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Recent Progress on Immobilization of Enzymes on Molecular Sieves for Reactions in Organic Solvents

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Abstract

Enzymes exhibit high selectivity and reactivity under normal conditions but are sensitive to denaturation or inactivation by pH and temperature extremes, organic solvents, and detergents. To extend the use of these biocatalysts for practical applications, the technology of immobilization of enzymes on suitable supports was developed. Recently, these immobilized biomolecules have been widely used and a variety of immobilization supports have been studied. The majority of these supports cover diverse kinds of materials such as natural or synthetic polyhydroxylic matrixes, porous inorganic carriers, and all kinds of functional polymers. Microporous molecular sieve, zeolite, has attracted extensive interest in research because of its distinctive physical properties and geochemistry. Recently, with the discovery of a new family of mesoporous molecular sieves, MCM-41, this series of materials shows great potential for various applications. Molecular sieves involve such a series of materials that can discriminate between molecules, particularly on the basis of size. As support materials, they offer interesting properties, such as high surface areas, hydrophobic or hydrophilic behavior, and electrostatic interaction, as well as mechanical and chemical resistance, making them attractive for enzyme immobilization. In this article, different types of molecular sieves used in different immobilization methods including physical adsorption on zeolite, entrapment in mesoporous and macroporous MCM series, as well as chemically covalent binding to functionalized molecular sieves are reviewed. Key factors affecting the application of this biotechnology are discussed systematically, and immobilization mechanisms combined with newly developed techniques to elucidate the interactions between matrixes and enzyme molecules are also introduced.

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Index Entries: Zeolite; molecular sieve; enzyme; immobilization; MCM-41; matrix.

Introduction

Enzymes are biocatalysts well known for their high substrate specificity and the mild temperatures in which they are able to catalyze selective transformations. The use of enzymes as catalysts in organic media is a promising field in organic synthesis. Advantages of enzymatic reaction in organic media include favorableness to hydrophobic substrates and products, mild reaction conditions, general lack of racemization, minimal side chain protection, and high regio- and stereoselectivity, all of which has led to many promising results for various synthetic applications *(1)*. These include selective acylation of castanospermine *(2)*; preparation of bioactive peptides as well as peptide mimics and enzyme inhibitors *(3–5)*; synthesis of such bioactive conjugates as peptidyl steroid, peptidealcohols *(6,7)*; and resolution of racemic compounds *(8)*. However, the natural characteristics of these biocatalysts, such as the short half-life of the enzymes, low stability, and low reusability in buffer, are often the cost-limiting factors in the practical application of enzymes. Moreover, some enzymes possess serious weaknesses in solution. For example, trypsin easily undergoes autolysis *(9)*, and water-soluble lipase has to be absorbed on a solid support in order to have significant catalytic activity *(10,11)*.

Fixation of enzymes on suitable supports is a normally accepted way to solve the aforementioned problems. Enzyme immobilization, utilizing a chemical or physical method to bind enzyme molecules to the surface of a matrix, usually allows the enzymes to enhance their stability and catalytic activities while inhibiting other undesired processes such as autolysis. Generally, the use of this strategy cannot only increase the productivity of the biocatalytic process by concentrating the catalyst and substrates on the reaction media but also increase the possibility of reuse by inhibiting the denaturation and autolysis. Furthermore, it can allow the catalytic transformation to be operated in a continuous reactor.

Recently, immobilized enzymes have been widely used to prepare enzyme electrodes and transistors for diagnostic purpose *(12,13)*, as supports in high-performance liquid chromatography separation *(14)*, as therapeutic agents *(15,16)*, for chemical synthesis *(17–20)*, for classic experimental enzymology *(21)*, and so on. With the development of these applications, a large variety of immobilization supports has been studied; Table 1 lists various immobilization supports and their characteristics. Owing to their diverse nature and interesting properties, molecular sieves have recently attracted much attention for study with the purpose of looking for new, excellent immobilization carriers. Generally, four immobilization methods are involved in enzyme fixation techniques: physical adsorption, entrapment/encapsulation, crosslinking, and covalent coupling. Adsorption is one of the easier and fastest methods to produce an immobilized enzyme.

