Naval Research Laboratory
Washington, DC 20375-5000

NRL Memorandum Report 6225

(2)

The Covalent Binding of Alkaline Phosphatase on Porous Supports and the Stability of the Immobilized Enzyme

DAWN D. DOMINGUEZ AND GAYLE F. FRIEDMAN*

Surface Chemistry Branch
Chemistry Division

**GEO-Centers, Inc.*
Fort Washington, MD 20746

August 11, 1988

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NRL Memorandum Report 6225	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Naval Research Laboratory 6b. OFFICE SYMBOL (If applicable) Code 6170	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Washington, DC 20375-5000	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Material Development Activity Fort Detrick 8b. OFFICE SYMBOL (If applicable) USAMMDA	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) Frederick, MD 21701-5009	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO

11 TITLE (Include Security Classification)
The Covalent Binding of Alkaline Phosphatase on Porous Supports and the Stability of the Immobilized Enzyme

12. PERSONAL AUTHOR(S)
Dominguez, Dawn D. and Friedman,* Gayle F.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
	FROM 1/85 TO 2/87	1988 August 11	44

16. SUPPLEMENTARY NOTATION
*GEO-Centers, Inc., Fort Washington, MD 20746

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Immobilized enzyme

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Alkaline phosphatase was covalently bound to a series of porous silicas and controlled-pore glasess using different silanization procedures and derivatization methods in order to determine the conditions that would lead to the highest immobilized enzyme (IME) activity and stability. Aqueous silanization was found to give more controlled surface coverage of the porous support than toluene silanization; aqueous silanization also resulted in more stable IMEs. Nearly identical IME activities were obtained with aqueous silanization and three different derivatization methods. The IME formed with diisothiocyanate derivatization had the greatest stability in cold, dry storage but the poorest stability in cold, alkaline buffer. IMEs formed with glutaraldehyde were consistently more stable than those IMEs formed with carbodiimide derivatization.

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Dawn D. Dominguez	22b TELEPHONE (Include Area Code) 22c. OFFICE SYMBOL (202) 767-2998 Code 6170

DD FORM 1473, 84 MAR

SECURITY CLASSIFICATION OF THIS PAGE

CONTENTS

A-1

THE COVALENT BINDING OF ALKALINE PHOSPHATASE ON POROUS SUPPORTS AND THE STABILITY OF THE IMMOBILIZED ENZYME

INTRODUCTION

Traditional methods for detecting biological toxins include bioassays,[1] chemical assays,[1,2,3] immunoassays[4-7] and various spectroscopic methods[1] of analysis. Bioassays are probably among the simplest and, hence, most popular methods of detection but they generally are semiquantitative at best, have low sensitivities (submicrogram range) and they are not very selective. Chemical assays involve reacting or derivatizing the toxic compound prior to analysis by absorption or fluorescence spectroscopy, thin layer chromatography or gas-liquid chromatography. The sensitivity (submicrogram - nanogram range) of these semiquantitative, chemical detection methods depends on whether the toxic compound of interest can be separated from interfering components in the sample. Immunoassays are sensitive (nanogram/mL) and selective ways of quantitatively detecting low concentrations of toxins in complex mixtures in a few hours. The reagents used in immunoassays are often expensive and unstable, however, and analysis procedures are not suitable for field use. Another detection method that is sensitive (nanogram/mL) and highly specific is gas chromatography/mass spectroscopy (GC/MS). GC/MS can provide rapid and precise identification of toxic compounds even when there are substantial interferences present. GC/MS is semiquantitative, however. GC/MS also requires sophisticated instrumentation that is not suitable for field use.

Due to the significant disadvantages of the traditional detection methods an alternate approach is needed if analysis for biological toxins is to be carried out in the field. One such approach combines the attributes of an immunoassay with those of a chemical sensor (small, rugged, highly sensitive, rapidly responsive, relatively inexpensive) into an immunosensor.

If the immunosensor concept is to be developed for field use the analysis procedure must be made simpler than the typical immunoassay and the immunological reagents must be stabilized. Both of these difficulties may, in principle, be

Manuscript approved March 4, 1988.

overcome by immobilizing one or more of the immunological reagents directly on the sensor surface or on a support that can be used in a reaction chamber ahead of the sensor. This eliminates awkward handling of sensitive and possibly hazardous materials and, at the same time, eliminates one or more of the procedural steps; it may also impart some of the stability required for the immunological reagent.

The immobilization of enzymes on electrodes and on porous silica surfaces with fairly good retention of activity (30-100%) is well documented.[8,9,10] Recently, some of these same immobilization methods have been used to bind antibodies to sensor surfaces.[11,12,13] While these later studies indicated that antibodies could also be immobilized without destruction of their active sites, a number of key issues still need to be addressed before the immunosensor approach might be considered a practical alternative for toxin detection and identification in the field. These issues are whether 1) enough active immunological material could be immobilized for the effective detection of a threat toxin, 2) the immobilized reagents are sufficiently stable for practical application, and 3) the preparation of the immobilized reagents is sufficiently reproducible.

This report begins to address these technical issues by examining the covalent binding of alkaline phosphatase on silanized porous silica (PS) and controlled-pore glass (CPG) using different derivatization methods. Covalent binding was specifically chosen as the method of attachment since it is expected to provide greater long term stability than other immobilization methods (i.e. physical adsorption or entrapment). Although the eventual goal of the immunosensor approach is to bind antibodies or protein toxins to sensor surfaces, an enzyme was used as a model protein in these binding studies for two reasons - 1) its activity could be used as an indication of the amount of active protein bound, and 2) the stability of the immobilized enzyme, both in storage and in use, could be evaluated by measuring its activity over time. Alkaline phosphatase was chosen as the enzyme in our studies since its molecular

weight (100,000) approximates those of an IgG antibody or a large protein toxin such as clostridium botulinum toxin.

