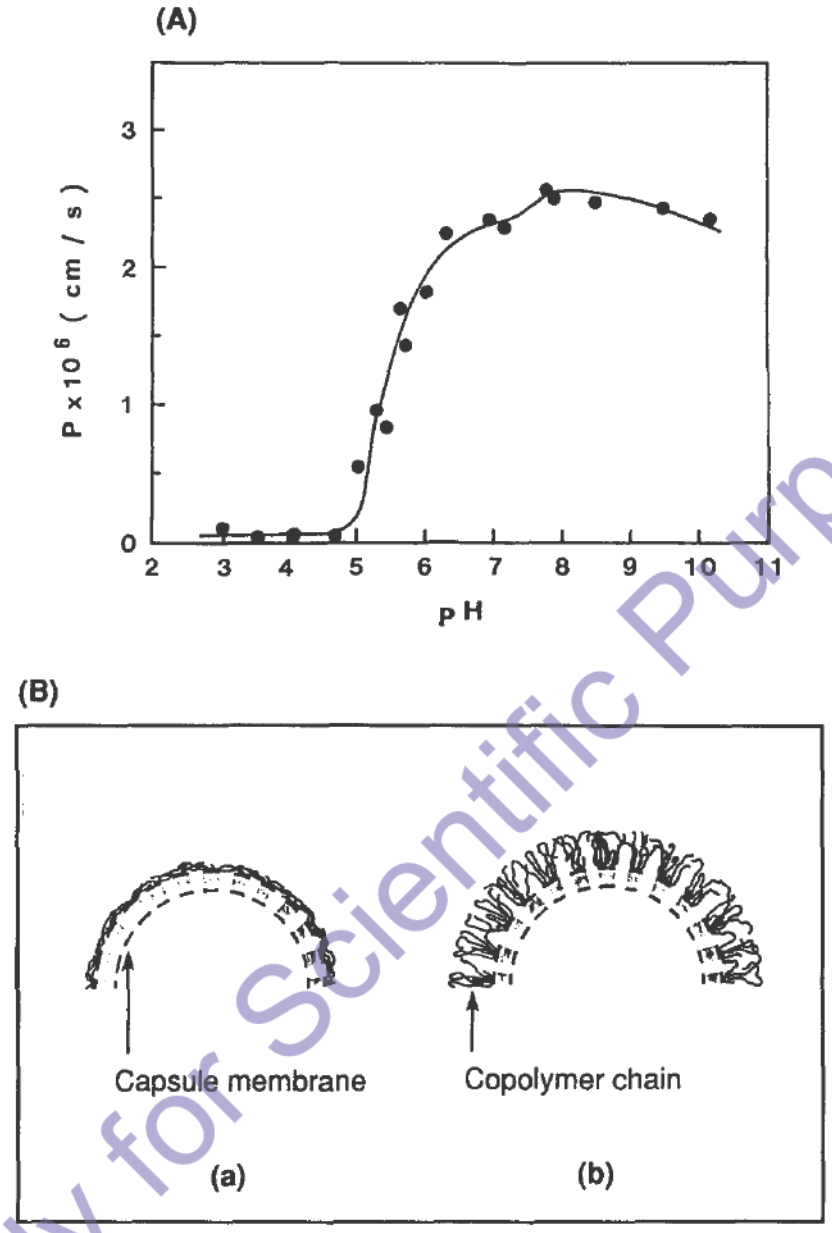


FIG. 9. pH-sensitive poly(styrene) microcapsules with surface-coating of alternative copolymer of styrene and maleic acid: (A) pH dependence of permeability constant estimated by use of *n*-propyl alcohol as permeate. (B) Schematic representations for understanding role of copolymer coating on outer surface of capsule membrane: (a) in the acidic pH range, permeability was reduced because the membrane surface was covered with tightly coiled chains; (b) in the neutral or alkaline pH range, permeability was enhanced because the membrane surface was covered with extended chains (from Kokufuta *et al.*[67]).

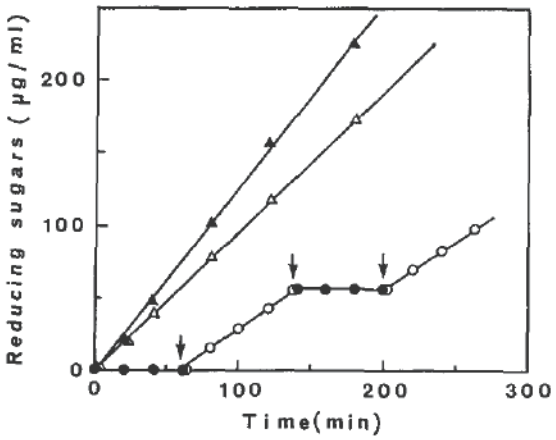


FIG. 10. Initiation–termination control of hydrolytic reaction of sucrose in aqueous suspension of copolymer-coated (○, ●) or uncoated (▲, △) pH-sensitive microcapsules containing invertase at pH 4.5 (▲, ●) and pH 5.5 (○, △). Arrows indicate pH adjustment made by rapid addition of a small amount of 2 M HCl or NaOH into the capsule suspension (from Kokufuta *et al.*[67]).

(Fig. 10).[67] With the uncoated capsules, the encapsulated enzyme catalyzed the hydrolytic reaction not only at pH 5.5 but also at 4.5, to form both glucose and fructose. In contrast, when the pH-sensitive capsules were employed at pH 4.5, the catalytic action of the loaded enzyme was almost or entirely depressed (the concentration of the reducing sugar was less than 0.1 μg/ml). Such on/off control could be repeated reversibly throughout a single run of measurements. In addition, repeated measurements over at least 8 days gave excellent reproducibility without damaging the capsules.

According to the same idea, Kokufuta *et al.*[66] succeeded in regulating the initiation and termination of the enzymatic hydrolysis of maltotriose in β-amylase-loaded pH-sensitive poly(styrene) microcapsules with a surface-coating of poly(iminoethylene) (see Refs 79–81 for the properties of this polyelectrolyte):

$$\text{⌐}\text{¬}\text{-(CH}_2\text{-CH}_2\text{-NH)}_x\text{-⌐}\text{¬}\text{-(CH}_2\text{-CH}_2\text{-N-)}_y\text{-⌐}\text{¬}\text{-(CH}_2\text{-CH}_2\text{-NH}_2\text{)}_z$$

$$x:y:z = 2:1:1.$$

In this case, a pH-induced transition of the conformation of the branched polycation in the solution (and also on the capsule surface)[66] was observed at pH 6.0. This is because the contracted and entangled chain resulting from the hydrogen bonding between the functional groups (NH_2-, $-NH-$ and $-N=$) changes very rapidly or discontinuously into the expanded one when the polyion charges are increased by the protonation of these functional groups. The concept introduced here, therefore, should be of general applicability, and a variety of enzyme reactions in microcapsules could be regulated if we could use or synthesize polyelectrolytes that undergo conformational transitions at different pH values.

3.5. *Control using temperature changes*

It is well known that in many polymer gels, a reversible and discontinuous volume change (i.e. volume–phase transition), often as large as several hundred times, occurs in response to changes in external factors such as temperature, solvent composition, pH, ionic strength, small electric field, and light (see Section 7.1). Such volume changes can be accompanied by variations in the mass-transfer rates of solutes from the outside to the inside of the gel, and vice versa. Thus, gels that undergo a large volume change in response to external stimuli may serve as supports for constructing various types of stimulus-sensitive immobilized biocatalysts. The gels studied so far represent only two kinds of the thermosensitive type,[68,82,83] both of which undergo sharp but not discontinuous volume changes with temperature. One is a lightly cross-linked copolymer gel consisting of NIPA and AAm which has been used by Dong and Hoffman[82,83] for the immobilization of asparaginase (L-asparagine amidohydrolase). From their detailed investigations on the temperature dependence of the activity of the immobilized enzyme, they proposed that the gel may be used to control the initiation and termination of immobilized enzyme reactions by temperature. However, this proposal was not realized by their experimental data.

The complete on/off control of enzyme activity using a thermosensitive gel was then performed by Kokufuta *et al.*,[68] who prepared a gel with immobilized exo-1,4-α-D-glucosidase by cross-linking an aqueous poly(vinyl methyl ether) solution with γ-ray irradiation. The resultant gel exhibited a thermosensitive characteristic: it shrank above 38°C and swelled below this transition temperature (T_c), as shown in Fig. 11(A). This behavior was reversible. The permeability of the gel for glucose thus changed dramatically above and below T_c: $2.0 \times 10^{-6}\,cm^2 \cdot sec^{-1}$ at 32°C and $7.3 \times 10^{-11}\,cm^2 \cdot sec^{-1}$ at 42°C. As a result, the immobilized preparation obtained displayed an excellent capacity for the on/off control of the enzymatic hydrolysis of maltose (see Fig. 11(B)). When the immobilized enzyme was utilized, glucose formation from maltose halted at 42°C, but immediately recommenced when the temperature was lowered to 32°C. Such initiation–termination control could be repeated reversibly throughout a single run of the measurements and reproduced without a serious loss in activity for at least 20 runs carried out with a freshly prepared substrate solution. In conclusion, this result clearly demonstrates the potential utility of thermosensitive gels in the on/off regulation of immobilized enzyme reactions.

Very recently, Inomata *et al.* have reported that the T_c of thermosensitive gels

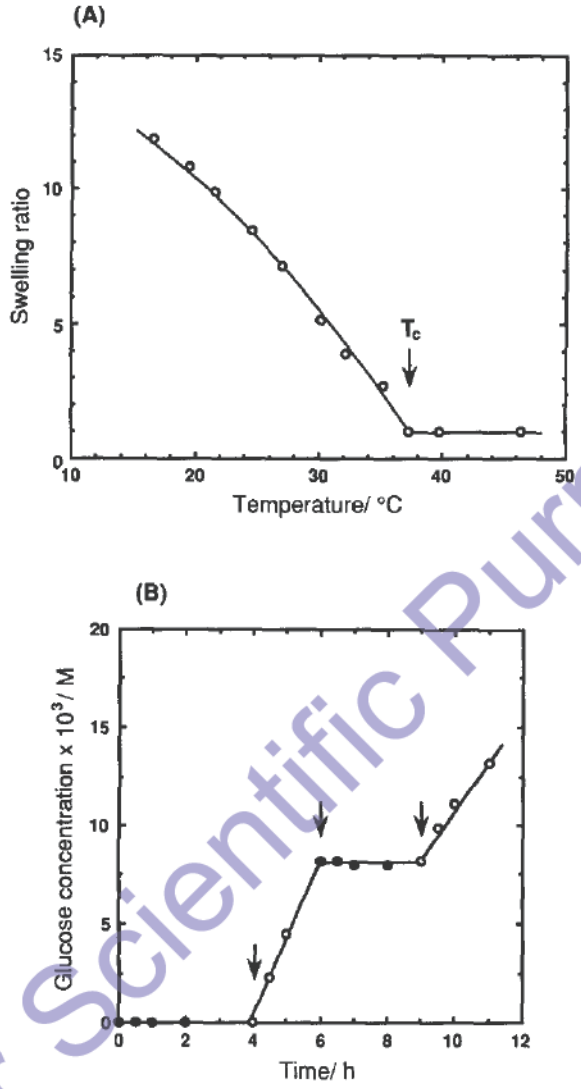
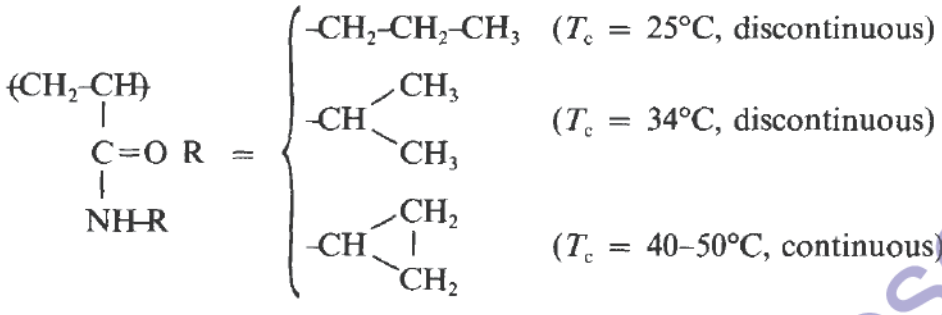