Table 1
Various Materials Used as Immobilization Supports

Supports	Characteristics	Drawbacks
Cellose *(22)*	Polyhydroxylic material, hydrophilic behavior	Not very uniform, not very mechanically strong
Agarose *(22)*	Polyhydroxylic material, rather uniform, easily cast in bead form	Not very mechanically strong
Chitin *(23)* Chitosan	Contains free hydroxyls available for enzyme coupling	Not very mechanically strong
Calcium alginate *(24,25)*	Suitable for entrapment of cells and organelles	
Collagen *(22)*	Can provide abundant groups to couple with enzymes, excellent for membrane or sheet uses	
Polyacrylamide *(26)*	Hydrophilic behavior, easily cast in beads, available in largest variety of pore sizes and internal surface	
Polystyrene *(22)*	Hydrophobic behavior, adsorption material, low cost	Nonporous, without large surface area
Hollow fiber *(27)*	Suitable for entrapment	Expensive
Nylon *(22)*	Excellent for membrane or sheet uses	
Ultrafiltration membrane *(28)*	Suitable for entrapment and chemical covalent binding	
Hydrogel *(29)*	Hydrophilic material, stimuli response	
Magnetic materials *(30)*	Potential applications	
Celite *(22)*	Contains many metal ions, rather large surface area	
Silica *(21)*	Porous inorganic materials, large surface area	Soluble in aqueous solution above pH 7.5
Controlled pore glass *(31)*		
Controlled pore silica *(22)*		
Molecular sieves *(32)*	High surface area, hydrophobic or hydrophilic behavior, mechanical and chemical resistance	

Entrapment and crosslinking are somewhat more laborious enzyme fixation methods but yield an enzyme that is least altered by the immobilization. As for the chemical covalent coupling method, although enzyme activity is damaged to some extent during the coupling process, it can effectively protect the immobilized biomolecules from leaching into reaction solutions. Since molecular sieves distinctly have interesting properties useful for enzyme immobilization and various types of these materials can be easily obtained through thermal or chemical treatment, it is promising to study such materials for use as enzyme immobilization supports.

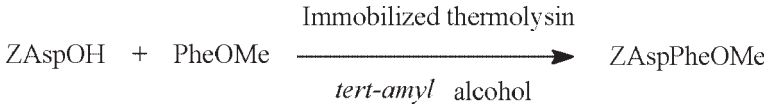
In systems containing enzymes immobilized on solid supports and the reaction was carried out in organic media, the characteristics of a support obviously have a significant influence on the total enzymatic activity and can even convert the reaction equilibrium (such as hydrolysis toward synthesis) *(11)*. Thus, the choice of a suitable support material is crucial for the successful use of immobilized enzymes in organic media. Key factors that influence the efficiency of immobilized enzymes include the water content in the reaction media, the pH value of the buffer used in the process of fixation, and the pretreatment of supports. In this article, recent progress in the immobilization of enzymes on molecular sieves is reviewed and various aspects that influence the binding efficiency as well as the activity of immobilized enzymes in organic media are discussed.

Enzyme Immobilization on Zeolite

Zeolites are crystalline aluminosilicates with microporous structures consisting of a highly organized three-dimensional structure of tetrahedral SiO_4 and AlO_4^- linked to each other by a shared oxygen *(33)*. Usually, they can be modified by thermal and chemical treatments such as cation exchange or dealumination. The modified zeolites give the possibility of creating and regulating acid-base, hydrophobic-hydrophilic, and selective adsorption properties that are responsible for their structural diversity and extensive applications in catalytic reaction. Until now, many enzymes have been studied to load onto this type of molecular sieve and diverse conversions have been realized using those immobilized enzymes.

Effect of Properties of Zeolite Matrix

Binding through physical adsorption is a common method used in the fixation of enzymes on zeolite supports owing to the small pore size of these materials, which makes it impossible to include enzyme molecules into the channels of the microporous structures. Therefore, the properties of zeolite support such as the acidity of the external surface of zeolites, the cation within the materials, and the composition of the zeolite framework are always the important parameters that affect the immobilization efficiency and activity of corresponding immobilized enzymes.



Scheme 1. Synthetic scheme of ZAspPheOMe with immobilized thermolysin as a catalyst. (Reprinted from ref. *36*.)