CPG was used as one of the supports for enzyme immobilization because -1) it is a homogeneous material containing about 96% SiO_2 (silica surfaces are common to many microsensors including the optical waveguide and the SAW device), 2) it is a porous material and, as such, it has a high surface area (10-200 m^2/g) which makes a high degree of protein loading possible, 3) it is relatively inert compared to organic supports and 4) it is rigid and not subject to deformation in flowing systems as are most organic supports. Porous silica was used as an immobilization support to see whether binding on this pure (100% SiO_2) material would result in greater immobilized enzyme stabilities.

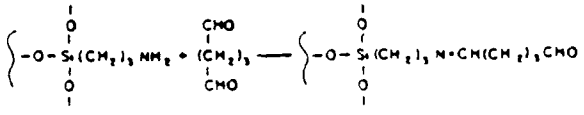
The total surface area of CPG and porous silica supports is inversely proportional to the average pore diameter of the support. Thus, for optimized protein loading on a porous support, the literature[14] suggests that the support with the smallest pore size accessible to the enzyme should be chosen to insure that the support with the highest available surface area is used for protein binding. Given the approximate molecular dimensions of alkaline phosphatase (100Åx60Åx50Å) determined from crystallographic data,[15] CPGs with 75, 240 and 500Å pore diameters were first examined with the intention of determining the optimum pore size from among these choices. Activity measurements and x-ray photoelectron spectroscopy (XPS) measurements were unable to discriminate among these, however. As a result, these pore sizes were all assumed to be too small to incorporate the enzyme in an active configuration and porous supports with larger average pore diameters (up to 4000Å) were added to our study.

Silanization of the support is known to be the most crucial step in enzyme immobilization since it provides the active sites for subsequent protein binding. Nevertheless, different silanization procedures are arbitrarily followed in the literature.

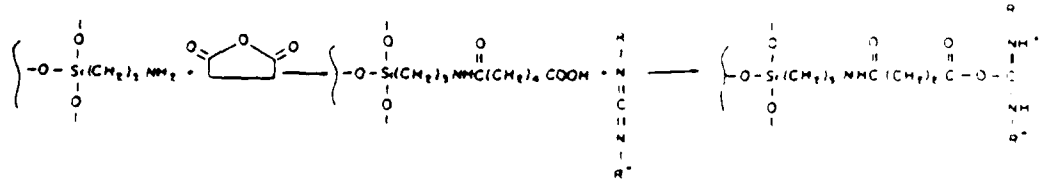
Two of the more common procedures involve heating the support in 1) an acidic solution of 3-aminopropyltriethoxysilane (APTS) and 2) a solution of APTS in refluxing toluene for 24h.[16] Both of these silanization procedures were examined and the silane concentration was varied in each to determine the optimum conditions for achieving monolayer surface coverage of the different supports. The extent of silane surface coverage was determined using XPS.

The covalent binding of proteins onto silanized supports is generally accomplished in two steps: derivatization of the silanized support followed by protein coupling. In this study, derivatization of the silanized supports was carried out using the glutaraldehyde (A), carbodiimide (B), and the diisothiocyanate (C) derivatization methods. The CPG-diisothiocyanate derivative was purchased directly since preparation of this intermediate involved handling the noxious chemical, thiophosgene. The other derivatives were prepared by literature procedures described in experimental section.

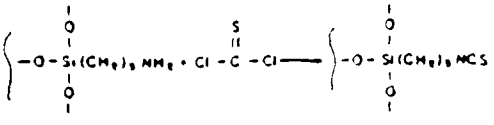
(A)



(B)



(C)



EXPERIMENTAL

1) Controlled-pore glass (CPG) - Uncoated 240Å CPG (#24835), the 75Å and the 500Å CPGs silanized with 3-aminopropyltriethoxysilane (#23538 and #23909, respectively), and the 75Å diisothiocyanate derivative (#22350) were purchased from Pierce Chemical Co. The surface areas and pore volumes of these CPGs are shown in Table 1. The 75Å and 500Å silanized CPGs contained 257 and 151 μmoles of silane/g of glass, respectively, according to the supplier; the diisothiocyanate (DITC) derivative contained 175 μmoles DITC/g of glass. In addition, a series of uncoated CPGs with average pore diameters (of 75-4000Å) was obtained from Sigma Chemical Co. The surface areas and pore volumes of these CPGs are shown in Table 2. Prior to silanization, all uncoated CPG surfaces were cleaned and activated by boiling in 5% nitric acid for 1 hour.

2) Porous Silicas (PS) - LiChrospher Si 500 and LiChrospher Si 4000 were obtained from Applied Science Laboratories, Inc. LiChrospher Si 100 was obtained from Alltech Assoc. The surface areas, pore volumes, and particle sizes of these PSs are also shown in Table 2.

3) Silanization - 3-Aminopropyltriethoxysilane (Petrarch #A0750) was used to silanize the uncoated porous supports by both the aqueous and the organic silanization procedures. The aqueous procedure involved heating the porous support in an acidic (pH 3-4) solution of the silane to 75-80^{o}C for 2 hours; triply distilled water (dH_2O) was used in this procedure and in all subsequent procedures requiring aqueous or buffered solutions. The organic method was carried out by heating a solution of the silane in refluxing toluene for 24 hours.[16]

4) X-Ray Photoelectron Spectroscopy (XPS) - XPS is a surface analytical technique that is used to determine the chemical composition of materials to a depth of approximately 20Å. The technique involves placing a sample in an ultra high vacuum (10^{-9} torr) chamber and exposing it to x-rays of known energy that can be absorbed and cause

Table 1. XPS Analysis of CPGs (Pierce) Silanized with APTS

Average Pore Diameter, Å	Average Particle Size, μm	Total Surface Area m^2/g	Pore Volume cm^3/g	Density g/cm^3	External Surface Area m^2/g	Silanization Solvent	% APTS	Atomic %C	Atomic %N
240	50	79	0.89	0.30	.40	dH_2O	1	4 ± 1	1 ± 1
							10	6 ± 1	1 ± 1
							20	6 ± 1	1 ± 1
							30	7 ± 2	2 ± 1
							40	13 ± 1	2 ± 1
							50	13 ± 1	1 ± 1
240	50	79	0.89	0.30	.40	toluene	1	14	3
							10	16 ± 2	4 ± 1
							20	25	7
							30	20 ± 3	5 ± 1
							40	13	3
75	50	182	0.47	0.59	.20	?	?	20	1
500	150	43	1.04	0.38	.11	?	?	23	1

