0273-2289/93/4103—0157$07.40

Preparation of Immobilized Proteins Covalently Coupled Through Silane Coupling Agents to Inorganic Supports*

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ABSTRACT

Enzymes were first immobilized on inorganic supports through silane coupling agents over 25 yr ago. Since that initial report, literally hundreds of laboratories have utilized this methodology for the immobilization of enzymes, antigens, antibodies, receptors, and other high and low mol wt compounds. Today silane coupling is one of the commonly used techniques in the arsenal of the biochemist for the binding of material of all sorts to inorganic surfaces. Inorganic materials come in a variety of shapes, sizes, and characteristics. Today silane coupling is one of the most used coupling methods for the preparation of biosensing devices. Sol-gel entrapped enzymes are also produced by the application of silane technology by the polymerization of the silane to form glass-like materials with entrapped protein. This review will discuss the general preparation and characterization of silane coupled proteins with special emphasis on enzymes and describe in detail the actual methods for the silanization and specific chemical coupling of proteins to the silanized carrier.

Index Entries: Silane; silane coupling; silanization; silica; metal oxide immobilization; protein coupling; inorganic supports; coupling efficiency; enzyme; enzyme kinetics; antigen; antibody.

*This review will also be published in The Proceedings of The Mosbach Symposium on Biochemical Technology, Held December 2–4, 1992 Lund, Sweden.

INTRODUCTION

Inorganic support materials have been used for chromatography for over 75 yr. However, it is only been within the last 25 yr that these materials have been applied to enzyme immobilization in which the protein is covalently bonded to the support (*1*). This review will cover the application of inorganic silicas and metal oxides for coupling of proteins. The review will not cover chromatography or deal with use of hydrocarbon and other organic solvents for immobilized enzyme catalysis.

Before choosing a material to be used as an enzyme support, one must answer several important questions:

1. Should the support material be porous or nonporous?
2. If the support material is porous, does the pore morphology permit the entry of the enzyme and the enzyme substrate?
3. Is the support material durable in the solvents utilized (acids, bases, high salt, organic solvents)?
4. Can the support material be conveniently handled?
5. Does the support material have high compression strength?
6. Can one attach enough enzyme to give adequate loading per unit volume?
7. Does the carrier tolerate maximum pressure drop?
8. How are all the above factors affected by particle size, flow rates, and particle shape?
9. Can the derivative be stored easily?

Each of the above questions must be answered in choosing an enzyme support material. In many cases, inorganic materials may fit the criteria closer than other materials. Inorganics have an advantage in that they come as particles of all shapes and sizes as well as in the shape of monolithic porous supports or filter materials. They can withstand extremely high pressure drops with no compression. They are not susceptible to microbial attack and they do not shrink or swell in organic solvents or with pH changes. In addition, and most importantly, they have loading capacities high enough for a variety of applications.

PREPARATION OF INORGANIC BIOCATALYST SUPPORTS

Pore Size

Most inorganic support materials consist of either porous or nonporous glasses, silicas, or metal oxides. Examples of some porous inorganic support materials are shown in Table 1. These support materials are all controlled-pore ceramics or glasses and can be prepared in a variety of pore morphologies and particle sizes (2). The controlled-pore glasses,

Table 1
Physical Characteristics of Several Ceramic Support Materials

Composition	Size (US mesh)	Pore diameter Range (Å)	Pore diameter Average (Å)	Pore volume (mL/g)
TiO_2 98%, MgO 2%	30/45	205–500	410	0.53
SiO_2 75%, Al_20_3 25%	30/60	205–575	435	0.89
SiO_2 89.3%, ZrO_2 10.7%	30/60	110–575	235	1.30
SiO_2 100%	30/60	185–700	435	0.76
SiO_2 100%	30/60	310–655	550	2.2
SiO_2 90%, ZrO_2 10%	30/60	185–700	435	0.76
SiO_2 75%, TiO^2 25%	30/45	875–205	465	0.76
Controlled Pore Glass	30/60	450–600	550	0.50

[a]Modified binder used.

Table 2
Durability Test Results for Supports

Material description	Static test (mg/m²/16 h) 1% NaOH	Static test (mg/m²/16 h) 5% HCl	Dynamic test (mg/m²/day) pH 4.5	Dynamic test (mg/m²/day) pH 7.0	Dynamic test (mg/m²/day) pH 8.2
TiO_2	0.2	0.8	0.05	0.05	ND[a]
ZrO_2	0.2	1.1	0.004	0.004	0.01
Al_2O_3	0.6–0.8	2.0	0.056–0.086	0.01	0.01
Al_2O_3-SiO_2	1.85	3.65	0.08–0.1	0.02–0.05	0.06
CPG[b]-SiO_2	1.3–2.0	0.2	0.03	0.7	0.7–0.09
CPG	3.06	0.08	0.02	0.5	0.3

[a]Not done.
[b]CPG, controlled-pore glass.

unlike the ceramics, can be prepared in pore sizes ranging from 30 Å to 2000 Å with narrow distribution ranges (+/−5%), rather than the much broader pore ranges as shown for the ceramic carriers (3).

Chemical Durability

Chemical durability is of importance for materials where exposure to solvents are prolonged or repeated. Table 2 shows the durability of several inorganic support materials under differing conditions of acid and base treatment (4–5). This data shows that glass is not the most durable material under basic conditions but the metal oxides are extremely durable under these conditions.

Capacity of Inorganic Supports

When using inorganic support materials as affinity carriers one must consider capacity for ligand binding. Nonporous materials in the 40–80 U.S. mesh size (177μ–420μ) range have an available surface area of only 1–5 m^2/g. Extremely small nonporous particles in the 5–10μ size range have surface areas from one to two orders of magnitude greater than the large particles. However, because of the packing densities observed with these fine materials, they are generally reserved for HPLC types of applications. For standard low pressure biocatalytic applications, high capacity can be achieved with larger particles by using porous materials. Table 3 shows the average pore diameter and available surface areas for several porous glasses. The surface area is inversely proportional to the pore diameter at constant pore volume. However, in choosing a pore diameter, one must also consider the size of the molecules one wishes to couple. In addition, the size of the enzyme substrate must be considered. The enzyme must have access to the surface for coupling and the substrate must have access to the enzyme within the pores of the support. Figure 1 shows the binding of amyloglucosidase to a porous support vs surface area and pore diameter. The data show that there is a pore diameter at which the protein is excluded from the pores of the support and couples only to the external surfaces. The figure also shows that there is an optimal pore diameter for the protein at which maximal coverage and activity are achieved.

PREPARATION OF INORGANIC BIOCATALYST SUPPORTS

The most often used method of preparing inorganic support materials for enzyme immobilization is to either adsorb an organic polymer to the support or silanize the support before coupling the ligand or protein to be used for purification. Silanization methods are of two types: aqueous and nonaqueous. Aqueous silanization offers the advantage of more even coverage and an apparently thinner silane layer on the support material. Organic silanization, whether in a water miscible or immiscible solvent, produces a thicker, uneven, more loosely-bound but higher capacity coating. Silane monolayers can be achieved by vapor deposition of freshly distilled monosilanes. However, for catalytic applications such support materials have poor durability.

Silane Coupling

Pretreatment

Before any inorganic material is silanized it should be cleaned to remove any adsorbed organics contaminants. This can be accomplished by either

Table 3
Comparison of Surface Area to Pore Volume
for a Controlled-Pore Inorganic Support

Pore diameter (Å)	Surface area[a] (m^2/g)	Surface area[b] (m^2/g)
75	249	356
125	149	214
175	107	153
240	78	111
370	50	72
700	27	38
1250	15	21
2000	9	13

[a]The pore volume for these calculations was taken at 0.70 mL/g.
[b]The pore volume for these calculations was taken at 1.0 mL/g.