FIG. 11. Application of thermosensitive poly(vinyl methyl ether) gel in the initiation–termination control of immobilized enzyme reaction: (A) Swelling curve of gel as a function of temperature, data normalized by dividing gel volume at an arbitrary temperature by volume of the completely collapsed gel at temperatures above 37°C. (B) Temperature-responsive initiation–termination control of glucose formation by gel-entrapped exo-1,4-α-D-glucosidase, demonstrating that the enzymatic reaction was depressed at 42°C (●), but commenced as the temperature jumped to 32°C within 2 min (○). Activity of the immobilized enzyme can be switched on and off repeatedly by rapidly decreasing the temperature from 42 to 32°C and rapidly increasing it from 32 to 42°C at the times indicated by arrows. The average rate of glucose formation in the two "on" states during a single run at 32°C changed from 58 ± 8 M · min^{-1} (initial run) to 45 ± 8 M · min^{-1} (final run) when the experiment was repeated in a series of 20 runs (results in (B) show initial run) (from Kokufuta *et al.*[68]).

consisting of the following *N*-substituted acrylamide derivatives varies depending on the species of the substituent groups:[84]



This suggests the possibility of various types of thermosensitive gel for use in altering the temperature range in the on/off regulation of immobilized enzyme reactions. Thus, the use of thermosensitive gels is expected to expand in the field of stimulus-sensitive immobilized enzymes.

Prior to these publications, Matsuoka *et al.* studied another type of thermosensitive immobilized enzyme using a liposomal membrane.[69] The membrane of a particular phospholipid such as dipalmitoyl phosphatidylcholine was known to undergo a phase transition at a specific temperture;[85] thus, the permeation of an aqueous solution containing substrates may be inhibited in the range below this phase transition temperature. Liposomes with immobilized urease were prepared on the basis of this idea using the following procedures: first, the enzyme was covalently bound with the aid of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate to the purple membrane isolated from *Halobacterium halobium*; the purple membrane-bound enzyme was then incorporated into liposomes of dipalmitoyl phosphatidylcholine by sonication. The preparation did not exhibit enzyme activity in the temperature range $< 42°C$, which was close to the phase transition temperature; but when it was heated above 42°C, the amount of activity gradually increased. The on/off control of the enzymatic decomposition of urea could thus be performed by switching the temperature from 36.5 to 45.6°C, and again to 36.5°C. In this immobilized system, however, the repetition of this temperature cycle brought about a slight "leak" of activity in the off condition (see Fig. 9 in Ref. 69).

4. ENHANCEMENT OF PARTITION OR DIFFUSION OF SUBSTRATES USING FUNCTIONAL POLYMER SUPPORTS

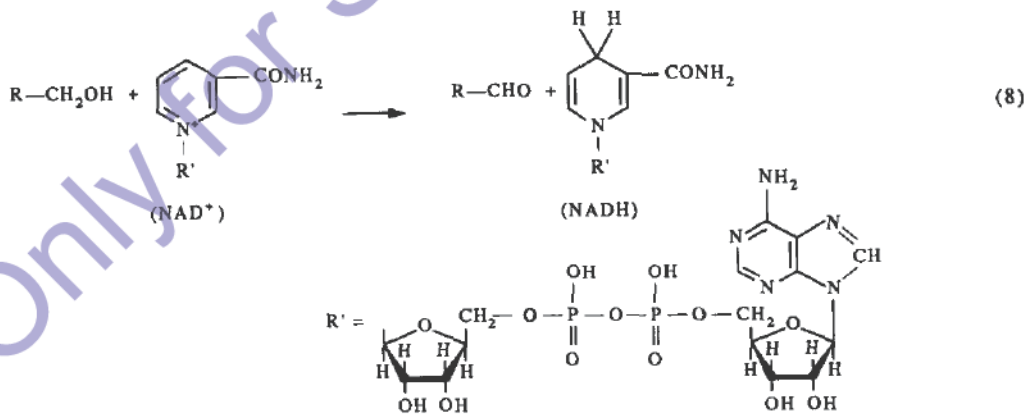
The main problem in the on/off control of immobilized enzyme activity was how to shut off the diffusion of substrates from the outside to the inside of the support. In contrast, the present section deals with approaches for enhancing the diffusion or partition of substrates. This is a very important subject in the research field of immobilized biocatalysts, because immobilization generally causes a fall in activity due to the external and internal diffusion limitations of

substrates. Three different principles have been used to resolve this diffusion problem.

4.1. *Supporting matrices with ionic or hydrophobic moieties*

A classic but important tool is the enhancement of the affinity of a supporting matrix towards a substrate by controlling the electric or hydrophobic interactions of the two. In the case of an electrostatic interaction between the support and substrate, both of which carry charges which are opposite in sign, the immobilized preparation exhibits an activity higher than that of the native enzyme under the same conditions of measurement. This is because the substrate concentration of the microenvironment around the immobilized enzyme is enhanced through the partitioning effect due to electrostatic attraction. As was previously shown in Fig. 4, therefore, K_m^{app} varies depending on the ionic strength of the bulk medium.[40] Such electrostatic activation of immobilized enzymes has been studied extensively and in detail by many authors.[38–40,47]

The effects of a hydrophobic microenvironment on the partitioning of a substrate were investigated by Johansson and Mosbach.[43] They used two polymer gels which were obtained from the polymerization of AAm or an AAm/methyl acrylate (MA) mixture (75 : 25 by weight) in the presence of MBA as a cross-linker. Hose liver alcohol dehydrogenase was then coupled to the cross-linked polymers using the glutaraldehyde method. In these polymers, AAm residues served as the hydrophilic component and MA residues as the hydrophobic component. Dehydrogenase activity was assayed by investigating the reduction of nicotinamide adenine dinucleotide (NAD^+) during the enzymatic oxidation of ethanol (hydrophilic substrate) and *n*-butanol (hydrophobic substrate).



(8)

As a result, an increase in the hydrophobicity of the support due to the copolymerization of MA caused a decrease in the K_m^{app} value of the bound enzyme for the more hydrophobic *n*-butanol substrate, whereas the K_m^{app} value for ethanol was essentially unaffected (see Table 1). The assumption that the hydrophobic microenvironment consisting of MA residues will preferentially adsorb the more

TABLE 1. Effect of matrix hydrophobicity on Michaelis–Menten constants for dehydrogenation of ethanol and *n*-butanol catalyzed by immobilized alcohol dehydrogenase at pH 8.0

Enzyme	Michaelis–Menten constant (mM)*†		Radioactivity of supernatant solution after equilibration‡			
	Ethanol	*n*-Butanol	[1-^{14}C]ethanol		*n*-[1-^{14}C]butanol	
			Supernatant (cpm/ml)	Adsorbed§ (fold)	Supernatant (cpm/ml)	Adsorbed§ (fold)
Soluble (native)	0.30	0.09				
Bound to polymer gel of acrylamide	0.57	0.16	11.140	1.0	37.575	1.0
Bound to copolymer gel of acrylamide/methyl acrylate (75 : 25, w/w)	0.54	0.04	11.210	0.96	33.350	5.9

*The NAD^+ concentration was kept constant at 2 mM and the alcohol concentration was varied from 0.16 to 5.0 mM for ethanol and 16 to 590 μM for *n*-butanol.

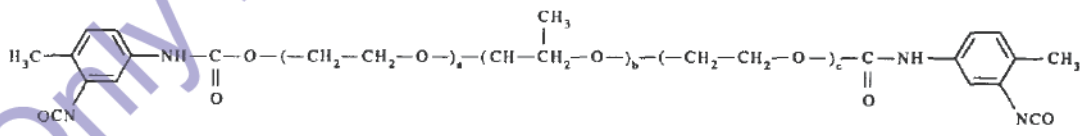
†Data represents K_m for native enzyme and K_m^{app} for immobilized enzymes.

‡Adsorption of the labelled substrate, ethanol (300 μM, 8 ml total volume) or *n*-butanol (100 μM, 8 ml total volume), to the glutaraldehyde-treated polymer gels (150 mg by dry weight) after 2 hr equilibration was determined by measuring the amount of radioactive alcohol remaining in the supernatant solution.

§Data was calculated assuming adsorption to be restricted to the volume occupied by the polymer gel (from Johansson and Mosbach[43]).

hydrophobic substrate was substantiated by equilibrium studies with *n*-[^{14}C]-butanol.

The following urethane prepolymers (as liquids) containing different amounts of ethylene glycol and propylene glycol units have been investigated for the purpose of controlling the hydrophobicity of supporting matrices.[86–90]



The prepolymers are miscible with water, but do not undergo the polymerization reaction ($2OCN{-}R + H_2O \rightarrow R{-}HNCONH{-}R + CO_2$) in an aqueous system under cooling.[86] The immobilization can be carried out without any cross-linker by raising the temperature after the prepolymers have been mixed with a chilled aqueous solution (or suspension) containing enzymes, microbial

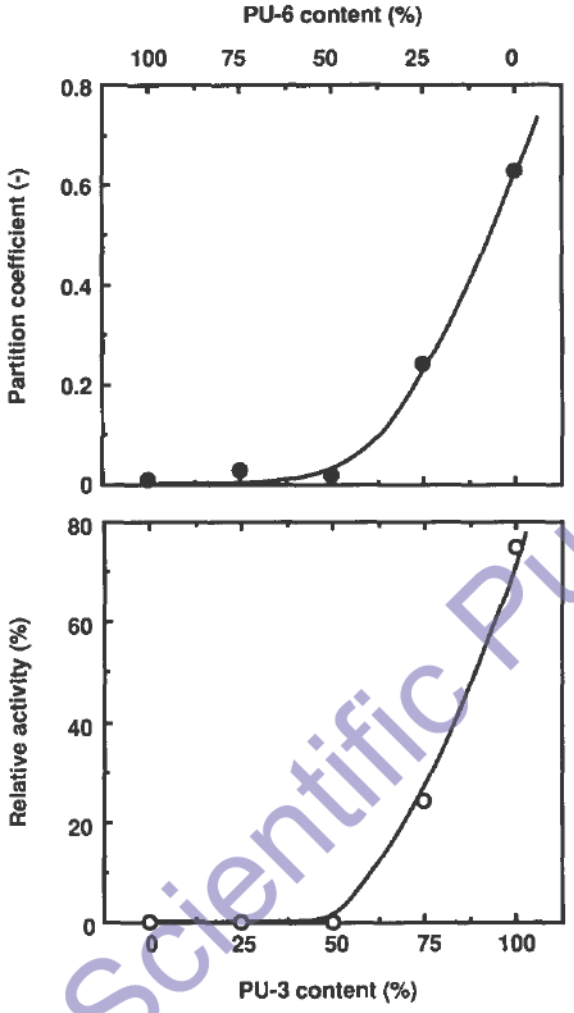
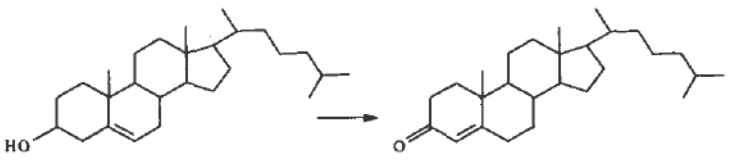


FIG. 12. Effect of hydrophobic PU-3 content of supporting matrix on cholesterol-transforming activity of immobilized *Nocardia rhodocrous* cells and on substrate partition between the bulk and matrix phases (from Omata *et al.*[87]).

cells or organelles. Figure 12 depicts a typical example of the hydrophobic microenvironment effect in the immobilized *Nocardia rhodocrous* cell-catalyzed transformation of cholesterol to cholestenone,[87]



(10)

in a water-saturated mixture of benzene and *n*-heptane (1 : 1 by volume). The cells were entrapped in a variety of gels with different ratios of the ethylene glycol

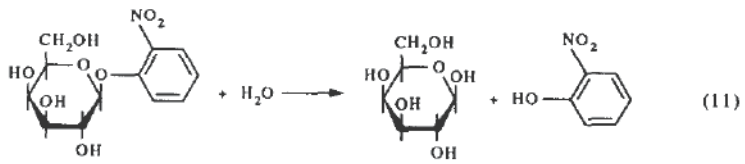
and propylene glycol units obtained from the two urethane prepolymers; hydrophobic PU-3 (M_w of polyol = 2529, NCO content = 4.2%, ethylene glycol content = 57%) and hydrophilic PU-6 (M_w of polyol = 2627, NCO content = 4.0%, ethylene glycol content = 91%). The activity of the immobilized cells was found to increase markedly when the content of hydrophobic PU-3 exceeded 50%. This result corresponds to the change in the partition coefficient with the PU-3 content, indicating that an increase in the hydrophobicity of the supporting matrices facilitated the partition of the substrate and raised the activity of the immobilized biocatalysts.

