Abstract: In this review, factors that contribute to enhancing the stability of immobilized enzyme membranes have been indicated, and the solutions to each factor, based on examples, are discussed. The factors are divided into two categories: one is dependent on the improvement of enzyme properties, and the other, on the development of supporting materials. Improvement of an enzyme itself would effectively improve its properties. However, some novel materials or novel preparation methods are required for improving the properties of supporting materials. Examples have been provided principally aimed at improvements in membrane stability.

Keywords: stability; long-term stability; enzyme-immobilized membrane; supporting materials; matrix

1. Introduction

There has been growing interest concerning immobilized enzyme membranes in the last two decades [1]. Immobilized enzymes can be applied not only to biosensing systems but also to bioreactors, medical analysis, and biofuel cells [2–7], and the demands for high-performance immobilized-enzyme membranes have been increasing. Development of immobilized enzyme membranes are expected to advance performances of analyzers and bioreactors, and to enlarge their coverage areas such as enzyme-based fuel cells. In these developments, one demand is for the improvement of immobilized enzyme stability. Naturally, the lifespan of apparatuses such as biosensing systems, bioreactors and biofuel cells could be extended by improving immobilized enzyme stability [8–10], and, as a result, the cost would be reduced.

The type of enzyme stability required should be fully considered. For example, heat stability should be required for the heat tolerance. If it is considered that the properties of the immobilized enzyme membranes consist of the combination of the properties of enzymes and supporting materials, heat tolerance would be mainly dependent on the enzyme properties. For example, Rueda et al. insist that chemical modification of enzymes and immobilization are considered as separate mechanisms to improve enzyme properties [11]. The relationship between key factors of stabilization on the immobilized enzyme membranes and constituent elements such as enzyme and supporting material is shown in Figure 1. It is assumed that properties of the appropriate elements are improved to enhance its stability. Concerning element enzymes, the developments of enzymes have been covered by other reviews, so immobilized enzyme membranes have been described, and supporting materials and immobilized enzymes have been detailed separately in this review.

Stability-enhancing techniques for key factors of each element have been introduced in this review. Generally, there are some reviews on the topic of immobilizations and immobilizing methods. In contrast, there are few reviews of stability on immobilized enzymes because evaluation criterion of stability is not simple. Regarding the types of stability, long-term stability was given more attention because improvement of stability leads directly to lengthening the lifetime of the apparatus.
In particular, a factor of long-term stability has been focused on in this review and new strategies have been introduced: the topic is unique and noticeable.

**Immobilized-enzyme membranes**

Key factors for stabilization:

- Supporting materials
- Stability against chemicals
- Stability against physical conditions
- Prevent from fouling
- Corrosion protection from bacteria

**+**

- Immobilized enzymes
- Heat stability
- Stability against physicochemical conditions
- Stability against hydrophobic solvents
- Stability of immobilization or entrapment

**II**

Total property of immobilized-enzyme membrane stability

*Figure 1.* Scheme of key factors for stabilization of immobilized-enzyme membranes and relation to the two elements, supporting materials and immobilized enzymes.

**2. Element One: Immobilized Enzymes**

In this review, immobilized-enzyme membranes have been described, which can be divided into two elements: immobilized enzymes and supporting materials. In this section, immobilized enzymes and their improvements for the enhancement of stability are introduced. By changing the enzyme itself, heat tolerance, pH stability and stability against the non-aqueous media of immobilized enzyme membranes can be improved; in the following subsections, the mechanisms on how the enzymes are changed and stability is improved have been discussed. For inducing mutations, protein engineering is a key technology [12,13]; examples are presented for improvements, such as pH stability [14] and heat tolerance [15]. Stability of enzymes produced via protein engineering can be examined using in silico prediction and actual measurement [16]. Enhancement of pH stability is not so easy because amino acids with electrically charged side chains exist at the enzyme surface and pH change makes slight changes of enzyme structure by charge distribution change caused by dissociation and association of the side chains. To solve the problem from pH change, accumulation of knowledge via protein engineering should be required. Since research is underway for the improvement of protein engineered-envelope stability, the information currently available has been discussed in this review.