Effect of Support Acidity

Usually, Brønsted acidity is presented at the external surface of zeolite owing to richly distributed OH groups on the zeolite, and the acid site density can be easily manipulated by chemical treatment. Since enzymes are sensitive species that always require strict conditions to maintain activity, it is conceivable that zeolite acidity influences the absorbed enzymes. Goncalves et al. *(34)* studied the effect of support acidity on the immobilized *Fusarium solani pisi* recombinant cutinase catalyzed hydrolysis reaction of tricaprylin. The set of zeolites they used covers a large range of acidity, from the rather basic forms of NaX and NaY to the highly acidic HUSY zeolite *(34)*. A progressive increase in acidity was also generated by progressive substitution of sodium cations with praseodymium cation in order to investigate the effect of external surface acidity on the reaction studied. As a result, the negative effect was observed with the increase in acidity. Even a quite weak acidity, like the one presented by the PrNaY series, significantly reduced the enzyme's catalytic ability. As to the strong acid sample HUSY, a very drastic reduction in activity is exhibited:

$$Pr^{3+} + H_2O \longrightarrow Pr(OH)^{2+} + H^+$$

Serralha et al. *(35)* observed the same negative effect in the alcoholysis reaction catalyzed by the immobilized cutinase with strong acid material NaHZSM-5 as support compared with the results with NaZSM-5 as a support. It is assumed that acidity of the support may induce some damage to the enzyme, through ion exchange with the buffer solution cations or direct interaction with the acid sites in the external crystallite surface during the adsorption process. A similar result was also reported by our group in the study of zeolite-immobilized thermolysin *(36)*. Four kinds of zeolites (microporous HY, NH_4Y, NaY, and mesoporous HNH_4DAY) were employed as matrixes to fix thermolysin, and an enzymatic coupling reaction between ZAspOH and PheOMe was selected to evaluate the activity of immobilized enzyme (Scheme 1). It was found that the activity of immobilized thermolysin by HY zeolite was very low and the coupling yield of dipeptide ZAspPheOMe was poor, indicating a drastic activity loss when the enzyme was adsorbed on the acidic HY zeolite. The exact pH value changes during the enzyme fixation (Table 2) were tested and it was found that owing to the strong acidity of HY zeolite, the pH value of the preparation solution decreased remarkably (from 6.98 to 3.22) after an hour's stirring in the immobilization process. Protease thermolysin is stable from pH 6.0 to 9.0, so the loss of activity in low pH value (3.22) was reasonable.

Table 2
The pH Value Changes During Immobilization of Thermolysin on Different Zeolites[a]

Zeolite	HY	NaY	NH_4Y	HNH_4DAY
Starting pH value of the enzyme solution	6.98	6.98	6.98	6.98
pH value of the water phase after adsorption on zeolite for an hour	3.22	7.10	6.76	6.84

[a]Reprinted from ref. *36*.

Table 3
The pH Value Changes During Immobilization of α-Chymotrypsin on Different Zeolites[a]

Zeolite	HY	NaY	NH_4Y	HDAY	HNH_4DAY
Starting pH value of the enzyme solution	7.95	7.95	7.95	7.95	7.95
pH value of the water phase after adsorption on zeolite for an hour	4.07	7.74	7.55	6.69	7.31

[a]Reprinted from ref. *36*.

However, HY zeolite–immobilized α-chymotrypsin still possessed a high catalytic activity partly resulting from the broader stable pH range (3.0–10) of α-chymotrypsin (Table 3) compared with that of thermolysin. It was then suggested that the external acidity was not the single factor responsible for the loss of activity. Thus, it was supposed that the intrinsic properties of enzymes were also the aspects that should be considered in the immobilization.

Effect of Composition of Zeolite Framework

There are several aspects concerning zeolite composition. The first aspect is related to its Si:Al ratio. In fact, the aluminum content of the crystalline network will determine the global framework charge, as well as the amount of cations in the structure, and will drastically influence the characteristics of the structure such as hydrophobic/hydrophilic and acid/base. Considering that the enzyme activity presented by a given preparation will probably depend on the enzyme-support interactions, as well as on the substrate partition between the organic solvent and the support surface, it is not surprising that the results for the immobilized enzyme are greatly dependent on the framework's composition. Goncalves et al. *(34)* tested several samples of nonacidic zeolites (to exclude the disturbance of acidity) with different Si:Al ratios (NaA, NaX, and NaY) as enzyme supports in the cutinase catalyzed hydrolysis reaction. The results showed