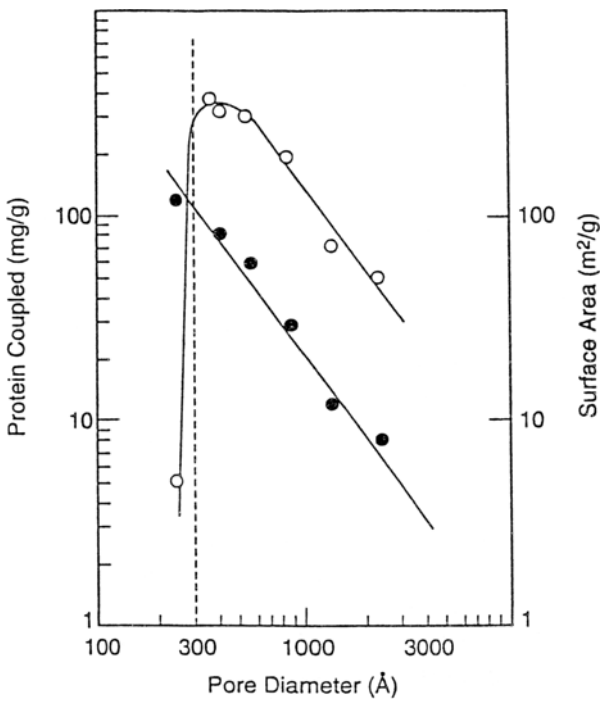

Fig. 1. Relationship of pore size to surface area and quantity of protein coupled. Closed circles represent surface area data; open circles represent protein coupled.

heating the material to a temperature that will burn off the adsorbed organics or by boiling it in 5% nitric acid solution for 45 min followed by exhaustive washing with distilled water to remove any residual acid. Chlorosilanes should always be used in organic solvents whereas the methoxy-and ethoxy-silanes can be coupled with either organic or aqueous methods.

Available Silanes

Available silanes include epoxysilanes, aminosilanes, cyanosilanes, sulfhydrylsilanes, phenylic silanes, and glycidoxysilanes, in methoxy, ethoxy, and chloro forms. In many cases, after silanization with one of the commercially available silanes, chemical modifications are performed to change the functional group for later coupling. Some of these methods will be discussed later in this review.

Organic Silanization

To 1 g of clean inorganic support material add 75 mL of 10% silane dissolved in toluene or similar solvent. Reflux for 12–24 h and wash with toluene followed by acetone. The product can be air dried. Stability can be improved by heating the dried product to 110°C for 4 to 8 h.

Silane Evaporative Coupling

The support material previously cleaned as described and dried is added to enough silane dissolved in acetone or other volatile solvent to cover the particles thoroughly. Generally a 1% solution of silane is sufficient for coupling. The solution is evaporated to dryness leaving behind the silane adsorbed and reacted to the support material. The support must be washed with the same solvent to remove unreacted silane and dried.

Aqueous Silanization

If the product has been fired, it must be thoroughly wetted before silanization. This is accomplished by boiling in distilled water for 30 min to 1 h before carrying out the silanization steps. The washed support is suspended in 75 mL of a 10% solution of silane dissolved in distilled water. The pH of the solution is lowered to 4.0 with HCl (usually 6*N*) and the pH adjusted solution heated in a water bath at 75°C for 3–5 h. The product is then filtered or decanted followed by washing with 2 vol of distilled water. The product should be dried overnight at 110°C to increase stability. With micron size particles drying should be avoided. Drying these samples causes irreversible clumping.

Curing

When proteins are coupled to silanized supports it may be necessary to remove loosely coupled or bound protein from the support material before it can be used. This "curing" process is accomplished by placing the coupled support material in a water bath at 56°C for several hours to several days or until no protein can be detected in the concentrated supernatant fluid.

General Comments on Silane Coupling

It is well known that the siloxane bound between the silanol residue and the silane coupling agent is reversible. Data in our laboratory and in other labs has shown that application of monoethoxy-, monomethoxy,- or

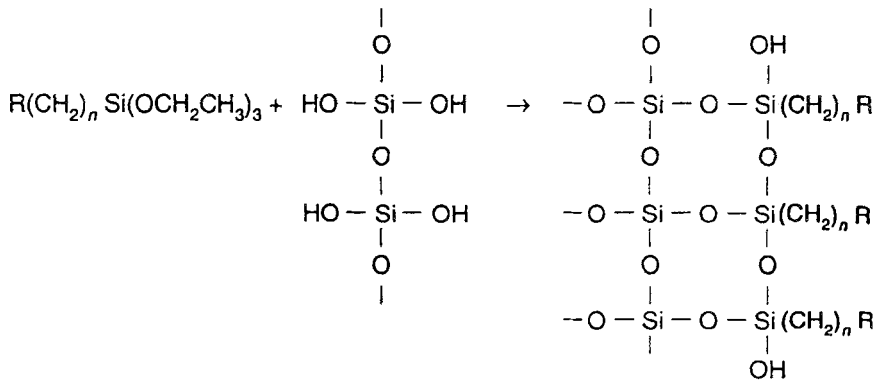

Fig. 2. Reaction between gamma-aminopropyltriethoxysilane and an inorganic support material. The ethoxy group reacts with the available silanol residues forming a silioane linkage. In addition, the silane appears to polymerize over the support surface. A product of this reaction is ethanol.

monochlorosilanes have poor stability on inorganic support materials and are lost rapidly over time. Trifunctional silanes, on the other hand, produce products that have greater stability. These silanes couple not only to the silanol residue on the support material's surface, but they bind to each other forming a polymer across the surface of the carrier. The heating step after silanization aids in the polymerization by driving off water. The reaction between a silica support and a triethoxysilane is depicted in Fig. 2.

Carrier Activation (Direct, Nonsilane Methods)

The silanol residues on the surface of glass and silica as well as metal oxides groups on ceramic surfaces appear capable of reaction with several organic activating agents. The activated carriers react with other organic functional groups and ligands forming permanent linkages.

Cyanogen Bromide (6)

To 1 g of support material suspended in 5–10 mL of distilled water cooled to 4°C is added 250 mg of solid cyanogen bromide (CNBr). The solid is added slowly with constant pH maintenance between pH 10–11 with NaOH solution. After the CNBr has been added, the reaction mixture is allowed to stand in the ice bath for an additional 30 min. The product is washed until the pH is near neutrality. The procedure should be carried out in a chemical fume-hood since the CNBr is highly toxic. The washed and activated support material is ready for coupling to a protein. It may be advisable to predissolve the CNBr in a water miscible solvent prior to addition. The solvent should not be reactive with CNBr. The mechanism of action is not understood. However it may be similar to that observed

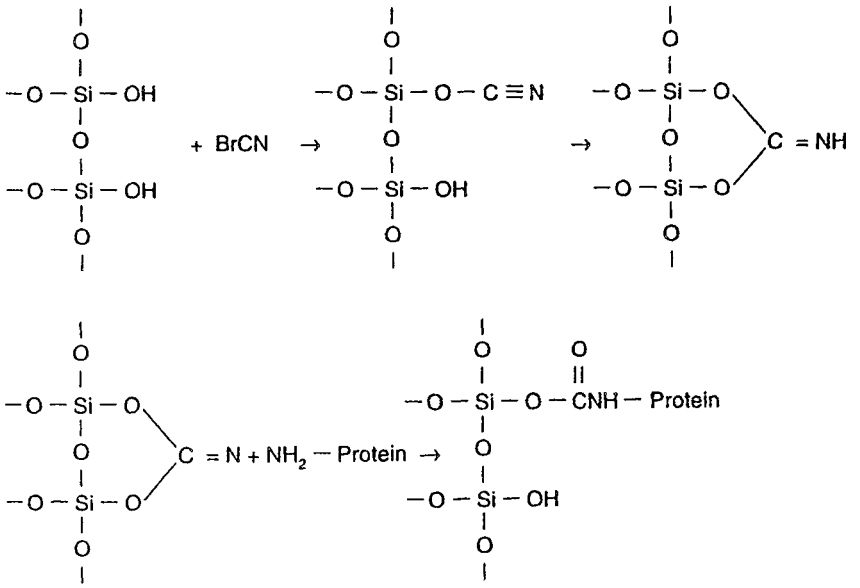

Fig. 3. A possible mechanism for the CNBr activation of silanol residues.

with conventional hydroxyl containing compounds that give reactive imidocarbonates with CNBr. In this case, however, the hydroxyl groups are in the form of silanol residues. A possible mechanism for the activation of CNBr is presented in Fig. 3.