**2.1. Strategy for Enhancing Heat Stability**

To enhance the heat stability of immobilized-enzyme membranes, heat stability of immobilized enzymes is the key factor; supporting materials usually have a greater heat tolerance compared with that of the enzymes. Immobilization enhances stability; however, the extent of the enhancement is unpredictable. To confirm the enhancing effect, it is important to show that improvement of the enzyme itself is necessary.

To obtain an enzyme with heat tolerance, the quickest way is to employ the enzymes from thermophilic microorganisms [12]: as the thermophilic microorganisms are alive at elevated temperatures compared with normal ones, enzymes from thermophilic microorganisms have a naturally higher level of heat tolerance. In general, the optimal operating temperature for moderate thermophiles is 60 °C and 95 °C for extreme thermophiles. However, there are two problems; one is the lack of these types of enzymes, and the other is their low activity at room temperature. Regarding
the first problem, appropriate enzymes should be found in thermophiles: ordinary enzymes would exist; however, there is no assurance that all the enzymes required would be present. Regarding the second problem, the rates of enzyme reactions obey the Arrhenius equation at the lower temperature of the optimum. Because the enzymes would be used at much lower temperatures than their optimum temperature, reaction rate at operational temperature would be quite lower compared with the specific activity of enzymes at the optimum. To solve these problems, researchers have turned to the technique of protein engineering.

Protein engineering based on genetic modification is a technology used to change the primary structure of proteins or to improve the rigidity of their structures by replacing amino acids to change protein properties [17–20]. Drobecq et al. changed the cysteine residue to alanine in small ubiquitin-like modifiers to improve their thermo-stability [17]. Several other papers have also reported that site-directed mutagenesis (replacing an amino acid with another) of enzymes enhanced heat stability [21,22]. To ensure the rigid structure of enzymes, the simplest way is to introduce a disulfide-bond at an internal chain of proteins [17–23]. This protein engineering technique has infinite possibilities [24]. However, knowledge of the relationships between protein structures and their properties is still lacking at this stage. Though thermo-stability prediction of enzymes combined with known protein sequences has been carried out with molecular dynamics simulations [25,26], and the complete design of amino acid sequences cannot be feasible.

Instead of the protein engineering technique, alternative techniques could be used for enhancing protein properties. One technique is the chemical modification method [12]. Fortunately, knowledge regarding chemical modifications has been accumulated, and an advantage applicable to enzymes has already been found. Siddiqui and Cavicchioli reported chemical modification with oxidized polysaccharides to lipase, and the modified enzyme showed enhanced thermal stability [27]. Cowan and Fernandez-Lafuente reviewed chemical modification and immobilization methods that enhance the properties of thermophilic enzymes [6]. As chemical modifications (and immobilization) suppress the structural changes by modifier, heat tolerance is enhanced.

2.2. Stability against Organic Media

As shown in the above section, chemical modification maintains the formation of the modified-enzyme. This effect has been applied to enhancing properties such as enzyme activity, heat tolerance, and pH stability [28–38]. Figure 2 shows one example of modified enzymes and schematic illustration of its’ enhancing mechanism. In the case of hydrophobic organic media, the modification method is effective to protect enzymes from denaturing by the media. As shown in this figure, an amphiphilic molecule was used to modify an ordinary enzyme. In aqueous media, the original enzyme and modified enzyme are soluble, and both are active. In contrast, in the organic media, the modified enzyme retains its activity while the unmodified enzyme loses its activity. This is because the enzyme is deformed by the hydrophilic/hydrophobic environment change i.e., the hydrophobic part of the enzyme, which is usually buried inside, is forced to the outside, and the hydrophilic part is forced to the inside. Modification would prevent deformation of modified-enzymes from the hydrophobic effects. The prevention would be mainly caused by the slight existence of water molecules at the enzyme surface. We synthesized a modified-enzyme, which consisted of an enzyme and an amphiphilic molecule, activated polyethylene glycol (PEG), as a modifier [29–35]; the modified enzyme was soluble in organic media and retained its activity in organic media. By applying the modified enzymes when constructing carbon paste-enzyme electrodes, high-performance biosensors can be obtained. Carbon paste contains oil and graphite, so the immobilized enzymes on or in carbon paste should be protected from the effect of organic media.
Similarly, amphiphilic materials can have an amphiphilic effect even if they do not bind to the enzymes. Cipolatti et al. prepared lipase-immobilized PEGylated polyurethane particles and showed a stabilizing effect for oil ethanolysis [36]; lipase was adsorbed on the polymer, and the protection of the PEG derivative against the oil environment was effective on the enzyme. Pazhang et al. showed the stabilizing effect of sorbitol and glycerol on trypsin in organic solvents [37]. As a result, immobilized enzymes were stabilized by the mechanism, wherein amphiphilic molecules are not required to bind covalently.