that for those different preparations, the enzyme's hydrolytic activity increased as the Si:Al ratio increased, probably owing to a slight decrease in the hydrophilic property of the surface, which can possibly increase the affinity of the substrate to the support. By contrast, Serralha et al. *(35)* reported the opposite tendency using zeolites NaX, NaY, NaDY, NaM, NaZSM-5, and NaHZSM-5 as matrixes, respectively, for loading of cutinase in the alcoholysis reaction in organic media. Their figure showed that zeolites with a high Si:Al ratio exhibit lower enzymatic activities. The same result was also obtained by our group in the study of the synthesis of the tripeptide ZTyrGlyGlyOEt, a protected fragment of Leu-enkephalin with immobilized α-chymotrypsin as catalyst. We compared two types of carrier with different Si:Al ratios, Y zeolite and DAY zeolite (dealuminized Y zeolite), in which Y zeolite possesses a lower Si:Al ratio. The data suggested that Y zeolite was a better carrier for the peptide synthesis *(36)*. Lie and Molin *(37)* evaluated both hydrolysis and esterification reactions with immobilized lipase on hydrophobic and hydrophilic zeolites. They concluded that hydrophilic zeolite was superior to hydrophobic carrier in the esterification, while hydrophobic zeolite was better than a hydrophilic one in a hydrolysis reaction, indicating that the optimum type of carrier during hydrolysis differed from that of the esterification reaction.

It seemed that enzyme-catalyzed esterification, alcoholysis, and peptide bond formation were affected by many factors. It is not easy to get a clear correlation between the Si:Al ratio of a zeolite with corresponding activity of an immobilized enzyme.

Another interesting aspect in relation to the zeolite's composition is the influence of the cation. Zeolite, being crystalline silica-aluminates, of which the aluminum is in a tetrahedral arrangement, requires one monovalent cation per each aluminum atom in the framework. These cations are easily exchangeable and affect very drastically the properties of the zeolites in their various applications. Serralha et al. *(35)* studied the nature of the cation on the specific activity of immobilized cutinase using zeolite A exchanged with different cations: Li^+, Na^+, K^+, Cs^+, and Ca^{2+}. The data revealed that the range of specific activities was not much different from one sample to another. Nevertheless, the optimal activity value was obtained for different amounts of water in the reactions; the optimum water amount increased in the following order: KA ≈ CsA ≈ NaA < LiA < CaA. This order indicated that the higher the charge density of the cation, the higher the water content for optimal activity will be. For zeolites containing cations with a higher charge density, which will bind water more tightly, higher water content in the reaction media will be needed in order to reach the optimal enzyme activity. Thus, the nature of cation in the zeolite framework influences the enzymatic reaction through the water content needed for the optimum activity. Since adding water beyond the essential water activity level will result in an increase in the competing hydrolysis reaction and, at the same time, a decrease in the alcoholysis reaction rate *(38,39)*, the nature of cation in zeolite carrier should not be neglected when selecting a

suitable support. Goncalves et al. *(34)* investigated the same effect using NaX or NaY zeolite as supports in which the sodium cation was totally or partially substituted by protons or by praseodymium trivalent cations. In these cases, they recommended that the influence of the nature of the cation is caused by the generated acidity *(34)*. However, a different behavior was observed when the sodium cations were substituted by ammonium ions. A simultaneous decrease in the coupling yield of the enzyme onto the support and the specific activity was found. In the case of the ammonium form of the dealuminated Y zeolite, the activity of the immobilized enzyme was too small to be measured. This result indicated that there are other factors influencing the enzyme-support interaction apart from the acidity in those cases.

Therefore, the cation in the carrier affected the immobilization process and the efficiency of immobilized enzyme in complicated ways. Furthermore, it should be noted that those different aspects of zeolite matrix do not exert their influences in an independent way; instead, the complicated interactions existed among them. For example, the change of cation in the carrier can directly cause an emergence of electric field as well as the acidity at the external surface; and all these mixed issues affect the enzyme-matrix interactions. It was proposed that elucidating these interactions will depend on some newly developed techniques such as powder XRD, ^{27}Al, ^{29}Si MASNMR, HEMS, Synchrotron radiation, as well as the help of computer modeling *(40–43)*.

Effect of Water Content in Reaction Media

Water content is one of the most important aspects to be considered in choosing reaction conditions for the immobilized enzyme–catalyzed reaction. Although the water contents in these systems are generally low, water plays a crucial role in modulating the catalytic properties of the enzymes *(11)*. A completely dehydrated enzyme is not catalytically active. Water increases the internal flexibility of the enzyme molecule, which is necessary for catalytic activity *(44)*. Excess water in the reaction media may generate unexpected processes such as autolysis of enzyme, reversal of alcoholysis to hydrolysis, and secondary hydrolysis of the product *(45)*.