Bis-diazotized Silica (7)

A solution of 0.01% 4,4′-bis(2-methoxybenzene diazonium) chloride (commercially available) is prepared in distilled water. To 500 mg of inorganic support material is added 2 mL of the 0.01% coupling reagent. The reaction is allowed to proceed at room temperature for 20 min, after which the liquid is decanted off the support material. The product is washed with distilled water. At this point it is ready for coupling at pH 8–9 by azo linkage to a protein. The mechanism, like the CNBr mechanism, is obscure. However one possible mechanism is presented in Fig. 4.

Aldehyde Derivative

A method for preparation of an active aldehyde derivative has been described by Bursecz (*8*). The product is prepared by mixing 6 mL of a 2% aqueous solution of epiamine per gram of support material for 1 h. The epiamine is prepared by heating ethylenediamine to 100–110°C and slowly adding, with stirring, epichlorohydrin homopolymer at a rate of 1 mol chloromethyl group to 6 mol ethylenediamine. These reactants are heated an additional 3 h with stirring. The mixture is neutralized with NaOH, filtered, and the excess ethylenediamine removed by vacuum distillation. After reaction of the support material with the epiamine, it is filtered and can be dried. This product can be reacted with glutaraldehyde for coupling to protein.

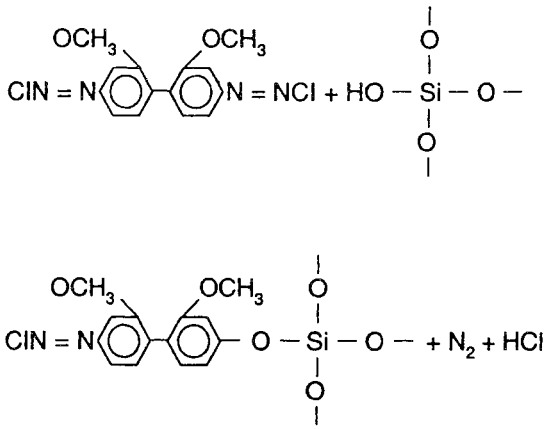

Fig. 4. A possible coupling mechanism of the bis-diazotized reagent coupling to a silanol residue.

A simpler approach is to prepare a solution of polylysine at a ratio of 100 mg/g of support material (*9*). This is allowed to adsorb at room temperature in 10 m*M* phosphate buffer at near neutral pH overnight. The product is washed with distilled water. It can be dried and stored for later activation with glutaraldehyde.

Metal Bridge Activation

Proteins can be covalently coupled to inorganic support materials through a metal bridge (*10,11*). The activated carrier is prepared by steeping the support material in a solution of the metal salt for a short time. It can generally be dried and stored for later coupling at neutral pH. The most successfully used metal salts are $TiCl_4$, $TiCl_3$, $SnCl_4$, $SnCl_2$, $ZnCl_4$, VCl_3, $FeCl_2$, and $FeCl_3$. Best results observed by this author using an inorganic support material has been with $SnCl_2$ (*12*). To 500 mg of inorganic support material is added 20 mL of a 1% solution of stannous chloride dihydrate solution at 37°C. This is allowed to react 45 min, decanted, and washed with 3 aliquots of 20 mL water. The product can be directly coupled at neutral pH. Like the other direct coupling mechanisms, this one is also unknown. A proposed mechanism is presented in Fig. 5.

ACTIVATION OF SUPPORTS FOR PROTEIN COUPLING

The previous sections of this review have dealt with characteristics of inorganic supports and silanization or direct activation of these inorganic support materials. For this section we will deal with activation and will assume that the molecules to be covalently coupled are proteins. Since proteins contain typical reactive groups, it is reasonable that methods used for protein coupling will be useful for the covalent attachment of other

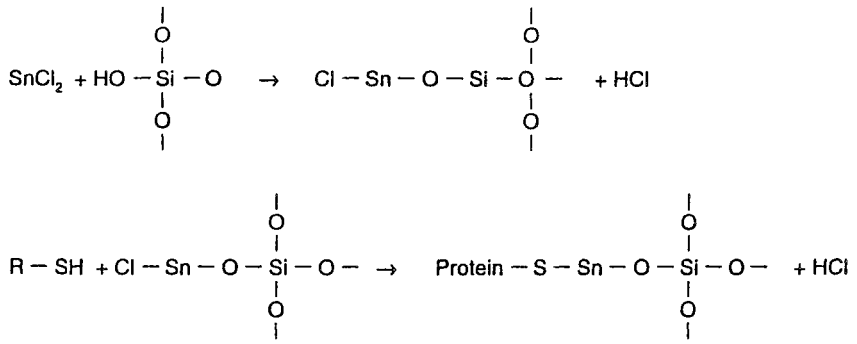

Fig. 5. A proposed mechanism for the Stannous Bridge coupling to a silanol residue.

types of molecules, including carbohydrates, lipids, nucleic acids, and low mol wt hapten or inhibitor molecules.

Proteins contain several types of reactive groups that can be used to form covalent bonds to solid supports. The most frequently used are the amino groups, followed by sulfhydryl, carboxyl, and aromatic groups. The functional group on the support material can be (among others): an aliphatic amine, sulfhydryl, aromatic amine, or epoxy group. Consequently, a considerable variety of methods are available for coupling proteins to inorganic supports. The methods vary in complexity from simple one-step processes to sequences involving three or more steps.

One step methods, primarily with derivatization of the support with a group capable of direct coupling to the protein, include use of oxiranes (epoxides) and imidocarbonates. Two step processes, most commonly used, employ either an initial activation of the functional group on the solid support, so as to react with the protein, or involve the preliminary attachment of a bifunctional "linker" substance to the support, followed by coupling to the linker. The linker may have the same reactive function on each end (homobifunctional) or the two ends may have different functional groups (heterobifunctional). In either case, the free group is capable of reacting with the protein. In some cases the linker is first coupled to the protein before attachment to the solid support. In this case it must be of a heterobifunctional type, since the free end must not be capable of reacting with a second protein, but only with the support.

In the three-step methods, the functional group on the solid support is first coupled to the linker molecule that is not capable of further reaction, but must be further modified. Alternatively, the protein may be modified so that it will react with the linker attached to the solid support.

The coupling methods described below, although in no way complete, are useful examples for the coupling proteins to porous and nonporous inorganic support materials.

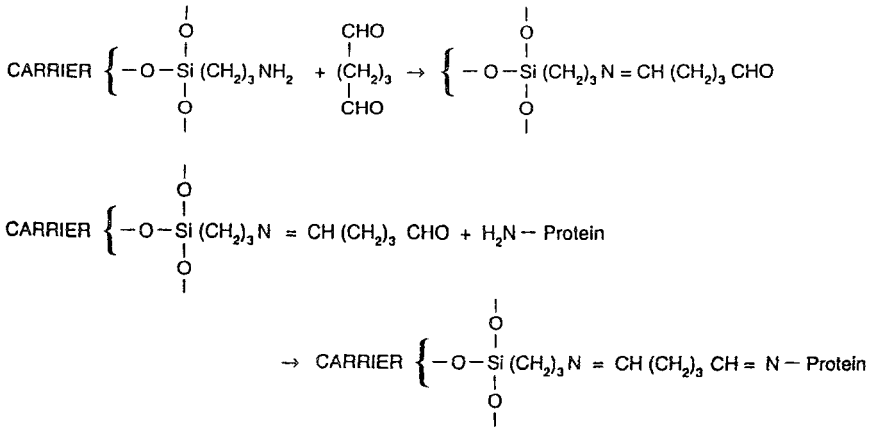

Fig. 6. The coupling reaction between an alkylamine support and glutaraldehyde followed by coupling of the activated reagent to protein. The glutaraldehyde reaction is somewhat more complicated, however, since commercially available glutaraldehyde is highly polymerized.