In non-aqueous systems, retaining enzyme activity and stability can be attained. In addition, new instances of enzyme stability in non-aqueous media such as ionic liquids have been found. Wang et al. reviewed concisely enzyme stability and activity in non-aqueous reaction systems [38]; this review deals with the structure and activity of enzymes in non-aqueous media such as organic solvents and ionic liquids. From the above points, it is apparent that modification by amphiphilic molecules retains enzyme structure.

For hydrophilic organic media, the prevention effect by PEG modification is not strong: the media can be miscible with water, and the mixed media could be reached and exchange water at the enzyme surface. As enzyme deactivation would depend on its structure and hydrophobicity of the media, stability against the media [39,40] and enzyme conformation [40,41] are examined. Some extent of stability enhancement would be expected by immobilization, so that some biosensors can be prepared by using native enzymes [42,43]. Enzymes with more stability have been obtained by chemical modification [44], cross-linking [45], and direct evolution [46] methods. Developments of these techniques would be opened for preparing stable immobilized enzyme membranes.
2.3. Stability by Immobilization

Immobilization methods for enzymes (proteins) are classified into two categories—one is the method that uses covalent bonds and the other is the method that does not use covalent bonds. The former method has the advantage of non-leakage of enzymes; if the enzymes are bound by covalent bonds, it is difficult for them to leave the matrix. In contrast, it would be of concern that there is some leakage of enzymes from immobilization matrices that use non-covalent bonding, such as adsorption of solid substrates and entrapment in polymers. Any leakage from the matrix would lead to loss of enzyme activity of the immobilized matrix.

Another factor contributing to loss of enzyme activity is deactivation during immobilization; deactivation would occur when the enzymes are adsorbed strongly on the substrate. Deactivation would be caused by the deformation of the enzymes by strong adsorption. On the contrary, weak adsorption would cause leakage of the immobilized enzymes. Xiaoyan et al. examined the relationship between surface conditions and immobilized enzyme stability on graphene [47]; graphene has unique characteristics including electrical, thermal, mechanical and chemical properties, and it would be interesting to immobilize enzymes on it. However, it has been reported that there are some problems regarding stability of enzymes upon immobilization. Information on the relationship would be important for constructing immobilized enzymes on a carbon-based matrix.

Immobilization methods such as adsorption or entrapment are required for immobilizing stability (i.e., to prevent leakage of the enzymes from matrix) as mentioned above. To prevent leakage, some alternations would be necessary such as changing the matrix materials and/or covering the immobilizing layer. When entrapping enzymes, penetration of substrate into the membrane should be considered. Substrates, which are smaller molecules compared with enzymes, should easily penetrate, and enzymes, which are larger molecules compared with substrates, should be prevented from leakage. The points that the matrix should be required to have are mentioned in another section of this manuscript.

Tight immobilization, which can be achieved by covalent binding, and retaining enzyme activity would be related as a tradeoff; the immobilization method used would be determined by the kind of enzymes and matrix used [48].