Table 2. XPS Analysis of CPGs (Sigma) and Porous Silicas Silanized with 30% APTS in Distilled Water

Porous Support	Average Pore Diameter, Å	Average Particle Size, μm	Total Surface Area m^2/g	Pore Volume cm^3/g	Density, g/cm^3	External Surface Area m^2/g	Atomic %C	Atomic %N
CPG	75	150	153	0.47	0.64	.06	6 ± 1	1 ± 1
CPG	240	150	94	0.85	0.40	.10	8 ± 1	1 ± 1
CPG	500	150	56	1.26	0.29	.14	9 ± 1	2 ± 1
CPG	700	150	25	.77	0.38	.11	7 ± 1	1 ± 1
CPG	1000	150	26	1.04	0.32	.13	9 ± 1	1 ± 1
CPG	1400	150	24	1.19	0.30	.13	9 ± 1	1 ± 1
CPG	2000	150	11	0.95	0.35	.11	8 ± 1	2 ± 1
CPG	3000	150	7	0.73	0.39	.10	6 ± 1	1 ± 1
PS	100	5	250	1.2	0.15	8	11 ± 1	3 ± 1
PS	500	10	50	0.8	0.17	4	11 ± 1	3 ± 1
PS	4000	10	6	0.8	0.15	4	6 ± 1	1 ± 1

ejection of electrons from different atoms in the sample. Analysis of the energy of the ejected electrons gives information about the chemical composition of the sample.

5) Silane derivatives - Three derivatives of the silanized CPGs were used to bind the enzyme. These included a carbonyl derivative formed by glutaraldehyde activation of the silanized support, a carboxyl derivative made by reaction of the silanized support with succinic anhydride, and a diisothiocyanate derivative purchased from Pierce. The glutaraldehyde derivatives were prepared by reacting the silanized support with 2.5% glutaraldehyde in 0.05M phosphate buffer (pH 7.0) at room temperature for 3h. The carboxyl derivatives were prepared by reacting 300 mg of the alkylamine CPG with 600 mg of succinic anhydride at pH 6 for at least 15 hours at room temperature.

6) Alkaline Phosphatase Immobilization - Alkaline phosphatase Type VII-L (#P-6772) was purchased from Sigma Chemical Co. as a lyophilized powder containing approximately 45% enzyme. The enzymatic activity of the free enzyme was approximately 900 units/mg enzyme protein at 37°C or 420 units/mg enzyme protein at 25°C. Immobilizations of alkaline phosphatase on the carboxyl derivatives of silanized CPG or porous silica were carried out in 0.05M phosphate buffer (pH 8) after activation for 1 hour with 0.1M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in distilled water at room temperature. Immobilizations of alkaline phosphatase on the carbonyl derivatives of silanized CPG were accomplished after washing to remove excess glutaraldehyde; the enzyme was added to the derivative in 0.05M phosphate buffer (pH 7.0). Immobilizations of alkaline phosphatase on the diisothiocyanate derivative of silanized CPG were carried out in 0.05M bicarbonate buffer (pH 9-10) All immobilizations were allowed to proceed overnight at 5°C.

7) Alkaline Phosphatase Assay - The activity of immobilized alkaline phosphatase was determined using the standard Sigma assay for serum alkaline phosphatase. The assay involved reacting 25 mg of the immobilized enzyme with 0.5mL p-nitrophenylphosphate substrate solution in 0.5mL of alkaline buffer at 37°C for 10 minutes. The mixture was then centrifuged and the solution was decanted into 10.0 mL

of 0.05N NaOH to stop the reaction and develop the color due to the p-nitrophenylphosphate produced. The absorbance of this solution was read at 400nm on a Perkin Elmer Lambda 5 UV-Visible Spectrophotometer with 0.05N NaOH as the reference.

8) Stability Studies - Immobilized enzyme stability studies were carried out in dry storage and in buffer solution. The former studies were done by measuring the activities of different immobilized enzyme samples from the same preparation after different periods of dry storage. The latter studies were done by making a series of sequential activity measurements on the same immobilized enzyme sample; between measurements the samples were stored in buffer solution.

RESULTS AND DISCUSSION

SILANIZATIONS OF POROUS SUPPORTS

The silanization of controlled-pore glass, CPG, was investigated by a) systematically varying the silane concentration in two different silanization solvents for a given CPG and by b) silanizing a series of CPGs and porous silicas having a range of physical properties with a fixed silanization procedure. Using this approach, the optimum procedure for covering the surface of the porous support with a monolayer of silane and the physical property of the porous support leading to maximum silane loading were determined. The optimum silanization procedure was determined by silanizing 240Å CPG (Pierce) with 1-50% solutions of 3-aminopropyltriethoxysilane (APTS) in distilled water and with 1-40% solutions of APTS in toluene as described in the experimental section; x-ray photoelectron spectroscopy (XPS) was then used to determine the relative amounts of silane present on the surface (top 20Å) of the silanized CPGs. This was accomplished by measuring the areas under the C1s, N1s, O1s and Si2p peaks in the XPS spectra of the silanized CPGs and calculating the atomic percentages of carbon and nitrogen present in each sample from the XPS area measurements.

The results of the XPS analysis of the silanized 240Å CPGs are shown in Table 1. As seen from the table, the measurements show that more silane binds to the CPG when the silanization is carried out in toluene; this is evident from the larger atomic percentages of carbon and nitrogen found in the toluene samples. The XPS measurements also show that when water is used as the silanization solvent, the amount of silane on the CPG surface increases as the silane concentration increases. Thus, the aqueous silanization procedure apparently allows more controlled coverage of the CPG than the organic procedure. Finally, the XPS data suggest that a saturation phenomenum occurs on the 240Å CPG with a 10-30% aqueous APTS concentration. In this case, saturation is likely to be monolayer surface coverage of the CPG with the silane. If this is true, the low atomic percentage of carbon found when the 240Å CPG was silanized with a 1% aqueous APTS solution can be attributed to incomplete surface monolayer formation while the high atomic percentages of carbon found when the 240Å CPGs were silanized with 40 and 50% aqueous APTS concentrations and with 1-40% APTS in toluene can be attributed to the formation of silane multilayers.