Alkylamine Containing Inorganic Support to Amino Group on the Protein

Two-step methods using homobifunctional linkers are presented first. Further in the review will be presented a three step procedure that utilizes an active ester that can react with amino groups.

Glutaraldehyde

To 500 mg of inorganic support material previously silanized with an alkylamine silane or aminated in some other fashion is added 50 mL of 2.5% glutaraldehyde in 0.1*M* phosphate buffer, pH 7.0. The reaction is allowed to continue for 60 min followed by extensive washing with phosphate buffer. The product is ready for immediate coupling at pH 6–8 (Fig. 6).

Disuccimidyl Suberate

Five hundred milligrams of alkylamine inorganic solid support is suspended in 50 mL of methanol containing 50 mg of disuccinimidyl suberate. The mixture is shaken for 60 min, washed twice with 50 mL of methanol, and immediately coupled to an amine containing protein (Fig. 7).

Succinylation: Formation of an Active Ester

One gram of alkylamine inorganic support material is suspended in 100 mL of 0.05*M* sodium phosphate buffer, pH 6.0. To this is added 0.3 g of succinic anhydride. The mixture is allowed to react 15 h at T_r followed by 3 washes of 100 mL each with water, twice with 100 mL of methanol,

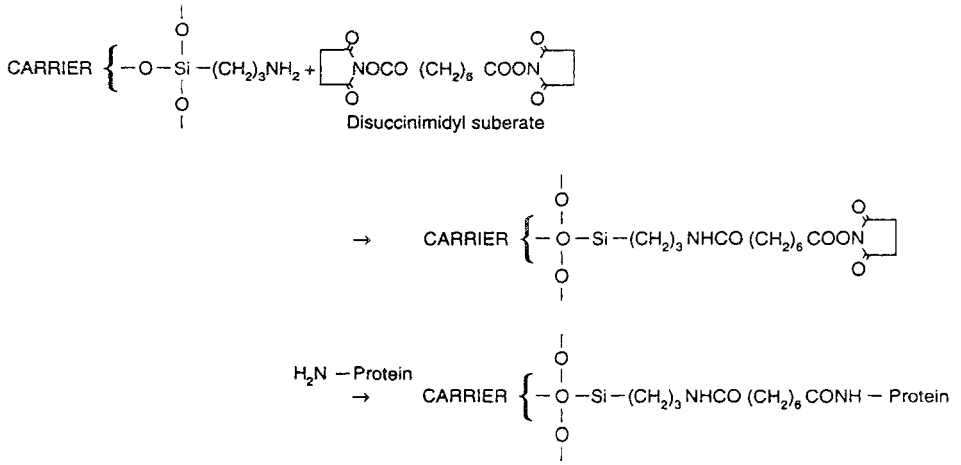

Fig. 7. The coupling of dissuccinimidyl substrate to an alkylamine inorganic support and subsequent coupling to a protein.

and once with 100 mL of dioxane. The particles are then suspended in 1.2 g *N*-hydroxy-succinimide (NHS) and 1.9 g 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in 100 mL of dioxane. This is shaken for 90 min at T_r, washed twice with 100 mL of dioxane, twice with 100 mL of methanol, dried, and stored in a moisture-free sealed container. The particles are ready for coupling to amino containing compounds generally in aqueous suspension at pH 8.0 (Fig. 8).

Alkylamine Inorganic Support to Sulfhydryl Groups on the Protein

These methods use heterobifunctional linkers.

Succinimidyl 3-(-2-pyridyldithio)proprionate (SPDP)

To 500 mg of inorganic alkylamine support material suspended in 50 mL of 0.1*M* phosphate buffer, pH 8.0 is added, dropwise, 50 mg SPDP dissolved in 5 mL of methanol. The suspension is agitated at room temperature for 60 min followed by three washes with 50 mL of 0.1*M* phosphate, pH 7.4, containing 1 m*M* EDTA. The product is ready for coupling to available sulfhydyl groups at pH 7.4 (Fig. 9).

Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)

To 500 mg of alkylamine inorganic support material suspended in 50 mL of 0.1*M* phosphate buffer, pH 7.0, at 30°C is added 50 mg of SMCC dissolved in 5 mL of dioxane. The reactants are agitated for 2 h followed by three washes with 50 mL of phosphate buffer, pH 7.4, containing 1 m*M* EDTA. The product is ready immediately for coupling to sulfhydryl containing proteins at neutral pH (Fig. 10).

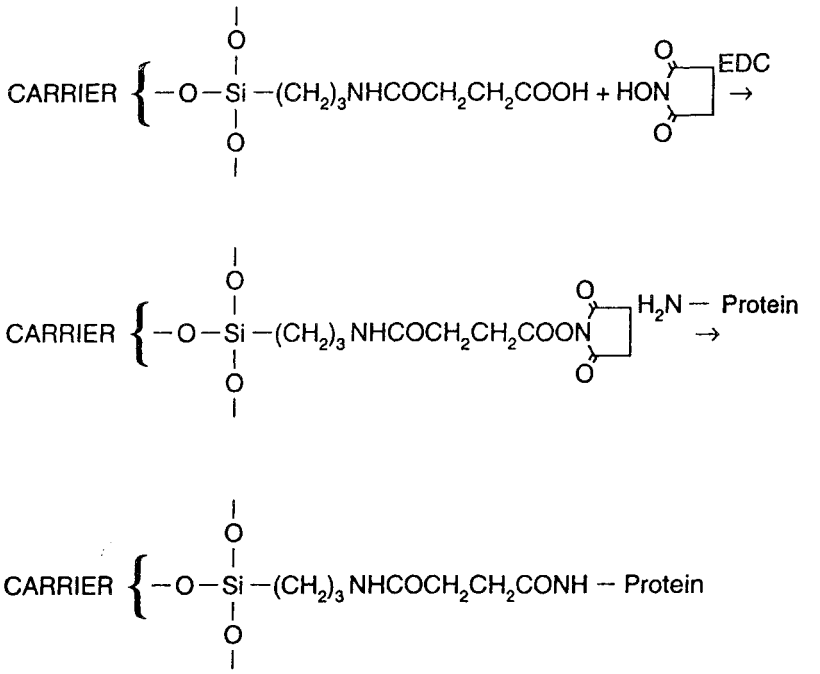

Fig. 8. The coupling of a carboxy-derivitized carrier to *N*-hydroxy-succinimide using a carbodiimide coupling reagent followed by coupling to a protein via the active ester.

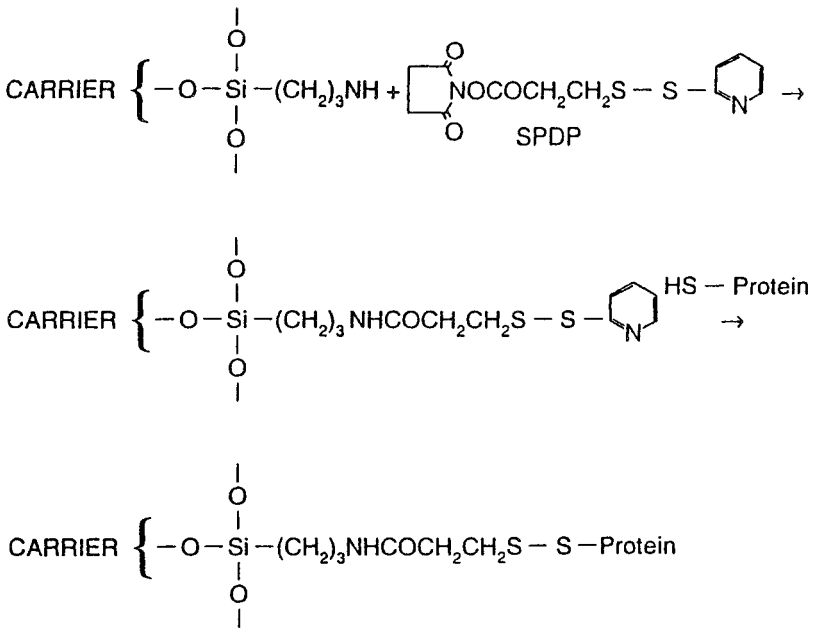

Fig. 9. The coupling of a protein to an alkylamine support via activation with SPDP.