3. Supporting Materials

In the past, the function of immobilized enzymes was expected to reserve the enzymes from reaction solutions. The process would be to cut the cost, and immobilized enzymes have been used for industry. Demand for higher-performance immobilized enzyme membranes has arisen: membrane with chimera function, which unites enzyme and supporting material functions, is an example [49]. In the last two decades, it has been required to exert immobilized enzymes performance fully. Especially for applying immobilized enzymes membranes to bio-fuel cells, immobilized enzymes are required to perform its enzymatic function completely [50]. The supporting materials of the membranes are also demanded for this purpose: immobilization should not lose enzyme function such as activity and stability. Recently, along with the progress of nanotechnology, nanocomposite membranes have been developed: it would be enabled to control enzymes at one molecular level. In these ways, function of the supporting materials is important for the immobilized enzyme membranes.

It is important when enhancing stability of enzyme membranes not only to stabilize enzyme properties but also to stabilize the membrane matrix. Generally, stability for pH [51–54], heat [52–58], and other properties [59–71] is improved by the immobilization of the enzymes. However, as mentioned above, improvements in enzyme properties such as heat tolerance and pH stability are effective in enhancing stability. To stabilize the membrane matrix effectively, several factors, as shown in Figure 1, can be pointed out. There is no given method for designing the materials of a matrix to address these factors because the factors are not fully clarified. Therefore, improvements for these factors are not based on scientific problems but on engineering solutions. From this viewpoint, it is preferable to introduce several examples in this manuscript.
To enhance the long-term stability of immobilized enzyme membranes, factors that decrease stability should be eliminated. The factors that affect the supporting materials can be classified into three groups [10]: (1) tight binding (or immobilization) to the materials; (2) fouling and contamination caused by proteins, lipids, and microorganisms in the samples; and (3) lifetime of supporting materials. Though these factors would complicedly conform to the property of the stability in the actual case, solutions to each factor are shown below.

The first factor, tight binding or covalent immobilization, would be a tradeoff against enzyme activity. As mentioned above, tight binding would reduce the loss of enzymes from substrates (or matrix) [72], but there is a fear that some chemical reagents can damage the enzymes. To view the tradeoff relationship, in some cases, it is useful to use tight binding, such as immobilization on nanomaterials [73], forming aggregates (binding itself) [74], and immobilization on a matrix with a sparse density [49,75,76]. Another method to prevent enzyme loss to the substrates is by covering it with a density membrane (Figure 3). Nafion [77], chitosan [78], ionic polymers [79], polyion complex membranes [80–83], and cellulose [10,84,85] have been used to cover the enzyme layer. These methods are effective, but the resulting membranes can become thicker, i.e., penetration of substrates can worsen.

![Figure 3](image_url). Schematic illustration of covered type-immobilization of the enzyme layer. Supporting material should prevent enzyme leakage and penetrate the substrate—i.e., if a polymer is used as the supporting material, mesh size of the polymer network should be greater than the enzyme substrate and smaller than the immobilized enzymes.

Solutions to the second and third factors are introduced in the following two sections.

3.1. **Prevention of Fouling by Supporting Materials**

Fouling on the surface of immobilized enzyme membranes can be caused by co-existing materials in the samples; there may be many co-existing materials, such as proteins, lipids, carbohydrates, and larger particles in the samples. The problem is that these materials adhere to the surface, and the analytes are prevented from reaching the immobilized enzyme. As a result, apparent enzyme activity is reduced with the passage of operating time. Córdova et al. described the fouling mechanism of micro-pore membrane surface as follows [86]: as the pores of the membrane are blocked by fouling, performance deteriorates. Deterioration can be prevented by reducing the amount of interfering materials and/or the use of supporting materials that reduce their adhesion. Because the former method cannot be applied to all samples, novel supporting materials should be developed.

To avoid the adhesion of proteins on immobilized enzyme membranes, different supporting materials including polyurethane [87] and polysiloxane [88–93] have been used as the membrane matrix. A hydrophobic supporting matrix would be suitable for the elimination of hydrophilic species.
such as proteins and electrochemically interfering species in the samples [91]. However, there is a concern that species with a hydrophobic structure might adhere to the matrix surface.