As discussed previously, there is always an optimal water content for a free enzyme–catalyzed reaction in organic media *(41)*. We have studied the effect of water content in dichloromethane on the zeolite-immobilized α-chymotrypsin-catalyzed peptide synthesis *(36,46)*. With HY zeolite-loaded α-chymotrypsin as an example, different amounts of water were added to the reaction to observe the trend, and the results were compared with those obtained with the corresponding free enzyme as a catalyst (Fig. 1). Interestingly, in the HY zeolite–immobilized α-chymotrypsin-catalyzed synthetic reaction, the optimum water content range was broader (0.25–1.0% [v/v]) in contrast with the corresponding value of 0.15% (v/v) in the condition of free enzyme–catalyzed reaction. This effect may be owing to the characteristic of the support. Since a molecular sieve itself can easily adsorb water

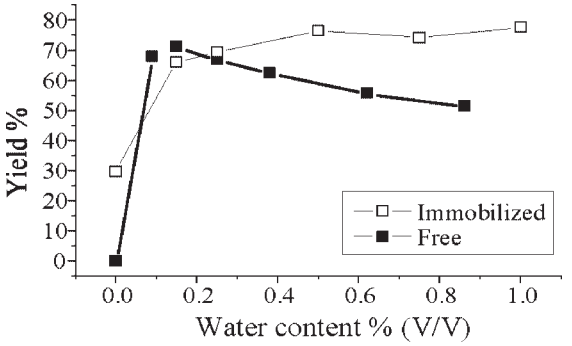


Fig. 1. Influence of water content on yield of ZTyrGlyGlyOEt catalyzed by HY zeolite–immobilized or free α-chymotrypsin. (Reprinted from ref. *36*.)

molecules that cannot be completely removed during the lyophilization for preparing the immobilized enzyme, this residual water can serve as the essential water for the enzyme to maintain its catalytic activity in organic solvent. As a result, the sensitivity of immobilized α-chymotrypsin to the water content decreased and the range of the optimal water content extended. Goncalves et al. *(47)* discussed this problem with different materials—NaX, NaY, NaUSY, and NaDY—as matrixes. Their results showed a general increase in activity with increasing values of water content, with the exception of NaUSY for high water content. In both cases, NaY presents the best effectiveness for the reaction. It was also found that there were some drawbacks with high amounts of water in organic media. For example, the reusability of HY-immobilized α-chymotrypsin with high water content decreased dramatically even though it exhibited high activity when used the first time. In addition, the emulsion and some enzyme desorption was observed by Goncalves et al. *(47)* in the condition of high amounts of water. Similarly, Adlercreutz *(11)* concluded that the water-adsorbing capacity of the complete preparation was the sum of the water-adsorbing capacities of the support, the enzyme, and the buffer salts.

Effect of pH of Preparation Buffer

The common procedure of a physically adsorptive immobilization of enzymes on zeolite supports involves the stirring of preparation buffer solution with certain pH value and ionic strength in which both matrix materials and the enzymes are contained. Thus, the pH value of the buffer used is often considered as an important factor influencing the efficiency of immobilization. Usually the amount of enzyme adsorbed on a support is maximum at its isoelectric point because the electric repulsion between the adsorbed enzyme molecules and support is at a minimum *(48)*. As an example, the maximum activity of NaY zeolite–immobilized cutinase was at pH 8.0 near the isoelectric point of cutinase *(37)*. It is also noticeable that since usually the acidity exists at the surface of zeolite, the isoelectric point of the enzyme should not be the only aspect to be considered.

In the process of immobilization of glucose oxidase on a type of β zeolite, considering the isoelectric point of glucose oxidase (4.3) as well as that of the matrix (4.05), the best result was gained when the pH range of the preparation buffer dropped from 4.0 to 4.3 *(49)*. Since the negative charge was expressed at the surface of β-type zeolite and the positive charge exhibited at the surface of glucose oxidase molecule at this range, the strong electrostatic interaction between the support and enzyme occurred and the efficiency of immobilization was reasonable.