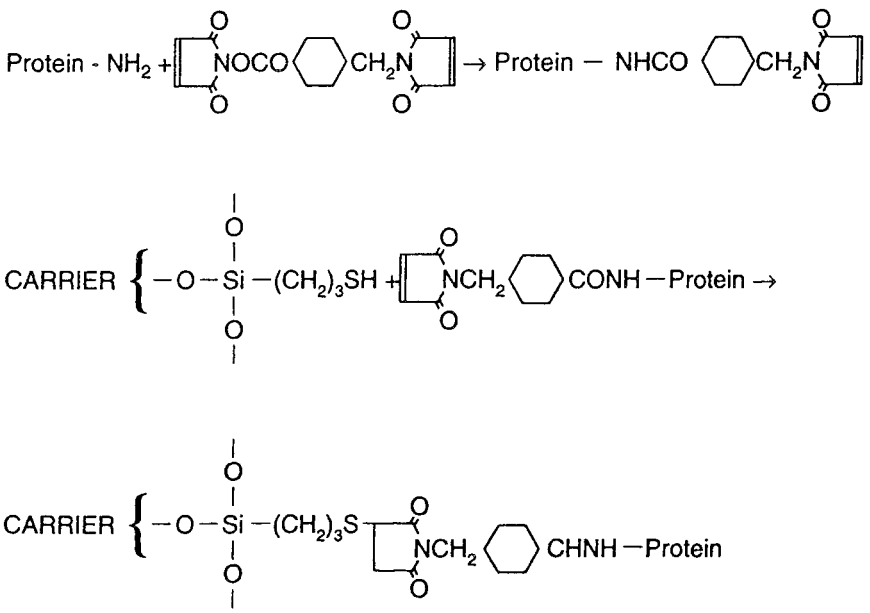

Fig. 10. The coupling of an alkylamine support by activation with SMCC followed by coupling to a protein.

Arylamine Inorganic Supports Coupled to Different Functional Groups (Diazotization)

This method utilizes principally tyrosine residues or other aromatic groups. However, histidine, lysine, cysteine, tryptophan, and arginine will also react to a lesser extent. The inorganic solid support can be converted to an arylamine derivative (*5*), or the ligand, if it contains a diazotizable amine, can be coupled to a tyrosine or similar group contained on the support material.

Diazotization is carried out as follows: To 500 mg of arylamine support suspended in 50 mL of 2*N* HCl in an ice bath is added 5 mL of a 1% solution of sodium nitrite. After 30 min the excess nitrous acid is eliminated by the addition of soid urea or sulfamic acid until starch-iodide paper no longer shows a purple color when a drop of the reaction mixture is placed on the paper. The product is washed with ice-cold water. It is ready for coupling to a protein at pH 8–10 (Fig. 11).

Sulfhydryl Inorganic Support to Sulfhydryl Protein

The method described below uses a homobifunctional linker. It should be remembered that sulfhydryl compounds are very susceptible to oxidation on storage and need to be reduced prior to carrying out the reactions described below. Reduce the carrier material by suspending it in 0.03*M*

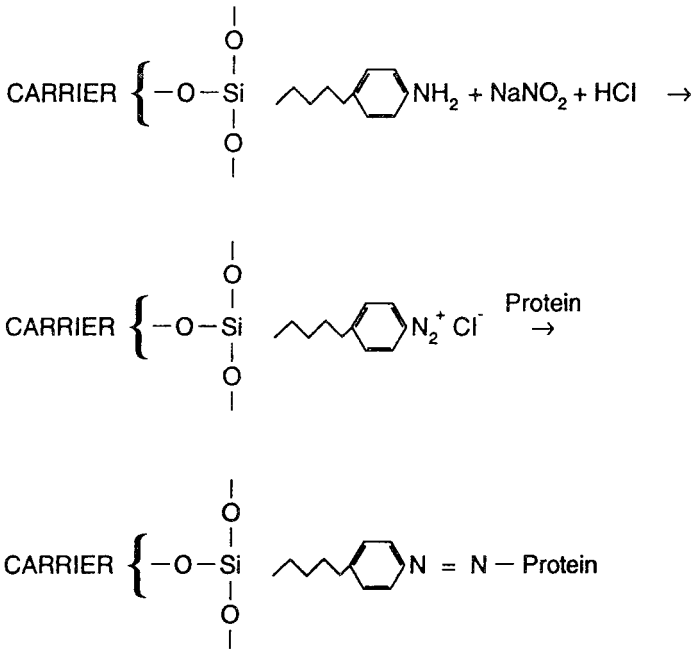

Fig. 11. The diazotization of an arylamine support followed by coupling to a protein.

dithiothreitol in 0.1*M* Tris/HCl, pH 8.0, containing 1 m*M* EDTA. Allow 30 min for reduction, followed by extensive washing.

1,6-Bismaleimidohexane (BMH)

To 500 mg of sulfhydryl inorganic support is added 50 mL of 0.1*M* phosphate buffer, pH 7.0, containing 1 m*M* EDTA. To this is then added 50 mg BMH dissolved in 5 mL of acetone. The reactants are agitated for 60 min followed by three 50 mL washes with the phosphate-EDTA buffer. The product is ready for coupling at pH 7.0 (Fig. 12).

2,2'-Dipyridyldisulfide (DPDS)

Fully reduced sulfhydryl inorganic support material is suspended in 50 mL of 0.1*M* Tris/HCl buffer, pH 8.0, containing 1 m*M* EDTA and 1.5 m*M* DPDS. The reactants are agitated for 30 min then washed repeatedly with the Tris buffer containing 1 m*M* EDTA. The product is ready for coupling at pH 8.0 (Fig. 13).

Sulfhydryl Inorganic Support to Amino Containing Protein: Sulfosuccinimidyl-4-(n-Maleimidomethyl) Cyclohexane 1-Carboxylate (Sulfo-SMCC)

To 1.5 mL of a 1% solution of the protein to be coupled dissolved in 0.1*M* phosphate buffer, pH 7.0, at 30°C is added, dropwise, 10 mg of

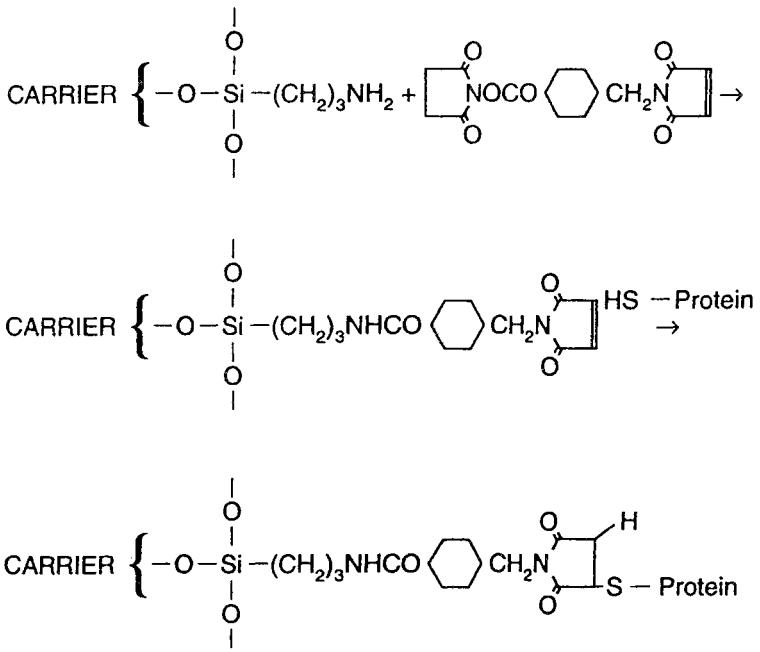

Fig. 12. The coupling of a fully reduced sulfhydryl support to protein via activation with BMH.