Also shown in Table 1 are XPS results obtained on two commercially silanized samples of CPG (Pierce). Measurements were made on these samples in an attempt to identify the commercial silanization procedure (the silanization solvent and the APTS concentration used to silanize these samples were proprietary) and to compare the silane surface coverage obtained commercially with those obtained on the 240Å CPG following literature silanization procedures. As seen from the table, the high atomic percentages of carbon present in the commercially silanized samples are consistent with toluene silanization but the low atomic percentages of nitrogen are consistent with aqueous silanization. Thus, on the basis of the XPS measurements, little could be concluded regarding the commercial silanization procedure nor could any comparison of silane surface coverages be made.

To determine which support variable(s) resulted in a maximum silane loading another

set of samples was silanized with 30% APTS in distilled water and analyzed by XPS. This set (shown in Table 2) consisted of a series of 150νm CPGs (Sigma) with average pore diameters of 75-3000 and of three porous silicas which had particle sizes of 5 or 10νm and average pore diameters of 100-4000. The surface areas of the CPGs ranged from 7-153 m^2/g and their pore volumes ranged from 0.47-1.26 cm^3/g. The surface areas of the porous silicas ranged from 6-250 m^2/g and their pore volumes were either 0.8 or 1.2 cm^3/g. The densities of the supports (shown in Table 2) were measured in order to calculate the external surfaces areas (also shown in the Table).

For this set of silanized samples, the atomic percentages of carbon and nitrogen determined by XPS appear to be independent of the total available surface areas of the supports and roughly proportional to the external surface areas sampled by the XPS. It should be noted, however, that this result may simply reflect the fact the XPS does not effectively sample the pores in the sample since the spot size is large (i.e. 300νm) and since the X-ray penetration depth is small.

IMMOBILIZED ENZYME ACTIVITIES

Following silanization, a portion of each preparation was used to bind alkaline phosphatase by the glutaraldehyde derivatization method (Glut) and a portion was used to bind the enzyme via carbodiimide coupling (CDI) after derivatization of the silanized support with succinic anhydride. The activity of the immobilized enzyme (IME) obtained in each case was assayed in duplicate using a standard assay procedure and the results were compared to see which preparations had the highest enzyme activities.

The IME activities measured for the commercially silanized (75 and 500) CPG samples are shown in Table 3. As seen from the table, when 11 mg of enzyme/g of CPG was offered for binding all of the IME preparations had reasonably high activities after six days of cold (5°C), dry storage. The samples prepared on the 500 CPG appear to have slightly higher activities than those on the 75 CPG and the 500 IME preparation

Table 3. IME Activities on Commercially Silanized CPGs (Pierce) and Silanized Porous Silicas After Cold Dry Storage

Porous Support	Average Pore Diameter Å	Binding Method	mg Enzyme g support/ Available	Activity (Abs/mg) day 3/6	Activity (Abs/mg) day 49	Activity (Abs/mg) day 91/92	Activity (Abs/mg) day 106	Activity (Abs/mg) day 207	Activity (Abs/mg) day 247
CPG	75	Glut	11	.067	--	.068	--	--	--
				.074	--	.077	--	--	--
CPG	75	CDI	11	.069	--	--	--	--	--
				.074	--	--	--	--	--
CPG	500	Glut	11	.094	--	.080	--	--	--
				.077		.082	--	--	--
CPG	500	CDI	11	.105	--	.070	--	--	--
				.103	--	.086	--	--	--
CPG	75	DITC	30	--	.105	.107	--	--	.108
					.108	.109	--	--	--
CPG	75	Glut	30	--	.108	.110	.091	--	.072
				--	.105	.103	.105	--	--
CPG	75	CDI	30	--	.026	.031	--	.027	.022
				--	.027	.027	--	--	--
CPG	500	Glut	30	--	.104	.086	.088	--	.084
					.109	.099	.088	--	--
CPG	500	CDI	30	--	.023	.021	--	--	.018
					.021	.022	--	--	--
PS	100	CDI	30	.099	--	--	--	--	--
				.103	--	--	--	--	--
PS	500	CDI	30	.108	--	--	--	--	--
				.107	--	--	--	--	--
PS	4000	CDI	30	.108	--	--	--	--	--

made by the carbodiimide method appears to have a slightly higher activity than the 500Å preparation made by the glutaraldehyde method.

When 30 mg of enzyme/g of CPG was offered for binding, the activities of the IMEs prepared by the glutaraldehyde derivatization method were higher (even after 49 days of cold, dry storage) than those in the previous series but the activities of the IMEs prepared by the carbodiimide method were lower. Degradation with time did not seem to be a likely explanation for the low activities of the carbodiimide samples since little activity (<25%) was lost when the carbodiimide samples prepared with 11 mg of enzyme/g of CPG were stored cold and dry for 92 days (see Table 3). Nor did it seem likely that the IME activity was inhibited by additional enzyme binding since IMEs made from both the glutaraldehyde and the diisothiocyanate derivatives with comparable amounts of enzyme had high activities (Table 3). The most probable explanation for the low activities of the carbodiimide samples in this set of IMEs is that the enzyme binding was unsuccessful.

The activities of IMEs prepared on the silanized porous silica supports were measured after three days of cold, dry storage; these activities are also shown in Table 3. The porous silicas were silanized with 30% APTS in distilled water and derivatized with succinic anhydride prior to enzyme binding; the latter was accomplished with carbodiimide coupling and with 30 mg of enzyme/g of porous silica. As seen from the table, all of the IMEs prepared on the porous silicas had activities that were comparable to those measured on the commercially silanized CPGs with the same amount of available enzyme successfully bound.