In addition, several photo-crosslinkable prepolymers[87–92] have been used in enzyme and cell immobilization as supporting matrices whose hydrophobicity can be controlled by changing the ratio of the ethylene glycol and propylene glycol units. In summary, the copolymerization of hydrophobic and hydrophilic monomers serves as a tool for controlling the activity of immobilized biocatalysts towards hydrophobic substrates, while in the case of ionic substrates, activity can be controlled by the copolymerization of ionic monomers.

4.2. *Polymer gels capable of facilitating pore diffusion of substrates*

One approach for enhancing the diffusion of substrates in immobilized reactions could involve the simulation of a biological transport system in which a variety of solutes penetrate membranes via a facilitated diffusion mechanism, which differs from the more usual simple diffusion. It is well known that a mobile carrier, often a protein with a high affinity for the molecule to be transported, plays an important role in the facilitated diffusion process (see, for example, pp. 266–275 in Ref. 93). On the basis of this idea, a model immobilized enzyme system has been designed and prepared[94,95] which is capable of promoting the pore diffusion not only of a low molecular-weight substrate but also of a polymeric substrate. Polymer gels were used as the support into which enzymes were coentrapped with carriers having a binding affinity towards the substrates.

A gel-entrapped β-D-galactosidase with the ability to facilitate the diffusion of a sugar substrate from the aqueous to the gel phase can be prepared by coentrapping a sugar-binding protein, caster bean lectin (CBL), as the carrier.[95] A polyelectrolyte complex-stabilized calcium alginate gel in the form of beads, which had already been studied by Kokufuta *et al.*[96] for the purpose of entrapping enzymes and cells (see Fig. 13), was used in order to avoid the leakage of the entrapped enzyme and lectin from the support. The following enzymatic hydrolysis was studied using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) as a model substrate for β-lactose,



(11)

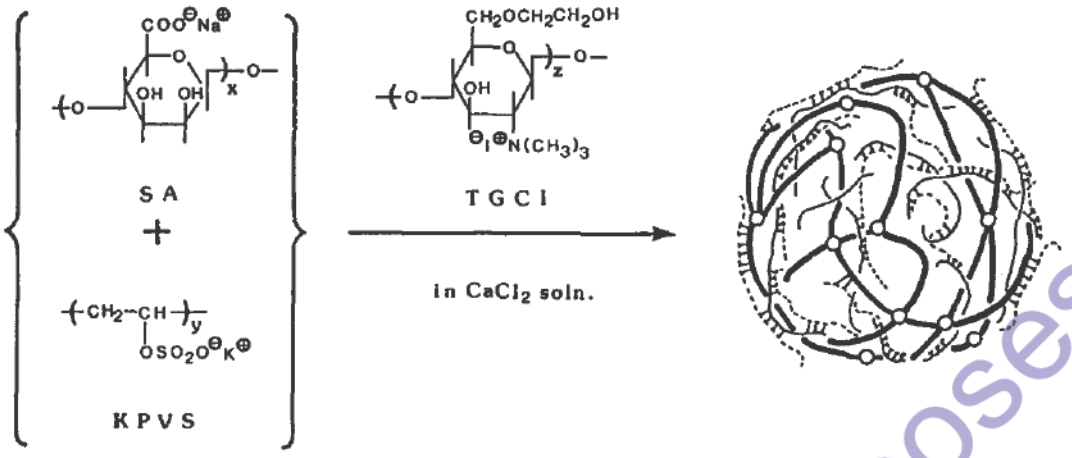


FIG. 13. Scheme for preparation of polyelectrolyte complex-stabilized calcium alginate gel beads: (=) alginate chain (——) KPVS chain; (– – –) TGCI chain; (ω,ω) salt linkages; (○) Ca^{2+} ion. A typical immobilization of enzymes (or cells) using the present method can be performed as follows: an aqueous 2% sodium alginate solution containing KPVS (10–75 mM) and an adequate amount of enzymes (or cells) is added dropwise to a gently stirred 100 mM $CaCl_2$ solution containing TGCI (10–75 mM), and the resulting gel beads are then cured in the same solution by stirring for 2–5 hr (from Kokufuta *et al.*[96]).

because CBL has been known to exhibit a high binding affinity towards β-lactose.[97] As shown in Fig. 14(A), the activity of the immobilized enzyme in the absence of the lectin carrier is about 35% that of the native enzyme. However, the presence of the carrier leads to a dramatic increase in activity, and at a lectin level ($[L]_g$) of 0.625 mg/ml gel, the activity of the immobilized enzyme exceeds that of the native enzyme by approximately 10%. From careful experiments, this lectin-induced activation was found to be due neither to a shift of the pH-activity profile upon immobilization nor to the formation of a complex between the enzyme and the lectin. The K_m^{app} and V_{max}^{app} values were thus estimated by analyzing the kinetic data obtained as a function of the substrate concentration and the lectin content. A rapid decrease in K_m^{app} with increasing $[L]_g$ can be seen from the results in Fig. 14(B), whereas the variation in the V_{max}^{app} (0.70 ± 0.05 μM/min) observed was independent of $[L]_g$ and within the limit of error in repeated activity measurements. In addition, an experiment using a gel which contained CBL but not the enzyme showed that at different levels of $[L]_g$, the equilibrium concentration of ONPG in the gel phase is usually higher than that of the aqueous phase. Therefore, the mechanism of the lectin-induced activation of gel-entrapped β-D-galactosidase was interpreted as shown in Fig. 14(C): the lectin as the carrier (C) in the gel attracts the substrate (S) from the bulk water to form a complex (C–S). When the resulting C–S complex is hydrolyzed by the catalytic action of the enzyme (E) to yield the product (P), the regenerated C again forms the C–S complex and the process is repeated. Even though the diffusion rates of the C–S and C, both of which have high molecular

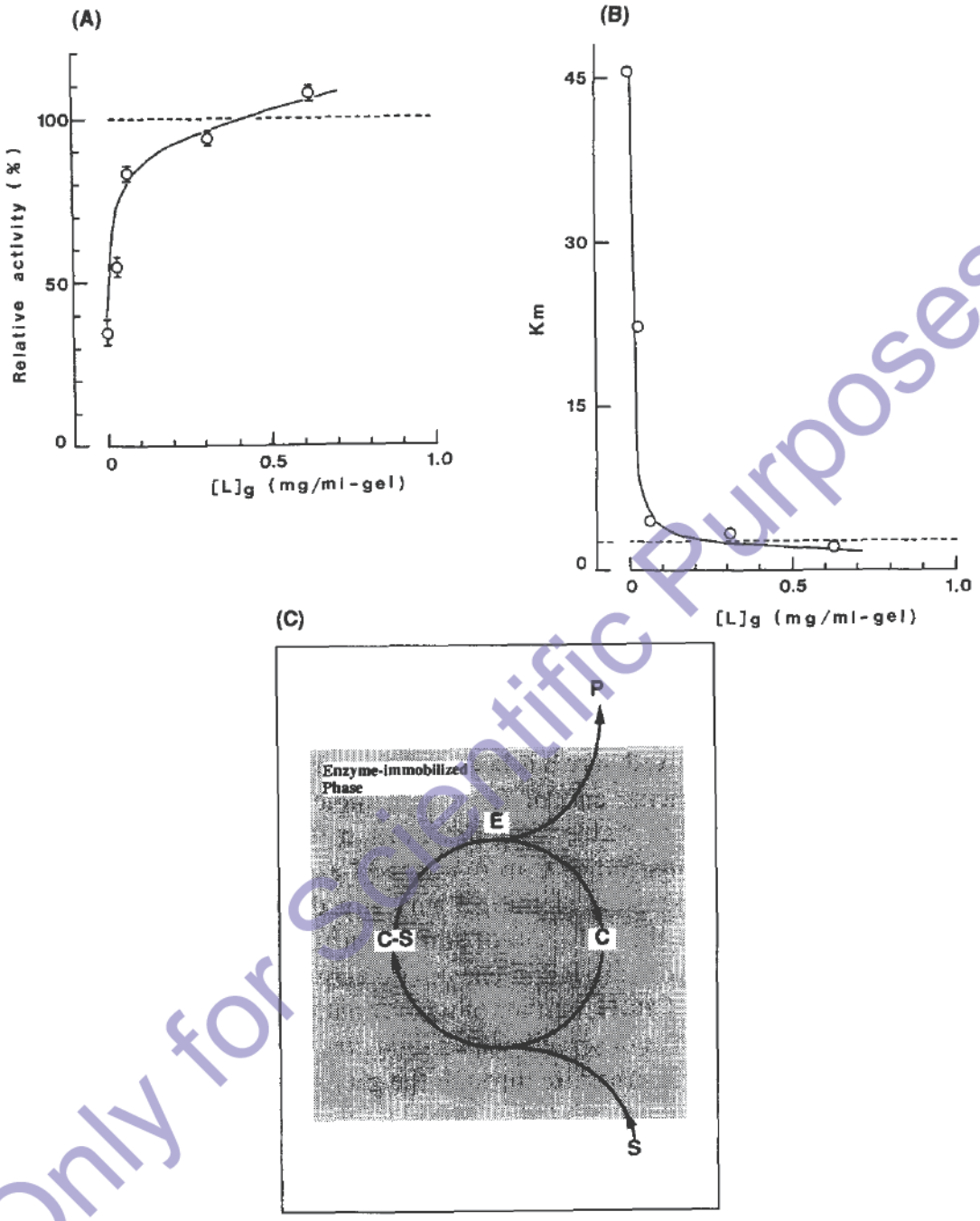


FIG. 14. Caster bean lectin carrier-induced activation of gel-entrapped β-D-galactosidase: (A) Change in immobilized enzyme activity with content ($[L]_g$) of coimmobilized lectin as a carrier. (B) Change in Michaelis–Menten constant with $[L]_g$. The assay system for the immobilized preparation consisted of 6 ml of substrate solution plus 4 ml of gel with 5 mg of entrapped enzyme, while the native enzyme was assayed in 10 ml of substrate solution including the same amount of the enzyme as used for the immobilized preparation. Substrate concentration, 0.08–4.0 mM; pH 7.25 (0.2 M Tris-HCl buffer containing 0.1 M NaCl plus 0.008 M KCl); 30°C. Dashed lines indicate activity and K_m for native enzyme. (C) Schematic representation for understanding the carrier-induced activation of immobilized enzyme (from Kokufuta *et al.*[95]).

weights, within the gel phase are small, the rate of the enzyme reaction can be increased by raising the carrier content to enhance the changes for collision between the C–S and E. In addition, the high binding affinity of C towards S seems to result in a large difference in the concentration of S between the outside and the inside of the gel because of a slight amount of S in the free form within the gel phase. These features appear to be similar to the carrier-mediated facilitated diffusion which is a common transport process in biological systems.