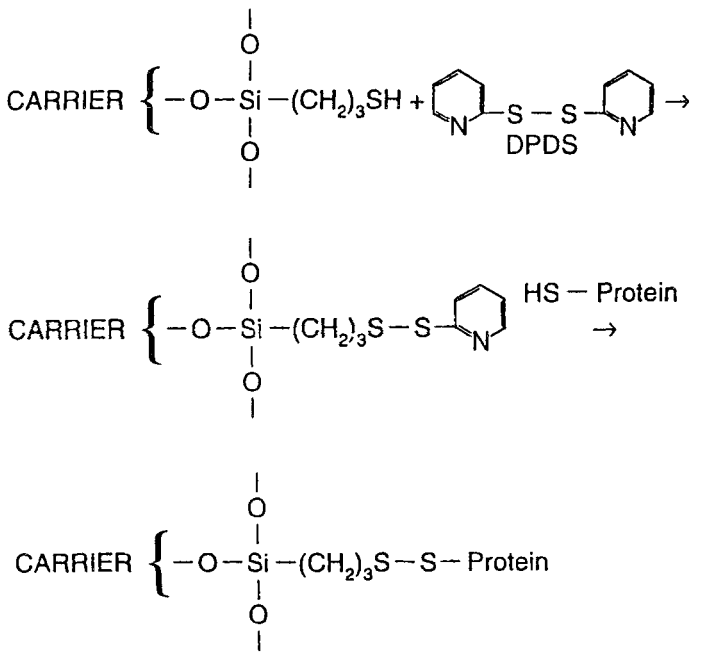

Fig. 13. The coupling of a fully reduced sulfhydryl support to protein via activation with DPDS.

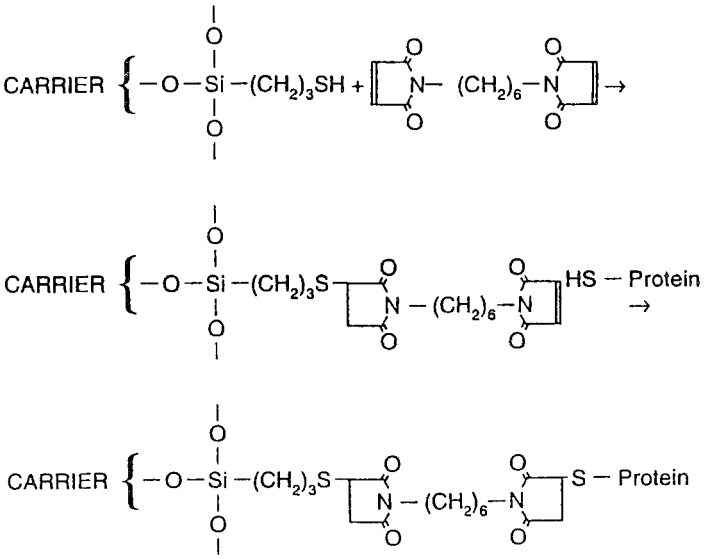

Fig. 14. The coupling of a protein to a fully reduced sulfhydryl carrier via activation with SMCC. In this example the protein is activated before the carrier.

SMCC in 1.5 mL dioxane, with stirring. After 2 h the solution is added to the sulfhydryl support and agitated for 16 h, washed, and stored. Since a protein is the agent to be coupled, one can add excess SMCC and separate the unreacted portion on a Sephadex G-25 column before further reaction (Fig. 14).

Oxirane Inorganic Support to Amino Group on Protein

The oxirane group is coupled to the inorganic support by direct attachment of the epoxysilane as previously described. Thus the oxirane group itself can be reacted directly with amino groups in a one step procedure. To 500 mg of an oxirane support is added a solution containing a large excess of the amino containing ligand or protein in 0.2*M* carbonate-bicarbonate buffer, pH 9.0. The mixture is agitated for 48 h at room temperature and washed with the same buffer. The product is ready for use in an affinity procedure (Fig. 15).

CHARACTERISTICS OF ENZYMES IMMOBILIZED ON INORGANIC SUPPORTS

The characterization of an immobilized enzyme encompasses not only the specific effects that immobilization has directly on the enzyme but the effects the carrier has on the microenvironment of the enzyme. In addition,

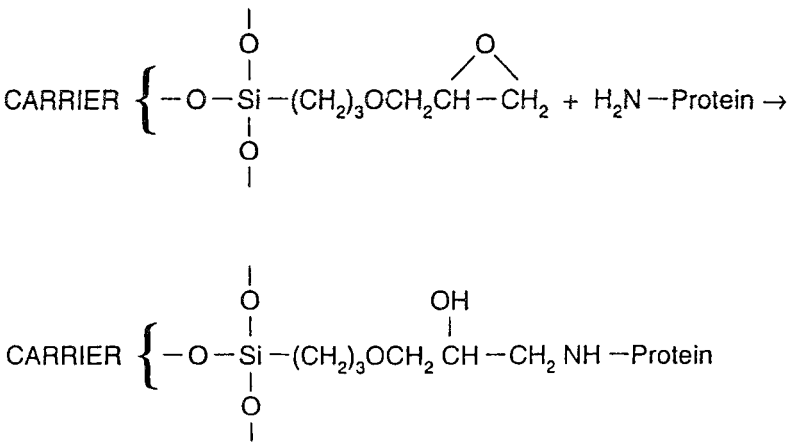

Fig. 15. The coupling of a protein to an epoxide (oxirane) activated inorganic support.

for situations where bioprocessing is involved it is important to consider a variety of other parameters as well. In this report we will consider the effects of coupling, the microenvironment, and only touch on the characteristics involved in bioprocessing requirements.

Coupling Efficiency

The covalent attachment of an enzyme to an inorganic support is not only dependent on the method of attachment but also on the quantity of enzyme offered. Attachment efficiency and activity recovery are not necessarily equivalent. As a typical example a series of experiments were carried out on three different inorganic support materials to determine the highest efficiency for the lowest enzyme levels offered (*13*). For this study the enzyme β-galactosidase was coupled to control pore glass, porous silica, and porous titanium oxide. The results (Fig. 16) show that highest coupling efficiencies are achieved at the lowest concentration of offered enzyme, even though the total bound activity may be higher at the highest concentrations of offered enzyme.

The phenomenon is most likely the result of decreased diffusion control of substrate and product. If the enzyme concentration is high enough, then the rate of substrate diffusion to the enzyme and product diffusion away from the enzyme may act as controlling steps on the overall reaction. Decreasing enzyme loading decreases the chances of substrate limitation or product inhibition controlling the reaction.

pH Profile

Immobilization of an enzyme on any charged support will generally cause a shift in the apparent pH optimum. The mechanism for this observed pH shift has been elegantly explained by Goldstein et al. (*14–15*). Using

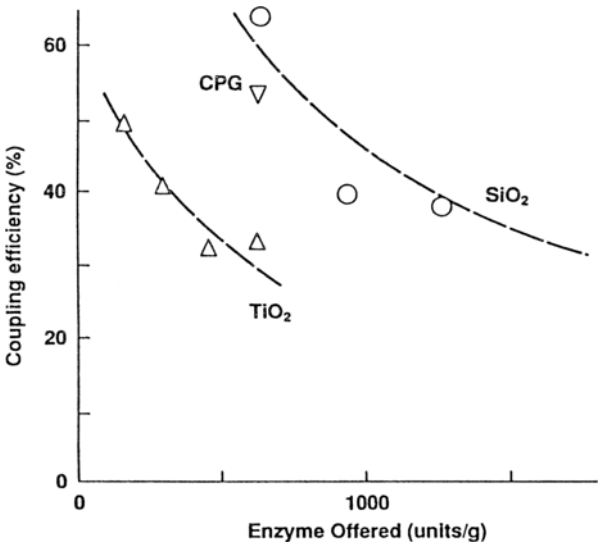

Fig. 16. Coupling efficiency of β-galactosidase to three inorganic supports. The figure shows that highest coupling efficiencies are achieved at the lowest concentration of offered enzyme, even though the total bound activity may be higher at the highest concentrations of offered enzyme.