MCM-41: A New and Promising Immobilization Matrix

Because of the microporous characteristic of zeolite, the common way for immobilization on this type of matrix is physical adsorption, which is the main weakness of desorption in the process of a continuous reactor. This drawback seriously restricts its application in industry. Fortunately, a new family of molecular sieve, MCM-41, was discovered by Mobil Technology *(50)*. The MCM-41 materials possess a regular array of uniform, unidimensional mesopores with a very narrow pore size distribution, which can be systematically varied in size from approx 20 to 100 Å. The MCM-41 materials have been used as hosts for photoinduced electron transfer reactions with bulky substrates and conducting polymers as well as metal complexes. The large pore dimensions of these mesoporous materials offer the possibility of accommodating small enzymes within the channels, which can be several hundred nanometers long. Furthermore, the pore openings of MCM-41 materials can be modified with organosilane groups, resulting in a reduction in the channel window diameter, which could effectively entrap a guest molecule. This approach to enzyme immobilization would resolve some of the disadvantages of other physical entrapment techniques such as the leaching of adsorbed molecules, the chemical degradation of the anchoring bond of covalently attached enzymes, and the barriers to diffusion of substrate and product encountered for large polymeric substrates in sol-gel preparations. The MCM-41 hexagonal phase could also be suitable in the evaluation of theoretical models of enzyme immobilization that assume a uniform pore size for the model supports, which is in contrast to most amorphous materials that possess a significant pore size distribution *(51)*.

Effect of Pore Size on Immobilization

The high dependence of the immobilization efficiency on pore diameters for the MCM-41 materials is determined by the fixation mode, which is different from that with microporous zeolites as matrixes. The immobilization of globular enzymes, such as cytochrome-*c* (bovine heart), papain (pagaya latex), and trypsin (bovine pancreas), in the mesoporous molecular sieve MCM-41 was tried by Diaz and Balkus *(51)*. A clear correlation between enzyme size and loading appeared, indicating the diffusion of enzyme molecules into the mesopores of MCM-41. For the MCM-41-immo-

bilized trypsin, a serious leakage (52%) occurred after being used one time owing to the large pore size. If the channel openings can be chemically reduced in size, then the enzyme would become physically entrapped. Thus, Diaz and Balkus *(51)* modified the MCM-41 through silanation, which proved successful in stopping leakage of the enzyme in basic solution. In addition, Gimon-Kinsel et al. *(52)* re-evaluated loading of the globular enzyme horse heart cytochrome-*c* (≈30 Å) on mesoporous all silica MCM-41 (≈32 Å), aluminosilicate MCM-41 (≈32 Å), all silica MCM-48 (≈34 Å), and Nb-TMS1 (≈33 Å) molecular sieves, and a clear dependence of adsorption efficiency on pore sizes of the molecular sieves was exhibited. After silanation of the molecular sieve's pore openings, a significant prevention of leaching even at high pH values was obtained. Takahashi et al. *(53)* systematically studied the correlation between pore diameter and adsorbed amounts of enzyme. As shown in Table 3, the loading amounts of enzyme improved with the increase in pore diameter in FSM-16 and MCM-41 series. Based on the data of nitrogen adsorption isotherms and XRD measurements, they proposed that horseradish peroxidase (HRP) molecules were adsorbed in the pore spaces of the FSM-16 materials with diameters of 51 and 89 Å while the HRP molecules only exist on the outer surface of the material with a diameter of 27 Å *(53)*. However, as for the effect of pore size on enzyme stability, an interesting result was exhibited. Enzymes (HRP and subtilisin) immobilized on FSM-16/51 expressed highest thermal stability among selected matrixes of FSM-16/27, FSM-16/51, and FSM-16/89, indicating that an immobilized enzyme showed excellent thermal stability when the average mesopore size matched the molecular diameter of the enzyme.

Mechanism of Immobilization of Enzymes on Mesoporous Molecular Sieve

Although a significant activity was obtained even after incubation of MCM-41-entrapped trypsin at 25°C for 1 wk, its catalytic activity value was moderate *(51)*. Diaz and Balkus *(51)* explained this effect with the orientation during the fixation, but they did not give the mechanism of interaction between enzymes and carriers. Takahashi et al. *(53)* applied the measurement of ionic pigment adsorption to determine why enzymes were selectively adsorbed to the mesoporous silica prepared with a cationic surfactant, such as FSM-16 or MCM-41 (Table 4). As a consequence, the adsorbed amounts of a cationic methylene blue for FSM-16 or MCM-41 materials were much higher than for SBA-15 materials (prepared with a nonionic surfactant). Since the use of a cationic surfactant may lead to enhancement of the anionic potential of the silanol groups on the surface to facilitate favorable adsorption of cationic molecules, they suggested that the ionic characteristics of the material surface determine their adsorptive selectivity to ionic compounds *(53)*. They investigated the diffusion of penicillin acylase into the channels of MCM-41. It was found that penicillin acylase immobilized on the MCM-41 in this way was more active than that