The same idea has been applied in the construction of an immobilized enzyme capable of promoting the pore diffusion of soluble starch as the polymeric substrate,[94] although it is general knowledge that immobilized enzyme activities are very low towards high molecular-weight substrates such as casein.[33,98,99] Preparation was carried out through the iterative freezing–thawing of an aqueous poly(vinyl alcohol) (PVA) solution including concanavalin A (Con A) which is a lectin with a binding affinity[100] towards the starch to be diffused through the gel porosity. Gel permeation chromatographic analysis of the starch remaining in an aqueous solution after shaking with a gel which contained only Con A showed that the starch concentration of the gel phase was effectively enhanced. This strongly suggests that an attractive force between the Con A within and the starch outside the gel facilitated pore diffusion. Thus, the Con A-loaded gel system seems to be effective in the enhancement of the hydrolyzing activity towards starch catalyzed by immobilized glucoamylase. The glucoamylase employed here has been known to hydrolyze mainly the α-1,4 linkage of the non-reducing end of the starch molecule.[101] The experimental results are summarized in Table 2. A preparation (CIGH) with immobilized human serum albumin instead of Con A was used as a control sample, since it was feared that an expansion of the gel porosity due to the entrapment of proteins would enhance the diffusion of starch and thereby the activity of the immobilized enzyme. A drastic fall in enzymatic activity is observed in Table 2 in the results for IG and CIGH, neither of which contained Con A. In each case, a large increase in K_m^{app} was observed, which indicates the great resistance to the diffusion of the polymeric substrate through the gel porosity. In contrast, CICG with Con A as the carrier exhibited a considerably restored enzyme activity, with a reduction of K_m^{app}. In addition, there were no distinguishable differences between the pH-activity curves and V_{max}^{app} values of the native and each type of immobilized glucoamylase. It can thus be said that Con A in the gel phase enhanced the starch concentration in the immediate vicinity of the entrapped enzyme molecules.

The binding affinity of the carrier (within the gel) towards the substrate (in the bulk solution) creates an attractive force between the molecules of both substances. It has been demonstrated that such an attractive force plays an important role in the enhancement of immobilized enzyme activities due to the facilitation of the diffusion of the substrate from the outside to the inside of the gel. The concept introduced here could be applicable in general, and various

TABLE 2. Effect of concanavalin A-mediated facilitated diffusion of soluble starch through pores of PVA gel on immobilized glucoamylase activity[a]

Enzyme sample	Abbreviation	Protein content μg/gel[b]		Relative activity (%)	K_m^{app} (w/v%)	V_{max}^{app} (mM/min)
		Total	Enzyme			
Immobilized glucoamylase[c,d]	IG	9.1	9.1	19.0	1.4	0.11
Coimmobilized glucoamylase with human serum albumin[c,d]	CIGH	511	9.3	19.3	1.4	0.10
Coimmobilized glucoamylase with concanavalin A[e]	CIGC	507	9.0	51.2	0.34	0.10
Native glucoamylase	NG			100	0.02[f]	0.11[g]

[a]Activity was determined under the following conditions: enzyme concentration, 9 μg/ml (regardless of free or immobilized state); substrate concentration, 0.125–1.0% (w/v); pH 5.0 (50 mM citrate buffer); and 37°C.

[b]All data are expressed by the weight of the initially prepared wet gel.

[c]The immobilization procedure was as follows: a viscous polymer solution composed of 12 g of 18% (w/w) PVA and 2 g of protein solution (containing 132 μg enzyme or 132 μg enzyme plus 7.2 mg albumin) was transferred into the cylindrical holes (3 mm diameter and 2 mm depth) of a plastic container, and frozen by soaking the container for 12 hr in *n*-hexane, precooled at −20°C, followed by thawing at room temperature for 3 hr; these freezing and thawing procedures were repeated 6 times.

[d]The sample for which the contents of the protein and/or the enzyme were very close to those of the others was chosen and used for the measurements, because the amounts of proteins released from the same kind of preparation differed somewhat.

[e]Immobilization was carried out using an aqueous polymer solution (12 g) containing 18% (w/w) PVA, 108 μg glucoamylase, and 6 mg Con A, because there was no protein released from the gel preparation during the washing process.

[f]Denotes K_m.

[g]Denotes V_{max} (from Kokufuta and Jinbo[94]).

types of biochemical or chemical binding affinities, such as antibody–antigen or receptor–substrate interactions, could be used for increasing the activities of immobilized biocatalysts.

4.3. *Polymer gels capable of absorbing substrate solutions*

The potential utility of thermosensitive gels in the on/off regulation of immobilized enzyme reactions has been demonstrated in Section 3.5. Here we will discuss the capability of thermosensitive gels for enhancing the diffusion of a substrate from the outside to the inside of the gel, which is based upon a possible absorption of the surrounding medium by a gel during a thermally controlled swelling process (see Fig. 15). This idea has been proposed by Park and Hoffman,[102–104] using the same thermosensitive gel described in Section 3.5.

The gel was prepared in the form of beads, with diameters ranging from 200 to 400 μm in the swollen state, by an inverse suspension polymerization using

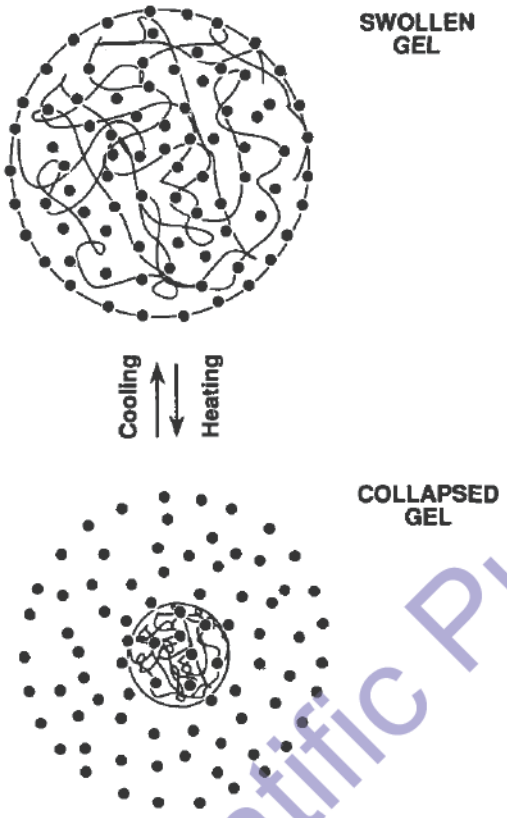


FIG. 15. Schematic illustration of cyclic pumping of surrounding medium (represented by ●) by thermosensitive gel during its thermally controlled swelling and collapse.

paraffin oil as a continuous phase and Pluronic L-81 as a surfactant. A typical enzyme immobilization was carried out as follows:[102] a 0.1 M sodium phosphate buffer (pH 7.4, 4.0 ml) containing NIPA (7.18 g), AAm (0.5 g), MBA (cross-linker, 0.32 g), ammonium persulfate (initiator, 50 mg), and β-D-galactosidase (1.8 mg) was immediately poured into 400 ml of paraffin oil including the surfactant (0.1 ml), and the polymerization was initiated by injecting *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMED, 0.5 ml) as an accelerator into the continuous organic phase where aqueous droplets of the solution, with both the monomers and the enzyme, had been formed by agitation (500 rpm). The reaction was performed in an ice–water bath, and nitrogen supplied continuously above the surface of the paraffin oil phase to avoid disturbing the beads with nitrogen bubbles. The gel obtained shrank completely when warmed above a critical temperature (38.5 ± 1.5°C), called the lower critical solution temperature (LCST), but reswelled when cooled below this LCST. This shrinking–swelling behavior was reversible. The enzymatic hydrolysis of ONPG (see eq. (11)) by the gel beads with immobilized β-D-galactosidase was tested in a packed bed column reactor (continuous and single pass mode). The operation was either isothermal at 30 or 35°C (both are lower than LCST) or cycled every

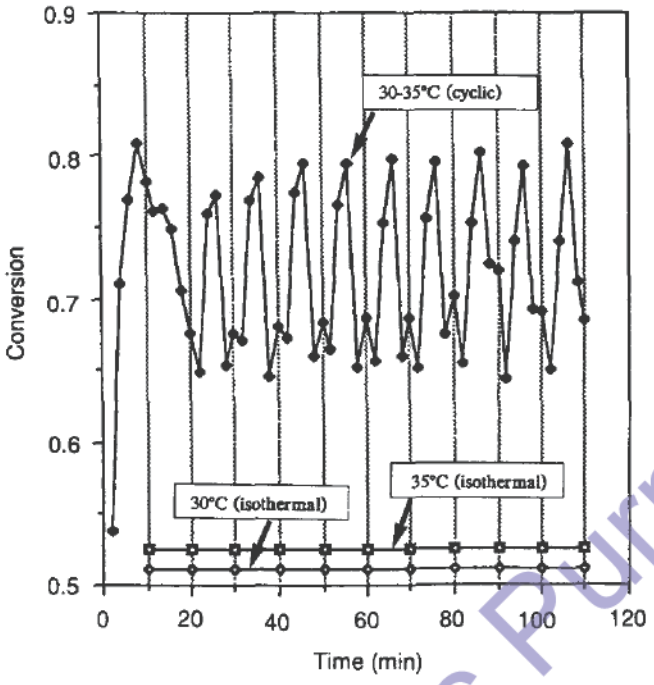


FIG. 16. Conversion as a function of time in packed bed reactor operated isothermally at 30 and 35°C or cycled between 30 and 35°C (from Park and Hoffman[102]).

10 min at 1°C/min between 30 and 35°C. To assay the activity of the immobilized enzyme, the conversion of the substrate into the products was estimated on the basis of the molar concentration ratio of the outlet product to the inlet substrate (Fig. 16). The conversion cycled in accordance with the temperature cycling, and was at all times higher than that for the isothermal operation at either 30 or 35°C. Also, the conversion appeared to maximize at 35°C and minimize at 30°C (each at 10 min intervals) during the thermal cycling operation. The shrinking of the gel upon heating squeezed out the product and any remaining substrate from inside the gel, leading to the observed increase in conversion, while the swelling of the gel upon cooling drew in the substrate along with any product in the surrounding fluid. Thus, mass transfer rates within the gel beads were greatly enhanced by the movement of water into and out of the gel. As a result, thermal cycling significantly elevated the overall reactor enzyme activity relative to the isothermal operation. On the other hand, a somewhat higher conversion at 35°C was observed in the isothermal operation relative to that at 30°C. At 30°C, the gel was significantly more swollen than at 35°C, and because of the greater volume and lower tortuosity of the pore structure, both the substrate and product diffusion rates into and out of the gel were expected to be higher at 30°C than at 35°C. In actual fact, however, the intrinsic diffusivities of both substrate and product, the intrinsic enzyme turnover rate, and the effective enzyme concentration within the gel phase were higher at 35°C than at 30°C. These various opposing factors tended almost to

balance, with the increase in reaction kinetics during isothermal operation at 35°C being more important than the decrease in diffusion rate, and this led to the higher conversion at 35°C.