highly charged enzyme supports they were able to show the microenvironmental effects of these supports on the enzymes' pH characteristics. Highly negatively charged carriers created a lower pH at the boundary layer between the carrier and the bulk solution. Thus, the enzyme finds itself in a more acidic environment as compared to the bulk of the solution. This being the case, it would require a higher bulk pH to raise the pH at the boundary layer to the actual pH optimum. The opposite result occurs with positively charged supports. Even in the case of uncharged supports, the overall charges on the enzyme may be sufficient to cause changes in the apparent pH optimum of the enzyme. Generally, however, the greater the charge on the support, the greater the effect, particularly with charged substrates. Figure 17 shows the shift in the apparent pH optimum for a yeast β-galactosidase covalently coupled to a porous ceramic support. Since the pH of the immobilized enzyme has shifted to the acid side, we can assume that the carrier was positively charged. The lower pH for the immobilized enzyme is necessary to bring the pH at the carrier surface, which is more alkaline, to the optimum for the enzyme, in this case, pH 5.0.

Enzyme Stability

When immobilized, enzymes can change their thermal characteristics. These changes generally cause apparent improvement in the thermal stability of the enzyme. Enzyme denaturation generally occurs as the result of changes in tertiary structure, oxidation of some labile groups at high

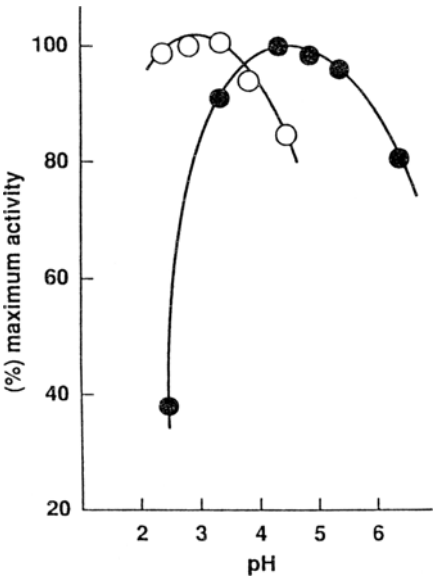

Fig. 17. The pH optimum of soluble lactase (filled circles) and the enzyme immobilized on porous silica (open circles).

temperature, or some other physical modification of the protein. In some cases, immobilization can increase thermal stability by increasing the molecular rigidity (*16*), thus preventing changes in the molecules tertiary structure on heating. An extreme example is the case of amyloglucosidase. When immobilized to porous silica, the enzyme has an operational half-life at 60°C of 20 d, whereas at 40°C the half-life is extended to over 1000 d (Fig. 18). In the case of this enzyme the carrier was porous silica, the substrate was 30% starch solids w/w, and the immobilized enzyme was operated by plug flow for over 150 d of continuous operation. It is obvious that not only does temperature play a role in the thermal stability of an enzyme, but so does the feed composition. Studies on β-galactosidase using different lactose solutions at constant temperature revealed different half-lives depending on the quality of the substrate and the lack of contaminants.

Enzyme Kinetics

In addition to thermal effects, immobilization can cause major changes in the apparent kinetics of an enzyme. Generally the apparent K_M of an immobilized enzyme is increased over that of a soluble form. This increase is usually related to a variety of parameters, including charge of the substrate and support, diffusion effects, and even the mode of operation. Table 4 gives examples of K_M values for several enzymes immobilized on inorganic supports. The mechanisms involved in causing the apparent changes in kinetic values are complex, but generally can be described via mathematical terms involving the usual engineering parameters covered in any good text on heterogeneous catalysis.

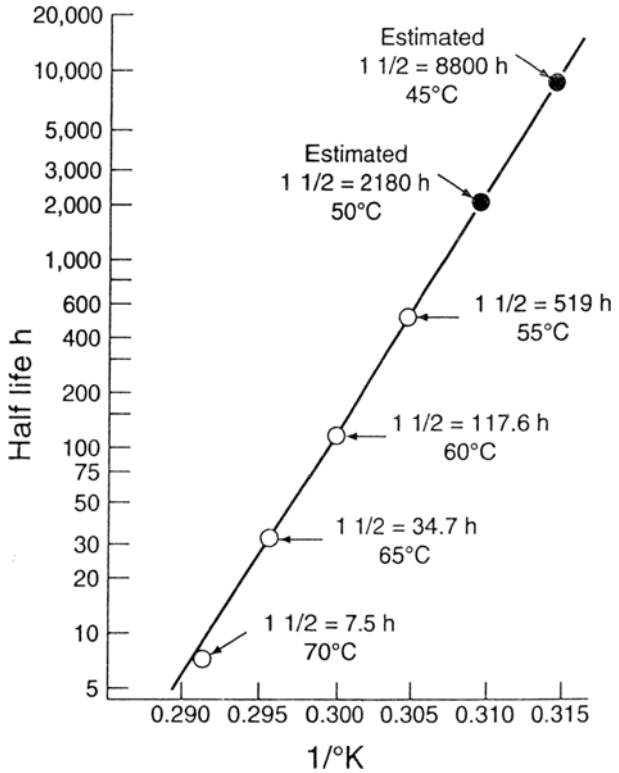

Fig. 18. Half-life values vs 1/*T* (K°) for amyloglucosidase immobilized on porous silica with 30% w/w starch solutions as substrate. The columns were operated on a continuous basis 24 h/d for over 100 d. Half-lives at 45 and 50°C were extrapolated by regression analysis from collected data at these temperatures.

Affinity Separations

As is the case with the organic polymeric support materials, inorganic supports have been used for the isolation and purification of a variety of compounds. In most cases the purity of the isolated products range from 70% to the high 90% range. The variation in range is determined by the nonspecific binding and release of nonspecific (NS) bound compounds. Generally, the quantity of NS components bound is a percentage of the total quantity of material offered during the affinity separation. On a well prepared support, where all sights have been filled or blocked, the nonspecific binding (NSB) ranges from 0.10% to 0.01% of the total offered. This means that if a 1-g affinity support column is offerred 1000 mg of protein, approx 0.1 to 1.0 mg of NSB will bind. Assuming that the compound of interest is at a concentration of 1.0 mg/g of starting material and it is totally bound by the support, then on elution the best one could expect would be 1.0 mg of specific component and 0.1–1.0 mg of NS protein. The purity, therefore could be as high as 90% or as low as 50%. Improvement in purity of the isolated component can be achieved by decreasing the

Table 4
Some K_m Values for Soluble
and Immobilized Enzymes in Inorganic Supports

Enzyme	Substrate	$K_m \times 10^{-3} M$ Support	Soluble	Immobilized	Reference
Lactate dehydrogenase	Lactate	CPG	8.0	5.9	(*35*)
Lactate dehydrogenase	Pyruvate	CPG	0.125	0.39	(*35*)
Invertase	Sucrose	CPG	0.49	0.49	(*36*)
Glucose oxidase	Glucose	Silica	7.7	6.8	(*36*)
Alkaline phosphatase	PNPP	Silica	0.10	2.90	(*36*)
L-aminoacid oxidase	L-Leucine	Silica	1.00	4.00	(*36*)
Arylsulfatase	PNPS	Silica	1.85	1.57	(*37*)
Aminopeptidase	LpNA	CPG	0.28	1.07	(*38*)
Trypsin	TAME	CPG	0.12	1.53	(*39*)

CPG = controlled pore glass; Silica = controlled pore silica; PNPS = *p*-nitrophenyl sulfate; LpNA = L-leucine-*p*-nitroanilide; Tame = α-tosyl-L-arginine methylester; PNPP = *p*-nitrophenylphosphate.