It has been reported that porous materials can be used as a supporting matrix for enzymes; since they have a high surface area, high enzyme loading can be expected. Usually, porous materials are made from inorganic substances that are stable. Fouling due to macromolecules would occur at the outer surface of the porous materials as described above; within the porous part, fouling can be avoided. On the basis of these ideas, porous carbon [94], microporous polymers [95,96], mesoporous zirconium [97], and mesoporous silica [98–102] have been developed. In particular, mesoporous silica has been given attention because it has designable pore size that can be utilized in implanting anchor molecules for covalent bonding. There is some possibility of fouling by small molecules, but it is difficult to make a smaller pore size to avoid this because the substrates would not diffuse into the deeper regions of the pores.

From the above examples, it appears that a material that avoids adhesion and has micro-(or nano-) pores is suitable for immobilizing enzymes. One of the materials that has both advantages is a polyelectrolyte complex membrane. Polyelectrolyte complex membranes are formed by the electro-static interaction between counter-charged polyelectrolytes. Unlike a bond between a cation and an anion, bound polymers between anionic and cationic polyelectrolytes are difficult to solve even in solvents (Figure 4). The bonds are formed at multiple-sites between polymers; thus, the complex is stable.

![Diagram of polyelectrolyte complex membrane](image)

**Figure 4.** Schematic illustrations of bindings between cations and anions. (a) bound cation and anion will dissociate, and it is unknown if the binding between the dissociated ions would occur again; (b) the bond between cationic and anionic polymers will break at one site, and the bond between the sites will be formed again because other bonds are not broken at the same time.

Polyelectrolyte complex membranes are divided into two groups based on structure: layer-by-layer films [103,104] and polyion complex membranes [105]. Membranes obtained by the layer-by-layer method are prepared by stacking layers of cationic polyelectrolyte and anionic polyelectrolyte, alternately. The enzyme surfaces have acidic and basic amino acid residues, resulting in positively and negatively charged sites. By the immobilization of enzymes in the layer-by-layer membranes, some perturbations of the polyelectrolyte layer appear around enzyme molecules. For strict layer formation of polyelectrolytes, enzymes should be covered to negate the surface charges or be stacked outside the layered region [106,107]. The structure of the polyion complex membranes resembles the mixture of cationic and anionic polyelectrolytes; formation of the membrane can be
achieved by mixing cationic and anionic polyelectrolyte solutions. If enzymes and other chemicals are required to immobilize into the membranes, the solution of enzyme and chemicals would be placed between the mixture of polyelectrolyte solutions (Figure 5). We prepared polyion complex membranes that immobilize glucose oxidase [83,108], alcohol oxidase [109], lactate oxidase [110,111], glutamate oxidase [81,82], pyruvate oxidase [112], NADH (reduced form of nicotinamide adenine dinucleotide) oxidase [113], choline oxidase [114], D-amino acid oxidase [80], D-fructose dehydrogenase [115], microperoxidase-11 [116] and peroxidase [117–119]. From the information obtained, polyion complex membranes can be applied to prepare the enzyme-immobilized membranes swiftly and easily.

The polyion complex membranes show unique characteristics including size exclusion effects and charge exclusion effects for enzyme substrates and products [105]. The former characteristic, i.e., the size exclusion effect, was applied to the perm-selective property of the membranes. When poly-L-lysine and polystyrene sulfonate were used for the preparation of a polyion complex membrane, the resulting membrane showed a size exclusion effect around a molecular weight (MW) of 110 [110], i.e., molecules smaller than MW 110 can penetrate the membrane easily, while larger molecules cannot go through. This is due to the small pores of the membrane. The pore is too small for species such as L-ascorbate and proteins to enter the membranes. A secondary characteristic, the charge exclusion effect, is shown by controlling the membrane surface charge to prevent penetration of the membrane [120]. As the component ratio of polyelectrolytes can be changed, the surface charge can be determined by the component.

By combining these two characteristics, prevention of fouling by proteins can be achieved [121,122]. Substances that cause fouling would be larger than the pore size of the membrane, and the surface charge would be altered to prevent fouling of the surface by controlling the component of the membrane. Polyion complex membranes would be stable materials for the prevention of fouling, and the enzyme-immobilized membranes can be used for a long period.