The present type of thermosensitive gel has been applied in the immobilization of *Arthrobacter simplex* cells, and the bioconversion of a steroid (hydrocortisone → prednisolone by 3-ketosteroid-Δ^1-dehydrogenase in *A. simplex*) was examined using the thermal cycling method.[104] In this case, the thermal cycling was also found to enhance the mass transfer of the substrate (hydrocortisone) into and the product (prednisolone) out of the gel, and thereby to increase the steroid conversion.

The cyclic pumping of the surrounding medium by the gel during its swelling–shrinking change may also be achieved using various stimulus-sensitive gels other than thermosensitive gels; for example, one can easily suggest that a photosensitive hydrogel[73] would permit the enhancement of immobilized enzyme activity through a possible photo-driven substrate-pumping mechanism after the successful entrapment of biocatalysts within the gel. In summary, stimulus-sensitive gels capable of cyclically pumping substrate solutions seem to be of considerable potential usefulness for the enhancement of the catalytic activities of a variety of immobilized biocatalyst systems.

5. REVERSIBLY SOLUBLE POLYELECTROLYTE COMPLEX-IMMOBILIZED ENZYMES

Oppositely charged polyions interact with one another in aqueous media to form polyelectrolyte complexes in either a soluble or an insoluble state. Several studies[49,105–109] have dealt with the immobilization of enzymes with polyions through the formation of polyelectrolyte complexes. Since enzymes carrying both acidic and basic groups behave as polyampholites, immobilization can be performed in two ways: complexation between oppositely charged polyions in the presence of an enzyme which produces a water-insoluble ternary complex composed of polyanion/enzyme/polycation;[107–109] and complexation of an enzyme with a polyanion or a polycation to yield a water-insoluble binary polyelectrolyte/enzyme complex.[49,105] As a result of the complexation (immobilization), significant changes are observed in the following enzyme properties: the optimum pH of the catalytic activity, the K_m^{app} and V_{max}^{app} values, the stability of the enzymes, and their resistance (protective effects) to metal ions. However, to the author's knowledge there have been no studies which have demonstrated that such water-insoluble enzyme/polyelectrolyte complexes provide any new functions in addition to the classical advantages of common immobilized biocatalysts.

When a non-stoichiometric interpolyelectrolyte complex (NIPC) is used in the enzyme immobilization, one can obtain a functional immobilized biocatalyst that undergoes a reversibly soluble–insoluble change depending on the pH and

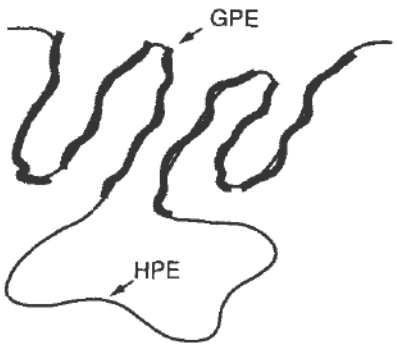


FIG. 17. Schematic illustration of a non-stoichiometric interpolyelectrolyte complex (NIPC) consisting of oppositely charged polyions (from Zezin *et al.*[111]).

ionic strength of the system,[110–115] i.e. a "reversibly soluble" immobilized enzyme system. The principal advantage of such an immobilized biocatalyst is that it can be separated by precipitation from a reaction mixture after homogeneous catalysis, a process in which the various obstacles to heterogeneous immobilized enzyme reactions (e.g. diffusion limitations by substrates) are not dominant factors.

An NIPC system, which was extensively studied by Kabanov *et al.*,[116–119] was composed of two oppositely charged polyelectrolytes (see Fig. 17): a "host" polyelectrolyte (HPE), which was either a weak polyacid or polybase with a relatively "high" molecular weight; and a "guest" polyelectrolyte (GPE), which was either a strong polyacid or polybase with a relatively "low" molecular weight. By mixing aqueous HPE and GPE solutions containing salts such as NaCl at a certain ratio (usually at [GPE]/[HPE] = 0.2–0.7, where the brackets signify the molarity based on the ionizable groups of each polymer), an NIPC was produced through a cooperative coupling reaction mechanism in which the GPE molecules formed salt linkages with the HPE to couple with several segments of an HPE chain. The other segments of the HPE molecule, which were not occupied by GPE molecules, held ionizable groups free of salt linkages with GPE. The resulting NIPC seems to have been a peculiar block copolymer molecule which contained both hydrophilic and hydrophobic domains. The solubility of an NIPC in aqueous media is due to its hydrophilicity, which depends on the degree of the dissociation of the remaining ionizable groups in the HPE. Thus, the precipitation of an NIPC from its aqueous solution, i.e. a phase separation, occurs when acids or bases are added to the system to reduce the degree of the dissociation. This phase separation, also occurring with the addition of salts, is due to the screening of the charges in the NIPC with counter ions.

Reversibly soluble immobilized enzyme systems based on NIPC have been examined using several enzyme samples: penicillin amidase,[113–115] α-chymotrypsin,[114] urease,[112] and alcohol dehydrogenase.[114] Immobilization was performed through the covalent attachment of enzymes to either the HPC or GPC,

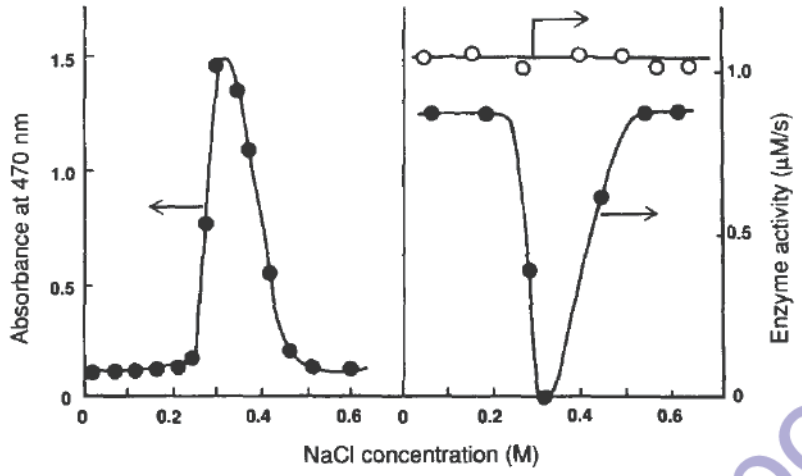
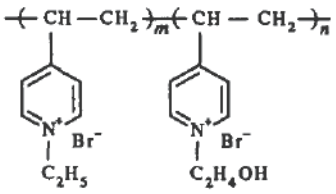


FIG. 18. Dependence of (a) turbidity and (b) activity on NaCl concentration for NIPC-immobilized penicillin amidase. Activities of the native (○) and immobilized (●) enzymes were assayed according to eq. (12). In the case of the immobilized enzyme, the assay was performed on the supernatant solution from which the precipitated complex-carrying enzyme had been separated by centrifugation (from Margolin *et al.*[115]).

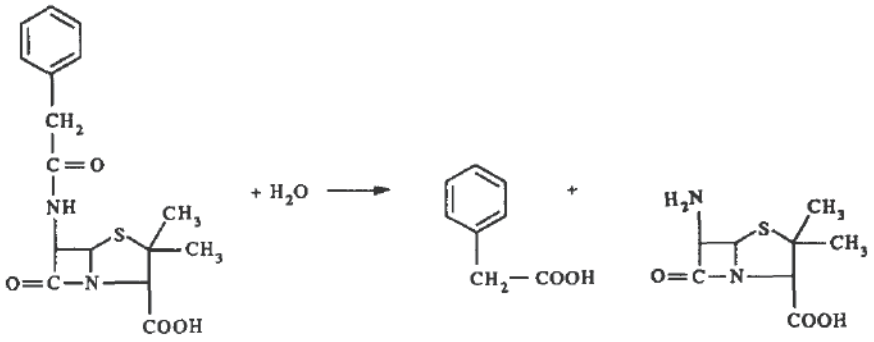
followed by the formation of an NIPC between the enzyme-bound HPC (or GPE) and GPC (or HPE). Poly(methacrylic acid) or its sodium salt was used as the HPE, while poly(vinyl pyridine) derivatives were employed as the GPC. A typical example of the soluble–insoluble regulation of NIPC-immobilized penicillin amidase[115] is shown in Fig. 18. The enzyme was covalently bound with the aid of cyanuric chloride to a poly(vinyl pyridine) derivative ($M_w = 4 \times 10^4$) of the following structure which was used as the GPE.



$m = 0.95–0.9$; $n = 0.1–0.05$

The enzyme-carrying GPE was then complexed with poly(methacrylic acid) ($M_w = 2.6 \times 10^5$; used as the HPE) to form an NIPC with a ratio of [GPE]:[HPE] = 1:3. The activities of the native and immobilized enzymes

were assayed by investigating the hydrolysis of benzylpenicillin (penicillin G).



(12)

As can be seen from the dependence of the turbidity on the NaCl concentration, the immobilized enzyme precipitated at a concentration > 0.25 M, but a redissolution occurred when the concentration > 0.5 M. It was pointed out that during the phase separation (0.25 M < NaCl concentration < 0.5 M), the NIPC rearranged into two species: an insoluble stoichiometric (1 : 1) complex consisting of the enzyme-carrying GPE and HPE, and the excess soluble HPE. Moreover, the redissolution observed at an NaCl concentration > 0.5 M was due to the dissociation of the insoluble stoichiometric (1 : 1) complex. These aspects may be summarized as follows;

$$(\text{E-GPE})_1 \cdot (\text{HPE})_3 \underset{-\text{NaCl}}{\overset{+\text{NaCl}}{\rightleftharpoons}} (\text{E-GPE})_1 \cdot (\text{HPE})_1 + 2\text{HPE}$$

$$\underset{-\text{NaCl}}{\overset{+\text{NaCl}}{\rightleftharpoons}} 1\text{E-GEP} + 3\text{HPE} \qquad (13)$$

where E-GPE denotes the enzyme-carrying GPE. As a result, the enzyme activity of the supernatant solution after the removal of the precipitated complex by centrifugation changed dramatically in the NaCl concentration range causing the phase separation.

A similar precipitation–dissolution control of NIPC-immobilized penicillin amidase was performed by the addition of acids and bases.[113-115] When the immobilized enzyme was precipitated, a marked increase in K_m^{app} was observed due to the diffusion limitations of the substrate. In a soluble state, however, the immobilized enzyme showed a K_m^{app} value approaching the K_m value of the native enzyme, indicating little influence by the diffusion limitation on the immobilized enzyme reaction.

In all the cases studied, the precipitation–dissolution was controlled by a small difference in pH or salt concentration. Each reiterated dissolving of the immobilized enzyme was followed by quantitative recovery of its catalytic activity. The recovered enzyme activity was not significantly different from that of the native enzyme because the NIPC-immobilized enzyme was used in a soluble state. These seem to be the principal advantages of NIPC-immobilized enzymes as functional immobilized biocatalysts in comparison with other known reversibly soluble immobilized systems.[120-122]

6. IMMOBILIZED BIOCATALYSTS CAPABLE OF CATALYZING MULTISTAGE REACTIONS

Most metabolic pathways in living systems consist of a series of reactions in which the product of one enzymic reaction acts as the substrate of another. The participating enzymes are usually bound to cellular membranes or organelles in such a way that consecutive reaction steps take place in proximity. The simulation of such metabolic pathways makes it possible for us to design and prepare immobilized biocatalysts that are capable of catalyzing multistage reactions in the same system and at the same time, i.e. immobilized "multibiocatalysts". This type of immobilized biocatalyst may facilitate the development of convenient and efficient processes in technological fields, such as analytical methods using enzyme electrodes (see pp. 68–70 in Ref. 22 for example). Also, a biocatalytic system consisting of two or more coimmobilized enzymes, called "immobilized multienzymes", serves as a refined model for the compartmentalization of intracellular enzymes, from which valuable information about the phenomena involved in the regulation of metabolic processes *in vivo* can be obtained.