The activities of the IMEs prepared on 240Å CPG samples silanized with 1, 10 and 30% solutions of APTS and with 30 mg of enzyme/g of CPG available for binding are shown in Table 4. These activities were measured after five days of cold, dry storage. As seen from this table, the IMEs prepared with glutaraldehyde derivatization and with succinic anhydride derivatization (carbodiimide coupling) had similar activities in most

Table 4. IME Activities on 240Å CPGs

IME Derivative w/30mg Enzyme/g CPG Available	Activities, (Abs/mg)	IME Derivative w/30mg Enzyme/g CPG Available	Activities (Abs/mg)
1% APTS in dH_2O, CDI	.031 .052	1% APTS in dH_2O, Glut	.035 .025
10% APTS in dH_2O, CDI	.103 .106	10% APTS in dH_2O, Glut	.071 .085
30% APTS in dH_2O, CDI	.101 .107	30% APTS in dH_2O, Glut	.087 .100
1% APTS in toluene, CDI	.107 .075	1% APTS in toluene, Glut	.104 .076
10% APTS in toluene, CDI	.075 .074	10% APTS in toluene, Glut	.032 .024
30% APTS in toluene, CDI	.086 .099	30% APTS in toluene, Glut	.063 .078

cases; these activities were comparable to those measured on the commercially silanized CPGs with the same amount of available enzyme (see Table 3). Also evident from Table 4 is the fact that the activities of the IMEs prepared on the 240Å CPG samples that were silanized in distilled water increase with the APTS concentration until the support surface is covered with a monolayer of silane while the activities of the IME samples that were silanized in toluene with one exception, are equivalent to those with monolayer silane coverage by the aqueous procedure. This result could be anticipated since only the top layer of silane should be capable of binding enzyme.

In addition to the above, the IME activity measurements were intended to 1) determine which support variable(s) had to be maximized in order to obtain maximum enzyme loading per gram of support and 2) identify the minimum pore diameter required for alkaline phosphatase penetration. However, both commercially silanized CPGs and all the porous supports that were silanized with 30% APTS in distilled water and that successfully bound 30 mg of enzyme/g of support had the same activity, 0.104± .005 Abs/mg· (see Tables 3 & 4). This was true although the total surface areas of the supports varied by a factor of 40, the external surface areas of the supports varied by a factor of 80 and the total pore volume of the supports varied by a factor of 3. Thus, from among the support variables examined, it appears that the IME activity obtained depends primarily on the total pore volume of the support. And, since all of the IME activities were identical, the minimum pore diameter that could accommodate the alkaline phosphatase enzyme could not be determined. Instead, all of the pore sizes examined (75-4000Å) were assumed to be accessible to the enzyme.

IMMOBILIZED ENZYME STABILITIES

IME Stabilities in Cold, Dry Storage

The activities of three of the IME preparations on commercially silanized CPGs with 11 mg of enzyme of CPG offered for binding were measured after three months of dry

storage at 5°C and these activities were compared to those of the freshly prepared samples. The results, shown in Table 3, suggest that the samples prepared by the glutaraldehyde method are more stable in cold, dry storage than those prepared by the carbodiimide method. In fact, after three months of cold, dry storage the glutaraldehyde samples retained around 95% of their initial activities while the carbodiimide samples retained only 75% of their initial activities in this time period.

The storage stabilities of the IMEs prepared on commercially silanized CPGs with 30 mg of enzyme/g of CPG offered for binding are also shown in Table 3. All of the IME samples in this series of preparations retained 100% of their initial activities after three months of cold, dry storage except for those prepared on 500 CPG with glutaraldehyde derivatization. The increased stability of the carbodiimide samples in this series of preparations might be attributed to their lower initial activities. After more than eight months (247 days) of cold, dry storage the IME preparation on 75 CPG with diisothiocyanate derivatization (DITC) appears to be the most stable; however, all of the IME samples in this series of preparations retained at least 70% of their initial activities in this time period.

The stabilities of the IMEs prepared on 240 CPG samples silanized with 1-30% APTS and with 30 mg of enzyme/g of CPG available for binding are shown in Table 5. From this table it can be seen that the samples prepared with the carbodiimide coupling retained at least 50% of their initial activities when stored dry at 5°C for 7.5 months (225 days); the carbodiimide preparations that had a monolayer of silane covering their surface (i.e. the 30% APTS in distilled water and the 1% APTS in toluene preparations) retained 100% of their initial activities in this time period.

The activities of three of the IMEs prepared on 240 CPG with glutaraldehyde derivatization appeared to increase by an average of 50% over 7.5 months of cold, dry storage. No explanation for this apparent increase can be offered at this time. One of the glutaraldehyde samples lost more than 2/3 of its initial activity during the same time.

Table 5. Stabilities of IMEs Prepared on 240Å CPG and Several Porous Silicas After Cold, Dry Storage

Porous Support	IME Derivative w/30mg Enzyme/g support Available	Average % Activity Remaining (Compared to Activity on day 3 or 6			
		day 57	day 69	day 84/85	day 225
240Å CPG	1 APTS in dH_2O, CDI	--	88	107	81
"	10% " " "	--	99	95	62
"	30% " " "	--	100	103	100
240Å CPG	1% APTS in dH_2O, Glut	--	177	133	133
"	10% " " "	--	105	117	176
"	30% " " "	--	98	--	--
240Å CPG	1% APTS in toluene,CDI	--	104	--	100
"	10% " " "	--	129	84	53
"	30% " " "	--	91	118	57
240Å CPG	10% APTS in toluene, Glut	--	53	50	28
"	30% " " "	--	146	153	174
Si-100	30% APTS in dH_2O, CDI	95	--	--	--
Si-500	30% APTS in dH_2O, CDI	91	--	--	--
Si-4000	30% APTS in dH_2O, CDI	98	--	--	--

The activities of the IMEs prepared on the silanized porous silicas with 30 mg of enzyme/g of PS available were measured after 57 days of cold, dry storage. Each of these IMEs retained 90% or more of its initial activity after nearly two months of cold, dry storage (Table 5).