Table 4
Physicochemical Properties of Mesoporous Silica Materials
and Amounts of Enzymes and Pigments Adsorbed to Mesoporous Silica Materials[a]

Porous silica	Pore diameter (ϕ)[b]	BET surface area (m^2/g)	Total pore volume (cm^3/g)	Adsorbed amounts			
				HRP (mg/g)	Subtilisin (mg/g)	Methylene blue (mmol/g)	ASAS (mmol/g)[c]
FSM-16	27	927	0.84	28	34	0.29	0.09
FSM-16	51	848	0.95	133	145	0.26	0.09
FSM-16	89	770	1.22	183	198	0.23	0.10
MCM-41	50	949	1.01	98	106	0.20	0.07
MCM-41	68	820	1.15	147	152	0.19	0.09
SBA-15	50	695	0.56	10	15	0.13	0.09
SBA-15	92	810	0.94	24	28	0.12	0.07
Silica-gel	20–200	451	0.85	49	52	0.11	0.08

[a]From ref. *53*.
[b]Calculated from the adsorption branch of the N isotherm.
[c]Anthraquinone-2-sulfonic acid sodium.

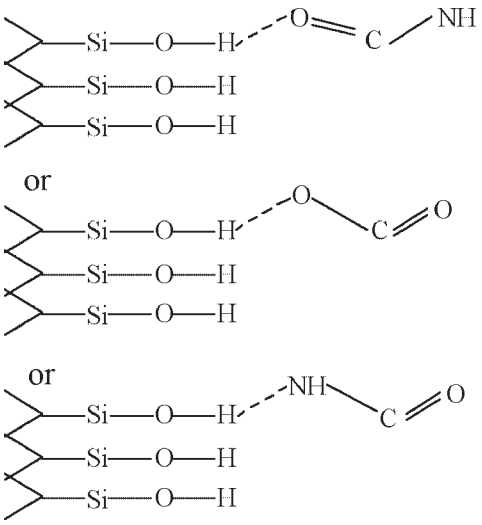


Fig. 2. Schematic representation of interaction between penicillin acylase and MCM-41. (Reprinted from ref. *54*.)

immobilized on other supports as well as free enzyme. They also studied the interaction between penicillin acylase molecules and the MCM-41 surface by infrared (IR) spectroscopy. Both the dramatic intensity reduction of the IR adsorption band at 3740 cm^{-1} that was assigned to acidic Si-OH groups and the appearance of the band at 3400 cm^{-1} that existed in the hydrogen bond region after immobilization clearly indicated the hydrogen bond interaction between hydroxyl groups and carbonyl or N-H groups in penicillin acylase molecules and the Si-OH groups at the pore inner surface of MCM-41 *(54)* (Fig. 2). In summary, the characteristics of the enzyme itself as well as the properties of the support's surface determine the interaction between them and the efficiency of immobilization. Different enzymes present different requirements for the properties of carriers in order to gain excellent enzyme activity and stability.

Immobilization of Enzymes with Chemical Covalent Bond

Even though fixation of enzymes through accommodating those molecules into channels of mesoporous molecular sieve has been shown to be an effective way to improve the stability and reusability of those biocatalysts, this method could not avoid the leaching problem. This was determined by the characteristic of weak interactions based on physical adsorption. Humphrey et al. *(55)* managed to immobilize trypsin into functionalized molecular sieves MCM-41, MCM-48, and SBA-15 (Fig. 3) through chemical covalent bonds. The leaching of enzymes into solution after being used twice was tested with mesoporous materials with different dimensions and structures as supports (Table 5). Trypsin loaded on is-Pr-SH-SBA-15 showed the highest activity and was also found to be recyclable