NSB or by increasing the concentration of the component to be purified, as a percentage of the offered protein. Similar results are observed in cases where low mol wt components are the products of the desired isolation. Improvements in purity are sometimes achieved by gradient elution such that the NSB elutes either over the entire range of the gradient whereas the product elutes as a sharp peak, or the product elutes before the NSB. Generally the former approach seems to work best.

ENZYMES

Inorganic supports are very useful materials for enzyme immobilization. Over the past 25 yr literally hundreds of papers have been published that use inorganic support materials. Table 5 presents several examples of publications over recent years that describe the immobilization of enzymes on several different inorganic support materials.

Many of these immobilized enzymes have been used in industrial, medical, and analytical applications. It is not within the scope of this review to cover these topics. For detailed coverage of these applications please refer to other sources that are available. One major source of information regarding applications of this technology may be found in the series of

Table 5
Some Recent Examples of Proteins, Enzymes, and Enzyme Containing Cells Covalently Coupled to Inorganic Support Materials Through Silane Coupling Agents

Support Material	Immobilized Component	Reference
Alumina	Mycobacterium	(40)
Alumina	Rhodococcus	(40)
Alumina	β-galactosidase	(41)
Alumina	Alkaline protease Glucose oxidase Alcohol dehydrogenase	(40)
Ca-Ti Phosphate	Invertase	(42)
CPG	Horseradish peroxidase	(43)
CPG	Acetylcholinesterase	(44)
CPG	Glucose oxidase	(45)
CPG	Fructosyl-trans enzyme β-fructofuranosidase	(46)
CPG	Amyloglucosidase	(47)
CPG	Acid phosphatase	(48)
CPG	Mammalian cells	(49)
CPG	FAD	(50)
CPG	Myeloperoxidase	(51)
CPG	Protocatechaute 3,4-dioxygenase	(52)
CPG	Naringinase	(53)
CPG	Glucose oxidase	(54)
CPG	Pronase	(55)
CPG (Ti-coated)	Amyloglucosidase	(56)
CPG	Phosphotransacetylase	(57)
CPG	Acetate kinase	(57)
Glass	Proteins	(55)
Glass	Monoclonal antibodies	(58)
Glass	*Lactobacillus sp.*	(59)
Glass	Avidin	(28)
Glass	Avidin	(28)
Glass fibers	*Catharonthus roseus*	(60)
Sintered glass	Amyloglucosidase	(61)
Ceramics	Mammalian cells	(62)
Chromium dioxide	Antibodies, protein antigens	(63)
Titanium IV-silica	Urease	(64)
Ceramics	Antibodies	(65)
Titanium oxide	Hydrogenase	(66)

(*continued*)

Table 5 (Continued)

Support Material	Immobilized Component	Reference
Silica	Mycobarterium	(*40*)
Silica	Rhodococcus	(*40*)
Silica	Bovine serum albumin	(*67*)
Silica	Antibodies	(*68*)
Silica	β-Galactosidase	(*69*)
Silica	Peptides	(*70*)
Silica	Monoclonal antibodies	(*24*)
Silica	β-Galactosidase	(*71*)
Silica	Bovine serum albumin	(*72*)
Silica	Antibodies	(*73*)
Silica	Concanavalin A	(*74*)
Silica	Cellulase	(*75*)
Silica	Glucose oxidase	(*76*)
Silica	Mammalian cells	(*77*)
Silica	Papain	(*78*)
Silica	Gramicidin S	(*79*)
Silica	Lipase	(*80*)
Porous silica	Fv antibody fragments	(*81*)
Porous silica	Carbonic anhydrase	(*82*)
Porous silica	Peroxidase, amyloglucosidase, urease	(*83*)
Porous silica	Glucose isomerase, β-Galactosidase	(*84*)
Porous silica	β-amylase	(*85*)
Porous silica	Peroxidase	(*86*)
Porous silica	Cellulases	(*87*)
Silica gel	Metalloenzymes	(*88*)
Silica gel	Peroxidase, urease, amyloglucosidase	(*89*)
Silica gel	Amyloglucosidase	(*89*)
Porous alumina	Proteases	(*90*)
Magnetite	β-Galactosidase	(*91*)
Magnetite	Cellulases	(*92*)

proceedings of the Enzyme Engineering Conferences (1971-1991). These proceedings from biyearly meetings held at locations throughout the world present an excellent source of information, and represent a good starting point for anyone interested in pursuing information on a specific aspect of immobilized enzyme technology.

ANTIGENS AND ANTIBODIES

Immobilized antibodies were first covalently coupled to an inorganic support by this author (*17*) for the isolation of antibodies and immumoglobulins. Since that successful attempt, antibodies on inorganic supports have been used for the isolation and purification of haptens (*18*), bovine serum albumin and immunoglobins (*17,19–20*), thromboxane B2 (*21*), Blood group A active oligosaccharide (*22*), arginosuccinase (*23*), lysozyme (*24*), EGR (*25*), receptors (*26*), viruses (*27*), antigen B27 (*28*), and cerbriosides and glycolipids (*29*).

NEW APPLICATIONS OF ENZYME IMMOBILIZED ON INORGANIC SUPPORTS

In recent years silane coupling has been extensively used for the attachment of proteins, in particular enzymes and antibodies, to electronic devices for the development of biosensors. Most devices have been made of quartz or ceramic materials. The coupling methods used have already been described in this paper. However, there are a few reports using silanes, in which the silanes were used in a somewhat different manner.

Self-Assembling Monolayers

Recently much has been reported regarding self-assembling monolayers and multilayer films. These films can be used in thin-film optics, sensors and transducers, protective layer, high-resolution imaging materials, and functionalized surfaces with specific chemical, biological, or adhesive properties (*30–33*). A very interesting report (*34*) describes the preparation of organosilane self-assembled monolayer films. Protein may be coupled to these films in a manner that permits the patterning of the films into areas with coupled protein and areas lacking coupled protein. Glass or fused silica slides, Pt films on Si substrates, and Si wafers with a native oxide coating were treated with various silanes to form film coatings. These coatings acted as precursors for the final coating of a silane that was used for the coupling reaction. Areas where protein coupling was to occur were masked. Deep UV irradiation modified the unmasked areas by bond scission making these areas more hydrophilic. If a sulfhydryl silane had been used for the second coating, the deep UV treated sample was no longer able to couple protein whereas the masked SH groups were available for coupling. The result was protein coupled in a desired pattern.

Silane Polymers

Other new applications of silanes on inorganic supports have been in the area of biosensors. Inorganic silanes are the most commonly used method of immobilizing an enzyme to an electrode surface. One recent report used a silane polymer as the backbone of a redox polymer incorporating ferrocene. This polymer in association with glucose oxidase was used for the quantitation of glucose in an electrode (*35*).

Sol-Gel Glasses

The most recent studies on silanization has been reported by Ellerby et al. (*36*). These workers prepared sol-gel glasses from tetramethylorthosilicate (tetramethoxysilane) monomer and entrapped proteins, including enzymes in these gels with retention of activity. The glasses were prepared by the acid catalyzed polymerization of the silane monomers. The material dries, forming a rigid, transparent glass with excellent thermal and dimensional stability.

Proteins, including bovine serum albumin and copper–zinc superoxide dismutase, were entrapped in this manner and appeared to retain activity. In our laboratories we are examining this procedure for the entrapment of light-sensitive proteins, including bacteriorhodopsin. Derivatives so prepared are now undergoing study for many analytical, biomedical, and industrial applications.

SUMMARY

Proteins can be immobilized on inorganic supports through silane coupling by a wide variety of methods. The supports can be chosen and modified to meet the specific needs of the application. Carriers can be prepared in different pore diameters, particle sizes, and compositions. Enzymes, antigens, antibodies or proteins, and peptides can be coupled through any of the molecules available functional groups.

Recently silanes have been applied to preparation of films on sensor devices and for patterned surface designs. Most recently silanes are being used for formation of sol-gel glasses containing active enzymes for a variety of new and unique applications in analysis.

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