IME Stabilities in Cold Buffer

The stabilities of the IMEs prepared on commercially silanized CPGs with 11 mg of enzyme/g of CPG and with 30 mg of enzyme/g of CPG offered for binding were examined in 2-amino-2-methyl-2-propanol buffer (pH 10.3) by making duplicate activity determinations on samples from each preparation several different times over 25 days. The samples were kept in cold (5°C), alkaline buffer during the entire test period except when the IME activities were determined. During this time the samples were allowed to warm up to room temperature; then they were centrifuged and separated from the storage buffer before fresh buffer and substrate solution were added. The IME samples were then incubated in the substrate solution at 37°C for 10 minutes and centrifuged. After centrifuging, the enzyme reaction was stopped by decanting the substrate solution into 10.0mL of 0.05N NaOH for the visible absorbance measurement at 400nm and by washing the CPG with the alkaline buffer. After centrifuging once more, fresh buffer was added to the IME samples and they were returned to the refrigerator for additional storage. The total time involved for each set of activity determinations was approximately 30-40 minutes.

The activities of the IMEs prepared on commercially silanized CPGs with 11 mg of enzyme/g of CPG were determined after six days of cold, dry storage; subsequent activity measurements were made on the same IME samples after storage in cold, alkaline buffer. These activities are plotted against time in Figure 1. As seen from the Figure, the activities of all of the IMEs decrease almost linearly during the test period. Thus, the stabilities of the IMEs in cold, alkaline buffer can generally be characterized as poor.

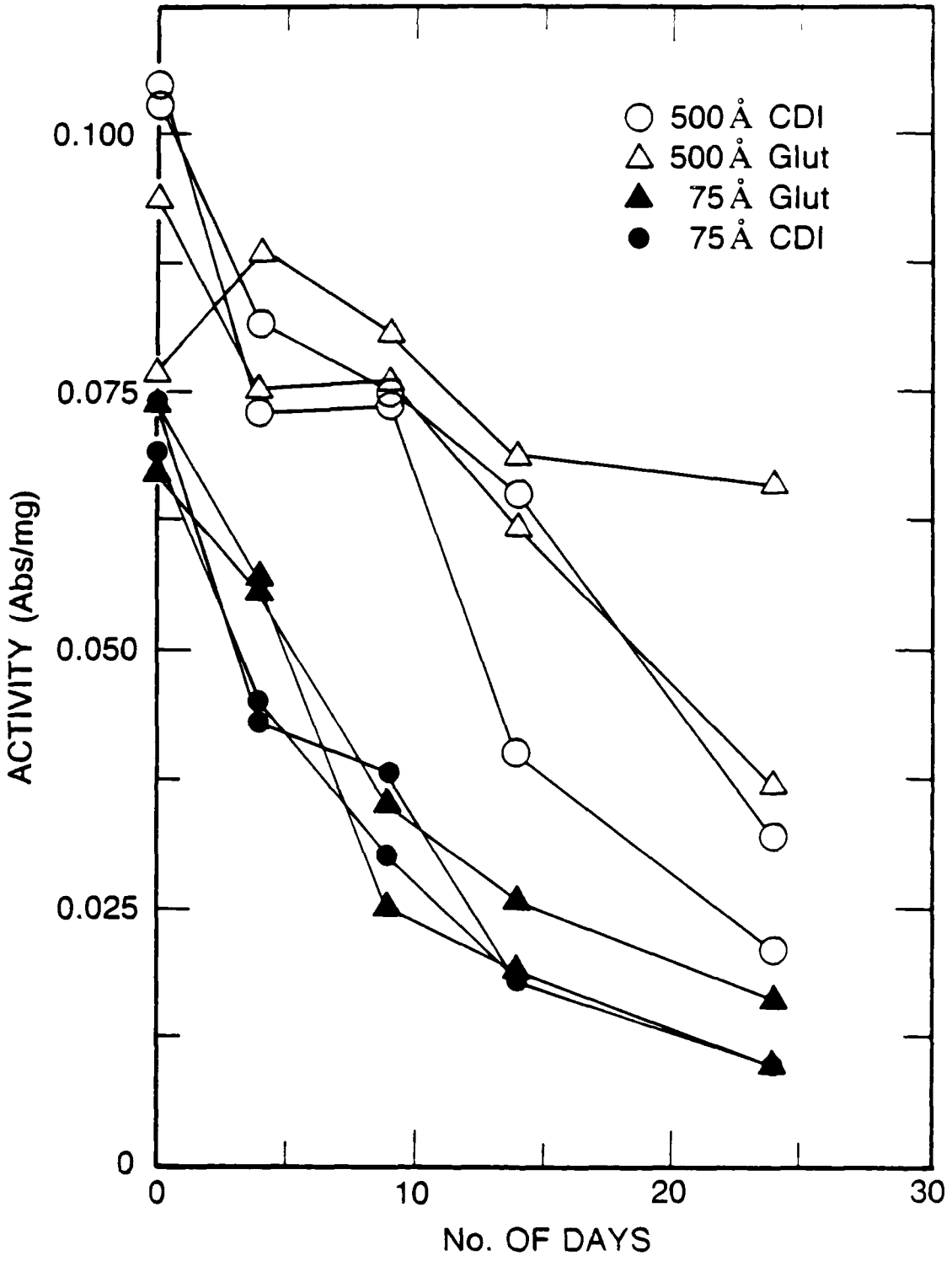


Figure 1. The stability of IMEs in cold, alkaline buffer. The IMEs were prepared on commercially silanized CPGs; they had 11 mg of enzyme/g of CPG available for binding. Measurement was begun after six days of cold, dry storage.

However, the samples prepared with glutaraldehyde derivatization are slightly more stable than those prepared with the carbodiimide coupling. For example, after 4 days of storage and two sets of activity determinations the glutaraldehyde preparations retained about 85% of their initial activities on the average whereas the carbodiimide preparations retained about 70% of their activities. After 25 days in cold, alkaline buffer and five sets of activity determinations the glutaraldehyde and the carbodiimide samples retained 40% and 20% of their initial activities, respectively.