In principle, immobilized multibiocatalysts can be prepared through the coimmobilization of different types of (i) enzymes, (ii) micro-organisms, or (iii) a combination of enzymes and micro-organisms. Extensive studies have been carried out upon these three combinations, using polymer supports involving gels, microcapsules, polyelectrolyte complexes, and so on. However, these supports have not been specially designed to provide immobilized systems with specific new capabilities. Much of the effort expended so far in constructing immobilized multibiocatalysts has concerned the problem of how to assemble the "original" biocatalysts for the purpose of catalyzing the multistage reactions required.[28,29] Thus, several typical examples are briefly reviewed here to discuss the basis of the design and construction of multibiocatalysts.

First, we consider immobilized multienzyme systems that can catalyze a series of consecutive reactions. The best known example[50] is the formation of citrate from malate using an AAm gel into which three enzymes, malate dehydrogenase, citrate synthase, and lactate dehydrogenase, had been coimmobilized via physical entrapment. The enzymic reactions proceeded in two stages, as shown in Fig. 19: the formation of oxalacetate (as the intermediate) from malate, which was coupled with the reduction of NAD^+ with the aid of malate dehydrogenase, was followed by citrate formation via a citrate synthase-catalyzed reaction of the intermediate (oxalacetate) with both acetyl coenzyme A (AcSCoA) and H_2O. In this system, NAD^+ was simultaneously regenerated from NADH during the oxidation of pyruvate, which was catalyzed by coimmobilized lactate dehydrogenase. The multienzyme system constructed was found to show about a two-fold increase in the steady-state rate of these overall reactions as compared to the rate catalyzed by the same enzymes in free solution. In general, an intermediate produced by one type of enzyme would

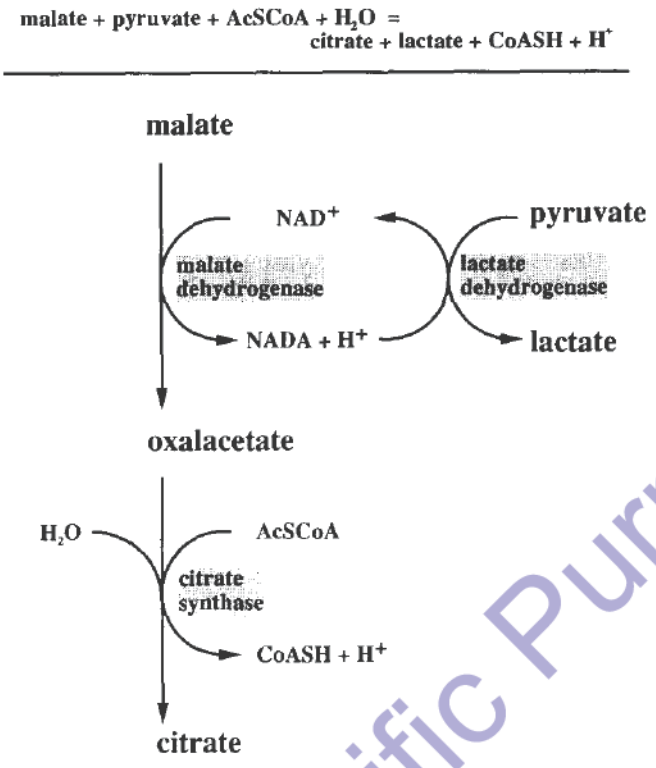


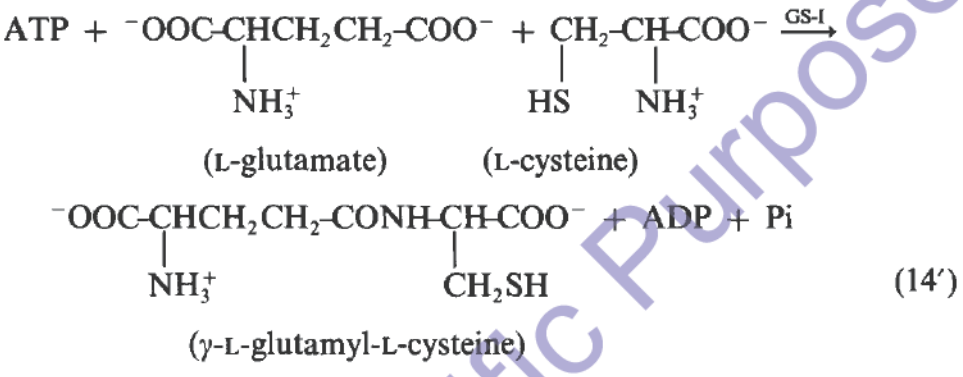
FIG. 19. Design of multienzyme system capable of forming citrate from malate through enzymatic multistage reactions (drawn on the basis of Srere *et al.*[50]).

encounter the next enzyme in the sequence more rapidly if the two enzymes were in close spatial proximity than if they were randomly distributed. Therefore, the coimmobilization of enzymes within the same support is profitable in enhancing the local concentrations of the reaction intermediates in the microenvironment of the enzymes, thereby resulting in a higher overall reaction rate.

The same conclusion has been drawn from the multienzyme systems previously studied; for example, the formation of 6-phosphoglucono-δ-lactone from lactose by the coimmobilization of three enzymes,[123] i.e. hexokinase, lactase (β-D-galactosidase), and glucose-6-phosphate dehydrogenase, and the formation of fumaric acid from arginine by the coimmobilization of five enzymes,[124] i.e. arginase, ornithine carbamoyltransferase, argininosuccinate synthetase, and argininosuccinate lyase (each of which constitutes the urea cycle), and inorganic pyrophosphatase, which was used to hydrolyze the inorganic pyrophosphate (PPi) produced from the hydrolysis of adenosine triphosphate (ATP), which simultaneously occurs in the urea cycle while arginosuccinic acid is formed from citrulline and aspartic acid with the aid of argininosuccinate synthetase.

Multienzyme systems coexisting within the micro-organisms under immobilized states can be used as immobilized multibiocatalysts as well as those prepared by the coimmobilization of enzymes, although a single enzyme in the

immobilized cells is often utilized to catalyze a reaction. For an example, the transformation of cholesterol to cholestenone (see eq. (10)) by gel-entrapped *Nocardia rhodocrous* cells[87] has been performed using a single enzyme (17 β-dehydrogenase) of the microbial cells, in which a number of other multienzyme systems coexist. In contrast, the production of glutathione from L-glutamate, L-cysteine, and glycine by AAm or κ-carrageenan gel-entrapped *Escherichia coli* B cells[125] utilized two enzymes, γ-glutamylcysteine synthetase (or glutathione synthetase I; GS-I) and glutathione synthetase (or glutathione synthetase II; GS-II), which catalyze the synthesis of glutathione:



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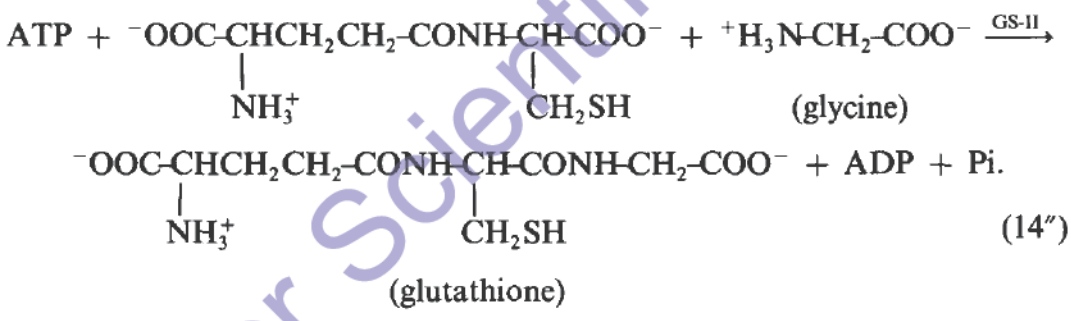
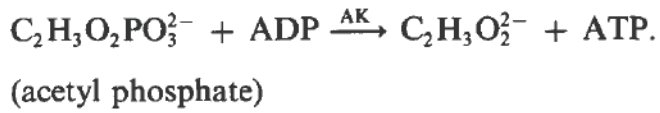


(14″)

In addition, acetate kinase (AK) in the same cells was used to regenerate ATP from ADP with the aid of acetyl phosphate, which had been added in a feed solution containing L-glutamate, L-cysteine, and glycine:



(14‴)

A more complicated multienzyme system has also been studied in which the metabolic pathways of the two coimmobilized types of bacteria were used for the purpose of catalyzing the oxidation of ammonia to nitrate, followed by the subsequent reduction of the resulting nitrate to gaseous products (primarily N_2), in the same locale at the same time.[126] It is known that autotrophic nitrifying bacteria, such as *Nitrosomonas europaea*, are capable of deriving energy for the synthesis of cellular materials from the oxidation of ammonia to nitrate under aerobic conditions,[127-129] i.e. nitrification ($NH_3 \rightarrow NH_2OH \rightarrow$

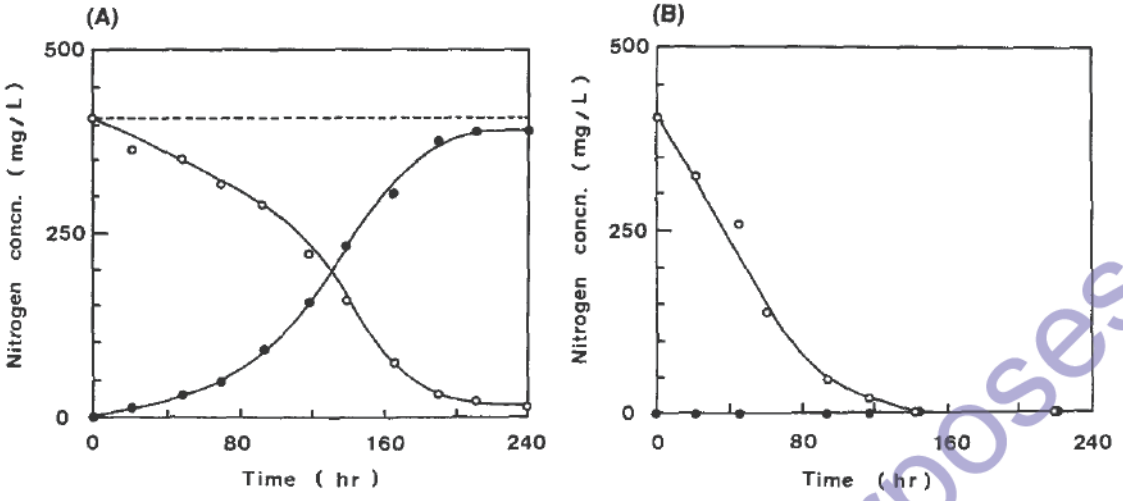


FIG. 20. Changes in nitrogen concentrations (○, NH_4^+-N; ●, NO_2^--N) during aerobic cultures of (A) singly immobilized *N. europaea* cells and (B) coimmobilized *N. europaea* plus *P. denitrificans* cells. Incubation was carried out at 30°C on 100 ml of medium containing $(NH_4)_2SO_4$ as a substrate and either 396 mg of the singly immobilized cells (98.4% complex and 1.6% nitrifying cells by dry weight) or 565 mg of the coimmobilized cells (67.6% complex, 31.3% denitrifying cells, and 1.1% nitrifying cells by dry weight) (from Kokufuta *et al.*[126]).