3.2. Chemically-Stable Material for the Matrix

To prevent fouling, it is important that the surface charge of the supporting material is as mentioned above. At the same time, it is important that another factor—stability against chemicals—is developed. In solutions in which the enzyme-immobilized membranes were used, reactive chemicals or chemicals that induce physicochemical states would coexist. Examples include radicals, strong acids, and bases. If the matrix reacts with these species, not only are the immobilized enzymes deactivated,
but the membrane condition would also worsen; as a result, the stability of the membranes is weakened. To overcome the problem, chemically-stable materials should be adopted for the supporting materials.

One of the candidate materials is cellulose, which is known to be stable against chemical reagents [123,124] because it can only be reacted with strong acids, bases and highly reactive chemicals. Moreover, it had been reported that only several strong solvents can dissolve cellulose. Recently, it has been reported that an ionic liquid can dissolve cellulose [125,126]; ionic liquids have unique properties such as low vapor pressure, non-flammability, high ion conductivity, and the ability to dissolve various materials. If strong solvents are used to dissolve cellulose, it is difficult to prepare enzyme-immobilized membrane by using cellulose because of solvent-induced damage to the enzyme. Thus, the discovery of ionic liquids can expand preparation methods involving cellulose.

As some ionic liquids can dissolve in water, the ionic liquids used to dissolve cellulose can be removed via water immersion of the cellulose-containing it. A preparation method for an enzyme-immobilized cellulose membrane is developed as shown in Figure 6 [84]; the enzyme layer was covered with cellulose-containing ionic liquid, and the whole membrane was immersed into water to remove the ionic liquid. After drying, an enzyme-immobilized cellulose membrane was obtained.

![Figure 6. Preparation method of cellulose-covered enzyme membrane [75]. Cellulose-containing ionic liquid coated the enzyme layer on the substrate, and whole substrate was immersed in water. During immersion, the ionic liquid diffused into the water. As a result, cellulose was deposited on the enzyme layer. Meshes of cellulose fibers have smaller pores compared with the diameter of the enzymes and larger pores compared to enzyme substrates.](image)

Long-term stability of enzyme-immobilized cellulose membrane is excellent [85]: apparent activity of immobilized enzymes was stable for four months after preparation, after which time activity gradually decreased. Although activity of the membrane was half the value of the initial activity 11 months after preparation, the linear range of calibration curve was not changed; it can be used even 11 months after preparation [85]. This indicates the protective effects of cellulose on immobilized enzymes against chemicals [85]. Cellulose nanoﬁber can be dispersed into water and a similar enzyme membrane can be obtained; however, similar results for stability cannot be obtained [127]. Mesh structure would differ from the membrane indicated in Figure 6, perhaps because the cellulose nanofibers are shorter in comparison or the membrane is perturbed by enzyme incorporation.

The above example using cellulose as a supporting material shows the improvement of stability of the membrane; chemicals cannot react with cellulose and thus membrane properties cannot be changed by them. There are other chemically stable materials, but they are difficult to handle and match for biological substances. At this stage, cellulose would be the best material for this purpose.
4. Conclusions

In this review, factors that contribute to enhancing the stability of immobilized enzyme membranes have been indicated, and the solution to each factor, based on examples, are shown. These factors are divided into two categories: one is dependent on the improvement of enzyme properties, and, the other, on the development of supporting materials. Examples and methods indicating how stability is improved are given. As varying enzyme sources and different supporting materials were used in each reported method, simple stability data, such as long-term stability period and heat tolerance, were not compared. However, it is expected that the strategies for addressing each factor would be understood.

To improve enzyme properties, the enzyme itself can be improved by protein engineering methods and chemical modification before being immobilized. This knowledge can be applied to various fields. In contrast, the development of supporting materials proceeds by the invention of novel materials, which requires the progress of material science.

Recently, through the progress of nanotechnology, the whole process can be measured and controlled at the molecular level. Today, a molecule can be immobilized into a molecular size cavity [2,128]. There have been reports on stability enhancement achieved by controlling the environment at a molecular level [129,130]. Through these immobilization techniques, enzyme-immobilized membranes that possess all the functions of the immobilized enzymes can be obtained, and it is believed that the membranes can be applied to various fields.

Conflicts of Interest: The author declares no conflict of interest.

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