The stabilities of the IMEs prepared on commercially silanized CPGs with 30 mg of enzyme/g of CPG offered for binding were determined after 91 days of cold, dry storage. The activities associated with these measurements are shown in Table 6. In this series of samples, as before, the samples prepared with glutaraldehyde derivatization have greater stability in cold, alkaline buffer than do the carbodiimide samples. After 4 days of storage and two sets of activity determinations the two glutaraldehyde preparations in this series of samples retained around 70% of their initial activities on the average. The same time in cold, alkaline buffer left the two carbodiimide preparations and the diisothiocyanate preparation with only 25% and 10% of their activities, respectively. After 25 days of storage in cold, alkaline buffer and six sets of activity determinations the glutaraldehyde samples retained 15% of their initial activities. In all cases involving the commercially silanized CPGs, the stabilities of the IMEs in cold, alkaline buffer appeared to decrease when more enzyme was available for binding. However, the stabilities of the IMEs in cold, alkaline buffer was dependent on the age of the samples. This age dependence will be discussed shortly.

The alkaline buffer stabilities of the IMEs prepared on 240Å CPG with 10-30% APTS and with 30 mg of enzyme/g of CPG offered for binding were examined after the samples

Table 6. Stabilities of IMEs After Storage in Cold, Alkaline Buffer

CPG Support	IME Derivative w/30 mg Enzyme/g CPG Available	Average % Activity Remaining (Compared to Activity on day 0) day 4	day 9	day 14	day 19	day 25
500Å	CDI	30	10	discontinued		
75Å	CDI	20	10	discontinued		
75Å	DITC	10	2	discontinued		
75Å	Glut	90	40	40	20	20
500Å	Glut	50	50	20	15	10
240Å	10% APTS in dH_2O, CDI	15	5	discontinued		
240Å	30% APTS in dH_2O, CDI	100	100	85	30	30
240Å	10% APTS in dH_2O, Glut	70	40	40	25	15
240Å	30% APTS in dH_2O, Glut	80	50	40	20	20
240Å	10% APTS in toluene,CDI	20	10	discontinued		
240Å	30% APTS in toluene, CDI	55	15	10	discontinued	
240Å	10% APTS in toluene, Glut	100	90	70	discontinued	
240Å	30% APTS in toluene, Glut	90	65	40	30	20

were kept in cold, dry storage for 69 days. As before, the activities determined for these samples are shown in Table 6. As seen from the table, after 69 days of cold, dry storage, 4 days in cold, alkaline buffer and two sets of activity determinations the glutaraldehyde and the carbodiimide preparations retained an average of 85% and 50% of their initial activities, respectively. These results compare reasonably well with measurements made on samples from the same preparations with only 5 days of cold, dry storage. Under these conditions, the samples retained an average of 85% and 70% of their initial activities, respectively.

A summary of the average percent activity remaining after 4 days of storage in cold, alkaline buffer for the IMEs prepared on 75, 240 and 500Å CPGs is shown in Table 7. As seen from the Table, the stabilities of the different preparations in cold, alkaline buffer appear to be dependent on the method of enzyme binding and on the age of the samples; they appear to be independent of the average CPG pore diameter and of the amount of enzyme available for binding. It is also apparent that IMEs prepared by the glutaraldehyde derivatization method are considerably more stable in cold, alkaline buffer than those prepared by the carbodiimide method and that IMEs prepared with diisothiocyanate derivatization are somewhat less stable.

A possible reason for the decreased IME activities in the cold, alkaline buffer was that the IMEs were deactivated at the high ($37^{o}C$) temperature of the assay. To see whether this was the case, the activities of three sets of IME samples prepared with carbodiimide coupling were determined with incubation times of 10, 30 and 60 minutes at $37^{o}C$. These sets were then stored overnight in the alkaline buffer at $5^{o}C$ as usual. The next day the activities of all of the IMEs were determined using the standard 10 minute incubation time and these activities were compared to those initial activities determined on the previous day with a 10 minute incubation. The results of these measurements, shown in Table 8, indicate that the time spent at $37^{o}C$ (and, hence, the number of previous activity determinations) did not alter the stabilities of any of the

Table 7. Average % Activity Remaining After 4 Days Storage in Cold, Alkaline Buffer (Compared to Activity on Day 0)

IME Derivative	75,500ÅCPG 11 mg Enzyme/g CPG Available 6 days old	240Å CPG 30 mg Enzyme/g CPG Available 5 days old	240Å CPG 30 mg Enzyme/g CPG Available 69 days old	75,500Å CPG 30 mg Enzyme/g CPG Available 91 days old
Glut	90	80	80	70
CDI	70	70	50	30
DITC	-	-	-	10

Table 8. Average % Activity Remaining After Heating to 37°C in Alkaline Buffer

IME Derivative w/30mg Enzyme/g CPG Available	Average % Activity Remaining After Heating 10 min	Average % Activity Remaining After Heating 30 min	Average % Activity Remaining After Heating 60 min
240Å 30% APTS in toluene, CDI	20	30	40
240Å 10% APTS in dH_2O, CDI	30	40	30
240Å 30% APTS in dH_2O, CDI	40	60	60
4000Å 30% APTS in dH_2O, CDI	70	80	65

IMEs prepared by the carbodiimide linkage. Although a similar determination was not carried out for the glutaraldehyde samples, it was assumed that these IMEs were affected even less than the carbodiimide samples since their stabilities in cold, alkaline buffer were consistently better.

Another possible explanation for the instability of the IMEs in the alkaline buffer solution was that the enzyme was being removed from the porous support. This could occur by breaking any one of the bonds between the enzyme and the support. One of the bonds common to both the glutaraldehyde and the carbodiimide IME preparations is the covalent bond formed between the silica support and the silane. The siloxane bond is susceptible to slow hydrolysis; this process was expected to occur more easily at high pHs since silica surfaces are more readily attacked under these conditions.