$NO_2NHOH \rightarrow HNO_2$), in which the enzymic transfer of electrons plays an important role. In addition, several denitrifying bacteria (e.g. *Paracoccus denitrificans*) are found to perform the dissimilatory reduction of nitrogen oxides (nitrate and nitrite) under anaerobic conditions,[127,128,130] i.e. denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), in which nitrogen oxides act as the terminal acceptor of the electrons coming from electron transport systems. In both biochemical reactions, however, the oxidation–reduction potential acts in opposite ways. Thus, two different operations under aerobic and anaerobic conditions are required in the microbial nitrification–denitrification processes.[128] In order to eliminate this inconvenience and to develop a novel immobilized biocatalyst capable of permitting simultaneous nitrification and denitrification in the same system, Kokufuta *et al.*[126] tried to coimmobilize both nitrifying and denitrifying bacteria. In the case of an immobilized biocatalyst consisting of living cells, it may be anticipated that by incubating an immobilized preparation in which both nitrifying and denitrifying bacteria were originally distributed in a "random" fashion, the density of the nitrifying bacteria in the "aerobic zones" in close proximity to the surface of a support becomes larger than in the "aerobic zones" of the interior side, which are hospitable to the denitrifying bacteria. The coimmobilization of *N. europaea* plus *P. denitrificans* cells was carried out using a polyelectrolyte complex composed of potassium poly(vinyl alcohol sulfate) and trimethylammonium glycol chitosan iodide or poly(diallyldimethylammonium chloride), the utility of which has been studied in the whole cell immobilization of several microbial cells.[131–134] In Fig. 20, the

time courses for ammonia consumption and nitrate formation by singly immobilized *N. europaea* cells within the polyelectrolyte complex were compared with those of the coimmobilized cells. "Aerobic" incubation was carried out in the investigations of their catalytic activities. There was a remarkable difference between both biocatalysts; that is, the singly immobilized biocatalyst quantitatively converted ammonia to nitrite, while the coimmobilized one consumed ammonia without forming nitrite. It was thus found that the nitrite formed from ammonia via oxidation by the nitrifying bacteria was immediately decomposed to gaseous terminal products via dissimilatory reduction by the denitrifying cells coexisting within the same support. In addition to these results, the following important characteristics were obtained from Fig. 20: (i) the initial rate of ammonia oxidation by the coimmobilized biocatalyst was about 3.4 times that of the singly immobilized biocatalyst, on the basis of 1 g *N. europaea* cells (dry weight) in both biocatalysts; and (ii) a complete oxidation of ammonia took place in the coimmobilized but not in the singly immobilized system. These advantages could have been related to the functional capability of the coimmobilized biocatalyst to decompose nitrite in the same system, because the accumulation of nitrite would inhibit the ammonia-oxidizing activity of the nitrifying bacteria,[133] and thereby the growth of the cells.[135]

As was demonstrated above, the coimmobilization of different kinds of micro-organisms is a tool for constructing multibiocatalysts capable of catalyzing multistage reactions that are very difficult to perform in the same system using immobilized multienzymes. The coimmobilization of micro-organisms with enzymes has been studied on the basis of the same idea; for example, the production of ethanol from cellobiose by a calcium alginate gel-entrapped multibiocatalyst, consisting of both *Zymomoas mobilis* and β-D-glucosidase,[136] which is capable of performing enzymic hydrolysis of cellobiose to glucose, followed by alcohol fermentation in the same system.

In many of the multibiocatalysts previously studied, however, the properties of the supporting matrices have not been looked upon as a dominant factor in controlling the functional capabilities of the catalytic systems. In fact, the use of a polyelectrolyte complex-stabilized alginate gel[96] (see Fig. 13) in the coimmobilization of *N. europaea* plus *P. denitrificans* cells, instead of the polyelectrolyte complex support, made it possible for nitrification and denitrification to occur simultaneously in the same system under aerobic conditions;[137] thus the primary requirement for the supporting matrix is that it provides aerobic and anaerobic regions which are habitable by the nitrifying and denitrifying bacteria, respectively.

More information about the enzyme reactions and the metabolic pathways in living systems, obtained from research by biochemists and enzymologists, should prove helpful in the design of multibiocatalysts as functional immobilized biocatalysts, but the introduction of new functions into the supporting matrix would enhance their abilities much more. For an example, a

support capable of facilitating the pore diffusion of starch[94] (see Section 4.2) may be available for the construction of a multibiocatalyst consisting of glucoamylase and *Z. mobilis*. Such a multibiocatalyst is expected to permit the direct production of ethanol from starch as the polymeric substrate, although it is ordinarily subjected to ethanol fermentation after chemical or enzymatic degradation. To our knowledge, nevertheless, little attention has yet been paid to the use of functional supports in the construction of multibiocatalysts. This is, therefore, an important subject for future research on immobilized biocatalysts.

7. GEL-ENTRAPPED ENZYME SYSTEMS WITH BIOCHEMO-MECHANICAL FUNCTIONS

The purpose of all the studies reviewed in the previous sections was to utilize immobilized biocatalysts in chemical conversion. On the other hand, new attempts have been made to directly convert the "energy" of immobilized enzyme reactions into mechanical work.[138,139] A system with such a biochemo-mechanical function can be prepared by entrapping enzymes into polymer gels that undergo volume–phase transitions in response to enzymatic changes. It is thus first necessary to describe the phase transition phenomena observed in polymer gels. In this section, a general paradigm for the design and preparation of biochemo-mechanical systems using the gel entrapment of enzymes is discussed after a brief description of the phase transition of polymer gels.

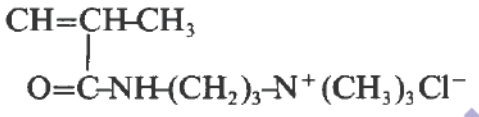
7.1. *Phase transitions of polymer gels*

The swelling of gels, such as that observed when rubber is placed in benzene, has long been a well-known phenomenon. In 1978, Tanaka[140] discovered the discontinuous volume change (i.e. volume collapse) of a covalently cross-linked AAm gel in an acetone/water mixture when varying the temperature or composition of the mixture. This phenomenon is now called the volume–phase transition of gels and is observed in many gels made of synthetic and natural polymers. The phase transition is accompanied by reversible, discontinuous (or in some cases, continuous) volume changes, often as large as several hundred times, in response to small variations in the solution conditions surrounding a gel.[141] Variables that trigger the transition include temperature,[140,142–145] solvent composition,[140,142,144,146] pH,[142,147,148] ion concentration,[148,149] small electric fields,[150–152] and light.[73,153]

Theoretical studies on phase transitions were attempted by Tanaka *et al.*[140,142,145,154] using the Flory–Huggins theory.[155] It has been shown theoretically that a gel undergoes either a continuous volume change or a first order discontinuous phase transition depending on the proportion of ionizable groups incorporated in the polymer network and on the stiffness of the polymer chains constituting the network. The counterions to the ionized groups and the stiffness of the polymer chains increase the osmotic pressure acting to expand the

polymer network, resulting in a discontinuous volume change. This situation is similar to a gas–liquid phase transition which can be either continuous or discontinuous depending on the external pressure exerted on the system.

Several other studies have also been made in an attempt to account for the phase transition theoretically in terms different from those of the Flory–Huggins theory. Otake *et al.*[156] thus proposed a theoretical model that takes hydrophobic interaction into account in explaining the thermally induced discontinuous volume collapse of hydrogels. In addition, Prausnitz *et al.*[157] proposed a lattice model, an improvement of which was made to explain the swelling curves of gels consisting of MBA-crosslinked copolymers of AAm with ((methacrylamide)propyl)trimethylammonium chloride (MAPTAC).[158]



(MAPTAC)

which were measured as a function of the degree of cross-linking and the concentrations of NaCl solution.

In order to apply the phase transitions of gels in technological fields, however, a more generalized explanation was required. Ilmain *et al.*[159] thus tried to account for the phase transition by hypothesizing a balance between the repulsion and attraction of the cross-linked polymer chains in their networks which arise from a combination of four intermolecular forces: ionic, hydrophobic, van der Waals and hydrogen bonding. When a repulsive force, usually electrostatic in nature, overcomes an attractive force such as hydrogen bonding or the hydrophobic interaction between network chains, gel volume should increase discontinuously in some cases or continuously in others. Conversely, a decrease in the volume may occur when the attractive force becomes dominant. The variables that trigger the transition influence these intermolecular forces and thereby the balanced state of the attractive and repulsive forces. This concept is extremely qualitative, but makes it possible for us to classify the swelling curves of gels observed under various conditions into four different types (see Fig. 21). The concept has also been applied in the preparation of a functional immobilized enzyme whose catalytic activity can be initiated and terminated by a change in temperature (see Section 3.5), as well as in the design of a gel system that is capable of converting immobilized enzyme reactions into mechanical work.

7.2. *Design and preparation of systems*

Mechano-chemical or chemo-mechanical systems which exert mechanical energies in response to chemical changes have long attracted interest among

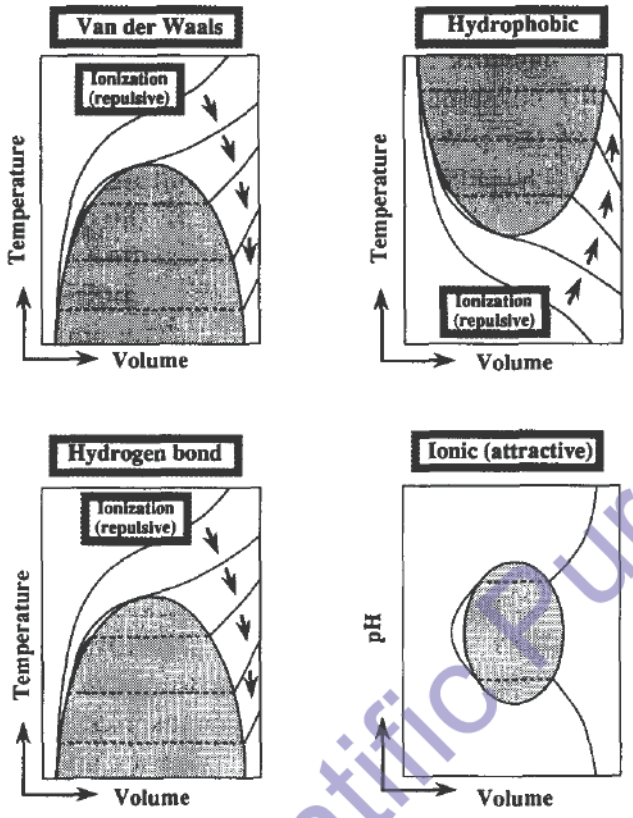


FIG. 21. Gel volume phase transitions induced by four types of intermolecular forces. The van der Waals interaction (attractive) causes phase transitions in gels in a mixed solvent system, in which a non-polar solvent influences the transition through a decrease in the dielectric constant. Gels, such as *N*-isopropylacrylamide (NIPA) gel, undergo phase transitions due to hydrophobic interaction (attractive) in pure water from a swollen state at low temperatures to a collapsed state at high temperatures. Gels with cooperative hydrogen bonding as the attractive force, such as the interpenetrating polymer network of acrylic acid (AA)/acrylamide (AAm) gel, undergo phase transition in pure water (swollen state is the high-temperature state). The repulsive ionic interaction determines the transition temperature and the volume change at the transition, whereas the attractive ionic interaction is responsible for pH-driven phase transitions, such as in gels consisting of AA/[(methacrylamide)propyl]trimethylammonium chloride (MAPTAC) (from Ilmain[159]).

scientists, medical researchers and engineers. Katchalsky and his co-workers[160–162] were pioneers in the development of such systems using polymer gels, and their work has been expanded further by various researchers (see Ref. 151).