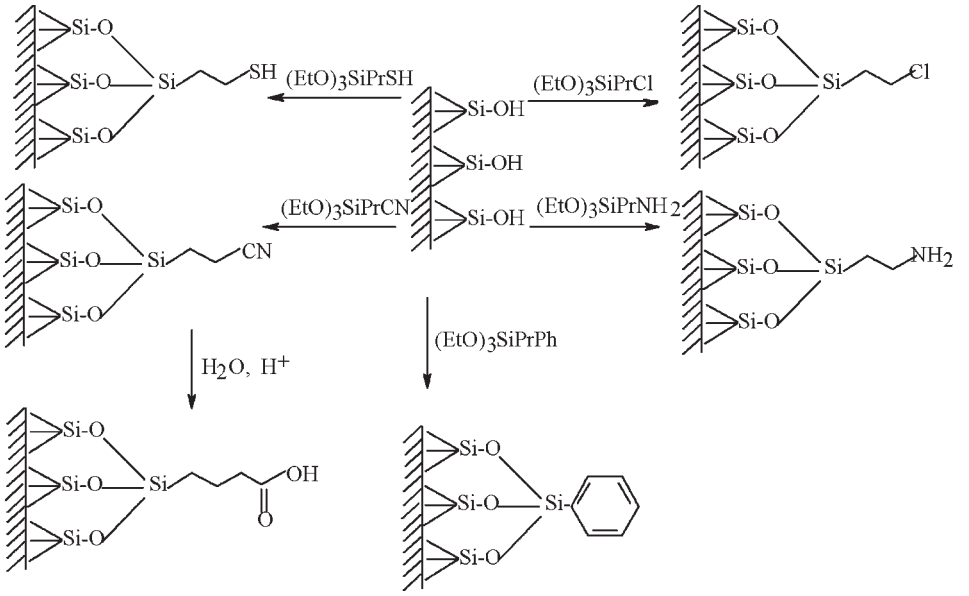


Fig. 3. Functionalization of surface of mesoporous molecular sieve. (From ref. *55*.)

Table 5
Immobilization of Trypsin
on Functionalized Mesoporous Molecular Sieve SBA-15[a]

Support	Immobilizing (%)	Leached (%)
SBA-15	100	52
Ps-PrCl-SBA-15	94	0
Ps-PrOH-SBA-15	73	16
Ps-Ph-SBA-15	88	55
Ps-PrNH_2-SBA-15	82	20
Ps-PrCN-SBA-15	72	28
Ps-PrCOOH-SBA-15	97	11
SBA-15 (extracted)	94	48
Is-PrCl-SBA-15	93	1
Is-PrSH-SBA-15	90	
Is-PrPh-SBA-15	91	19
Is-PrCOOH-SBA-15	70	2

[a]From ref. *55*.

(55). Another enzyme, α-amylase, used for hydrolysis of starch, was also successfully immobilized by Mody et al. *(56)* on amino-functionalized surfaces of silica supports (named SBA-15, silica-N, and silica-AC) by covalent bonding through glutaraldehyde. The resulted specific activity of α-amylase was observed to follow the order silica-AC > silica-N > SBA-15 while they exhibited higher operational stability in the order of SBA-15 > silica-

AC > silica-N. In addition, the comparatively lower specific activity than that of free enzyme was found. Since this fixation method through chemical covalent bonding may bring some damage to enzymes, the degree of loss of enzymatic activity was reasonable.

In summary, enzymes immobilized on mesoporous molecular sieves have shown great potential for practical applications. Although their lower resistance to mechanical and chemical treatment may prevent them from replacing microporous zeolite in practical use, further improvement of the characteristics of this kind of material may lead to its common use in the future. Furthermore, there is still much work to be done on the mechanism of immobilization both on zeolites and mesoporous molecular sieve.

Conclusion

The favorable characteristics of zeolites and MCM-41 molecular sieves demonstrate great potential as the immobilization matrixes for different enzymes. Numerous factors influence the immobilization efficiency and the activity of corresponding bioactive species. However, it must be noted that no single immobilization method or immobilization support is best for all enzymes or all applications of any given enzyme; although this article focuses on an adsorptive or entrapment method based on the characteristics of molecular sieves, the covalent coupling method is also valuable. The choice of a given immobilization method, an appropriate matrix, or even the best enzyme to use is highly dependent on the application requirement as well as on the deep insight into the interactions between enzyme molecules and supports. With the help of newly developed techniques such as powder XRD, ^{27}Al, ^{29}Si MASNMR, HEMS, Synchrotron radiation, and computer modeling, the interactions between small molecular substrates-matrix have been elucidated fairly clearly. Therefore, it is reasonable that the development of biotechnology, material chemistry such as the nanomaterials *(57)*, and surface chemistry will lead to advances in the understanding and practical application of molecular sieves as enzyme immobilization supports.

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