Biochemo-mechanical systems, in which biochemical changes such as enzymic reactions are used in place of the usual chemical changes for creating mechanical energies, are taken to be one development of chemo-mechanical systems. Since biochemical reactions are generally believed to be uniquely specific, the creation of mechanical energies in a biochemo-mechanical system is expected to respond to a specific kind of molecule. This aspect is not only technologically

important, but will also provide a basis for a better understanding of the marvelous energy-converting mechanisms available only in biological systems.

One approach for constructing biochemo-mechanical systems may be the enzymatic control of the phase transition of gels. Among the many variables that trigger phase transitions, solvent composition or solution pH seems to be controlled enzymatically. Also, it seems, based on the concept described in the last paragraph of Section 7.1, that the phase transition occurring as the result of a change in the balance between the ionic (repulsive) and hydrophobic (attractive) forces can be regulated through an enzyme-induced change in pH or solvent composition. Two gel systems have been designed according to these considerations: a copolymer gel[139] of acrylic acid (AA) and NIPA into which urease has been entrapped; and a NIPA gel with immobilized esterase.[138]

The details of the experiments and the results for the copolymer gel with immobilized urease are as follows: The gel was obtained by gelling an aqueous solution (1 ml) containing NIPA (75.6 mg), AA (2.3 mg), MBA (cross-linker, 1.33 mg), and urease (20 mg). Ammonium persulfate (0.4 mg/ml) and TMED (1.85 mg/ml) were used as the initiator and accelerator, respectively. The gelation was carried out at 0°C for 1 hr in a test tube into which glass capillaries with an inner diameter of 0.1 mm had previously been inserted. After the gelation was completed, the gels were taken out of the capillaries and thoroughly washed with an NH_4Cl/HCl buffer solution (0.2 M; pH 4.0). All the gel samples were cut into cylinders of ca. 2 mm length and stored at 3°C before use. Swelling curves were measured in the aforementioned buffer solutions, both with and without urea as the substrate (Fig. 22(A)). Gel diameters were determined using a microscope with a calibrated scale, the temperature being controlled to within 0.01°C between 25°C and 40°C. In the absence of urea, the gel underwent a discontinuous phase transition at 32.4°C (phase transition temperature, T_c). In contrast, the presence of urea bought about a continuous transition, and the transition temperature was shifted to a higher range. Taking these results into account, the reversible regulation of the gel volume was attempted by means of the alternative immersion of the gel into buffer solutions with and without urea (Fig. 22(B)). At a temperature of 33.4°C, which was higher than the T_c, the gel shrank in the absence of urea. The gel began to swell as soon as the aqueous solution in which it was immersed was replaced by the urea solution, and the swelling became complete within 15 min. The swollen gel collapsed again when the ambient solution was replaced with the urea-free buffer. The time needed for a complete collapse of the gel-size employed here was approximately 90 min. The swelling and collapsing were repeated several times with satisfactory reproducibility. The results obtained can be interpreted as shown in Fig. 22(C). The immobilized enzyme catalyzed the hydrolysis of urea into ammonia and carbon dioxide (see eq. (6)). The carbon dioxide produced was soluble in water and partially turned into HCO_3^- and CO_3^{2-}. The gel phase was saturated with the following species: NH_4OH, NH_4^+, HCO_3^-, CO_3^{2-}, and Cl^-, in which the

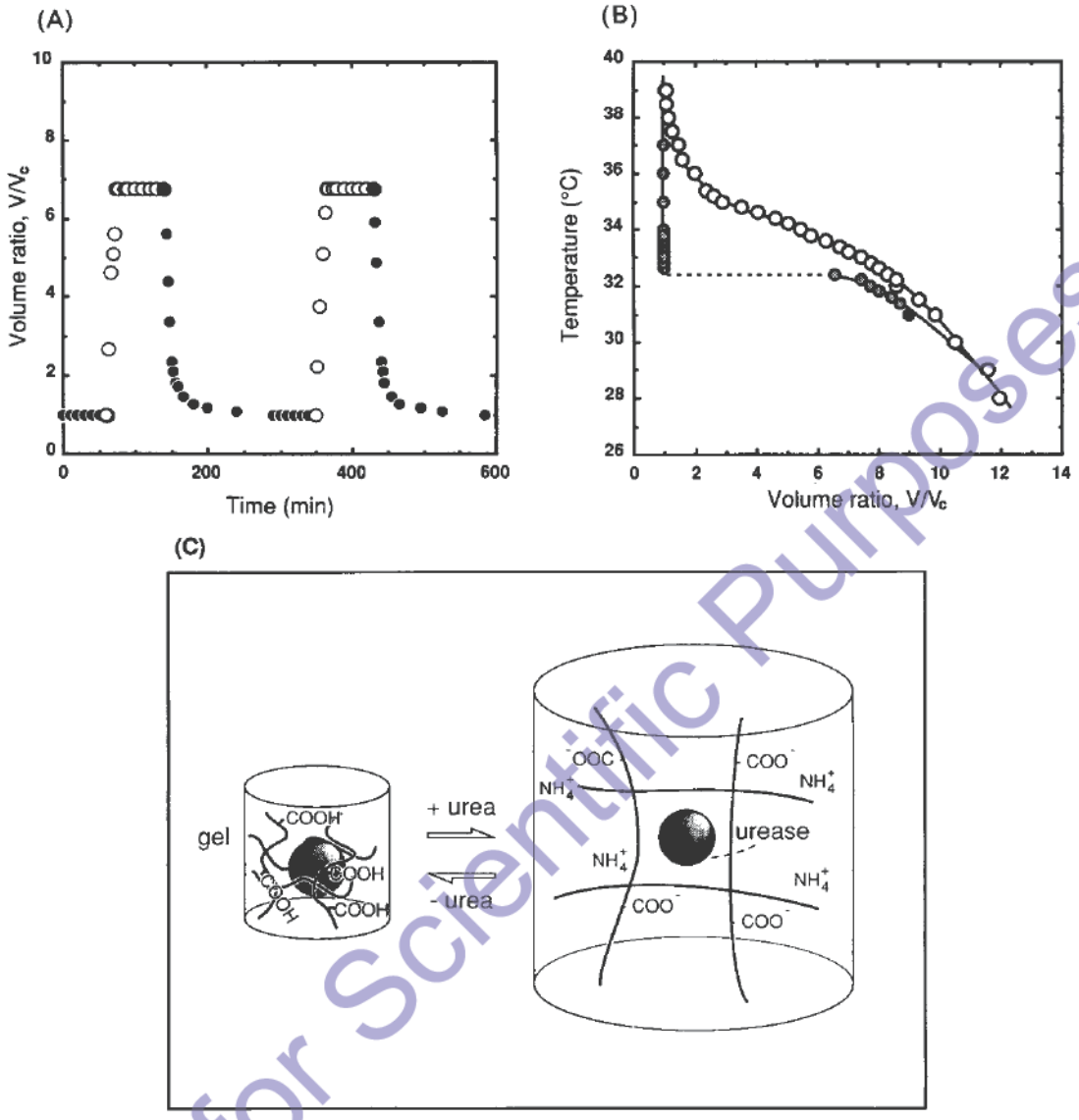


FIG. 22. Biochemo-mechanical function of *N*-isopropylacrylamide(NPA)/acrylic acid (AA) gel with immobilized urease: (A) Swelling curves of urease-loaded gel in 0.2 M ammonium buffer (pH 4.0) containing (●) and not containing (○) 1 M urea as the substrate. The completely collapsed volume (V_c) for each sample was determined at 50°C. (B) Repeated swelling and shrinking control of the urease-loaded gel at 33.4°C by the alternate use of urea-free (○) and urea-containing 0.2 M ammonium buffer solutions (●) at pH 4.0. The aqueous phase (80 μl) in the cell into which the gel sample was immersed was quickly replaced by 4 ml of another aqueous solution within 2 min. To avoid temperature change during replacement, both aqueous phases were kept at the same temperature. (C) Schematic illustration of the present biochemomechanical system, in which ammonia enzymatically produced from urea raises the pH of the gel phase and dissociates carboxyl groups even when the ambient pH is kept at a low level (4.0) in the acidic range. This brings about the swelling of gel, whereas the gel collapses in the absence of the substrate (from Kokufuta *et al.*[139]).

NH_4OH, NH_4^+, and Cl^- originated in part from the buffer solution. The reaction naturally increased the pH of the gel phase due to an increase in the ammonia concentration. The carboxyl groups of the gel were then dissociated, and the electrical force arising from the dissociation of the carboxyl groups overcame the hydrophobic interaction between the network chains. This, of course, brought about an increase in the transition temperature, as was previously expected. As a result, the gel was swollen when a certain amount of urea was present, but collapsed in its absence if the temperature was kept at a suitable range above T_c.

In summary, a large volume change (volume–phase transition) in gels with immobilized enzymes may be available for the biochemical creation of mechanical energies when coupled with enzymatic changes within the gel phase. In the design of such immobilized enzyme systems, the concept of controlling the phase transition threshold by changing the balance of the repulsive and attractive forces between the polymer chain networks which constitute the gel is accepted as a general rule.

This concept has also been applied in the construction of a gel system with immobilized concanavalin A (not enzyme),[163] in which it was possible to convert the difference in the biochemical affinities of this lectin towards two sugar molecules, α-methyl-D-mannopyranoside (neutral) and dextran sulfate sodium (ionic), into mechanical energy using the phase transition.

At the end of this section, the author wishes to note that prior to these studies, two different research groups[164,165] had attempted to control the volume of a gel or gel-like polymer membrane enzymatically. However, their purpose was not to convert the energy of enzyme reactions into mechanical work, but to control the release of chemicals through the gel porosity. The phase transition was not taken into account in their design and preparation; thus, the swelling ratio of the matrices used in their release control experiments was only a few percent.

8. SUMMARY AND CONCLUSION

The immobilization of enzymes, organelles, and microbial cells within appropriate supporting matrices is a very useful tool in the improvement of their abilities and utilities as biocatalysts. Immobilization techniques and the characteristics of immobilized preparations have been studied extensively, and the results of these studies utilized in industrial applications.

In order to renew interest in this research field, which has now reached a relatively mature stage, the present review has dealt with the current topic of "functional immobilized biocatalysts," which may be defined as immobilized biocatalyst systems with some beneficial functional capability other than the usually credited advantages obtained upon immobilization. Based on a considerably detailed survey of previous articles dealing with the immobilization of biocatalysts, the types of these functions have been classified as follows:

(i) controlling immobilized enzyme reactions, especially the initiation–termination of a reaction, by externally applied stimuli; (ii) enhancing the activity of a biocatalyst via the regulation of the partition or diffusion of substrates; (iii) permitting reversibly soluble–insoluble changes; (iv) catalyzing multistage reactions; and (v) converting the energy of immobilized enzyme reactions into mechanical work. All these functions, except for (iv), rely on especially designed supporting matrices, such as stimulus-sensitive polymers and gels, copolymers consisting of hydrophobic and/or ionic monomers, gels with entrapped specific carrier compounds, or reversibly soluble non-stoichiometric interpolyelectrolyte complexes.

Several of the functional immobilized biocatalyst systems discussed here were taken from articles published in the 1970s, when many researchers and engineers were actively studying the immobilization of biocatalysts under the still not-well-established concept of functional immobilized biocatalysts. On the other hand, most of the recently studied systems have taken the developing tools of the polymer and material sciences into consideration in order to impose special functions upon these systems. Further developments in the fields of polymer chemistry and material sciences would make much more important contributions to the functionalization of immobilized biocatalysts. However, we shall call attention here to events in the biochemical fields; thus, the concept of biomimetic engineering, which has focused on the simulation of natural biofunctions in the design and construction of artifical materials, plays an important part in the design of functional supports.

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