To determine whether the siloxane bond was broken during buffer storage, several silanized 240Å CPG samples were stored in alkaline buffer at 5°C for 20 days. The dried samples were then examined by XPS and the results shown in Table 9 were compared with those in Table 1. These measurements indicated that no appreciable amount of silane was lost from the samples that were silanized in distilled water. The samples silanized in toluene lost a considerable amount of silane, however. The greater hydrolytic stability of the samples silanized in distilled water can be attributed to likelihood that individual silane molecules formed more siloxane bonds with the support surface in these preparations.

Since the siloxane bonds formed in the distilled water preparations proved to be hydrolytically stable for at least 20 days in cold, alkaline buffer, IMEs were prepared on a series of CPGs that had been silanized in distilled water. These IMEs were used to further examine the cause of the alkaline buffer instability. This was accomplished by determining the enzyme activity remaining on the CPGs and in the storage buffer after different periods of cold storage with no previous test for IME activity. IMEs prepared by the carbodiimide method were used in this study since they were less stable than the

Table 9. XPS Analysis of Silanized 240Å CPGs After 20 Days Storage in Cold, Alkaline Buffer

Silanized CPG	Atomic %C	Atomic %N
10% APTS in dH_2O	11	2
30% APTS in dH_2O	8	1
10% APTS in toluene	9	2
30% APTS in toluene	9	2

IMEs prepared by the glutaraldehyde derivatization method. After 10 days in cold storage in the alkaline buffer and no previous test for IME activity approximately 20-25% of the initial IME activity (measured on separate samples) could be accounted for; 1/3 of this activity was found in the storage buffer while the other 2/3 remained on the CPG. After 20 days of cold storage and no previous test for IME activity only around 15% of the initial enzyme activity remained. This time, 2/3 of the activity was found in the storage buffer. These results indicated that both enzyme deactivation and cleavage of the bound enzyme occur; enzyme deactivation appeared to be the primary cause of buffer instability, however.

For comparison, the stabilities of the IMEs prepared with 30 mg of enzyme/g of CPG available for binding were also determined in cold, phosphate buffer (pH 7.8) and these results are shown in Table 10. As seen from this Table, the stabilities of the IMEs in cold, phosphate buffer are much worse than those in cold, alkaline buffer. For example, after 91 days of cold, dry storage and 4 days in alkaline buffer the glutaraldehyde and the carbodiimide samples prepared on commercially silanized CPGs retained 70% and 30% of their initial activities, respectively (see Table 7). After 49 days of cold, dry storage and 4 days in phosphate buffer samples from these same preparations retained only 10% of their initial activities. A similar decrease in IME activities was also observed with the 240Å samples (compare Tables 6 and 10).

IME Stabilities at Elevated Temperatures

The activation energies for IME deactivation in dry storage and in alkaline buffer were determined by measuring activities of three identical IME samples after storage at 5, 22 and 37^{o}C and by determining the slopes of the lines generated in Arrhenius plots. The activation energies for the deactivation of dry samples were determined after 45 days of storage; buffer deactivation was determined after 18 hours of storage.

Table 10. Stabilities of IMEs After 4 Days Storage in Cold, Phosphate Buffer

CPG	IME Derivative w/ 30 mg enzyme/g CPG Available	Activities, (Abs/mg) day 0	Activities, (Abs/mg) day 4	% Activity Remaining
240Å	10% APTS in dH_2O, CDI-1	.110	.008	10
	-2	.107	.006	10
240Å	30% APTS in dH_2O, CDI-1	.108	.048	40
	-2	.103	.038	40
240Å	10% APTS in dH_2O, Glut-1	.085	.009	10
	-2	.079	.011	10
240Å	30% APTS in dH_2O, Glut-1	.111	.008	10
	-2	.117	.011	10
240Å	1% APTS in toluene, Glut-1	.099	.007	10
	-2	.086	.006	10
240Å	10% APTS in toluene, Glut-1	.039	.009	20
	-2	.032	.005	20
240Å	30% APTS in toluene, Glut-1	.064	.002	5
	-2	.056	.009	20
500Å,	CDI-1	.023	.001	5
	-2	.021	.004	20
75Å,	Glut-1	.108	.010	10
	-2	.105	.013	10
75Å,	CDI-1	.026	.001	5
	-2	.027	.002	10
500Å,	Glut-1	.104	.015	10
	-2	.109	.016	15
75Å,	DITC-1	.105	.005	5
	-2	.108	.005	5

Table 11 shows the activation energies determined for IME deactivation in alkaline buffer. The Table includes IME samples prepared on commercially silanized CPGs (75 and 500) and on 240 CPG silanized with 1-30% APTS in both the aqueous and the organic procedures. The IME samples examined in this study included those prepared by both the glutaraldehyde derivatization and the carbodiimide binding methods.

As seen from Table 11, the activation energies for IME deactivation in alkaline buffer range from 1 to 4 Kcal/mol. IME samples prepared with glutaraldehyde derivatization have an average activation energy of 3 $\pm$ 1 Kcal/mol; samples prepared by the carbodiimide method have an average activation energy of 1 $\pm$ 0.5 Kcal/mol. Thus, IME samples prepared by the glutaraldehyde method are more stable in alkaline buffer than IME samples prepared by the carbodiimide method. This finding is in agreement with the activity measurements discussed previously.

Table 12 shows the activation energies determined for IME deactivation in dry storage. The Table includes several 240 CPG samples silanized with 1-50% APTS in distilled water and with 1 - 40% APTS in toluene; it also includes several porous silica samples with average pore diameters of 100-4000 that were silanized with 10-30% APTS in distilled water. All of the IME samples examined in this study were prepared by the carbodiimide binding method.

As seen from Table 12, the average activation energy for IME deactivation in dry storage is about 6 Kcal/mol. IME samples prepared on 240 CPG with toluene silanization have an average activation energy of 3 Kcal/mol while those prepared on the porous silica supports and on the 240 CPGs with aqueous silanization have an average activation energy of 7 Kcal/mol. Thus, for maximum IME stability, dry storage is preferable to storage in alkaline buffer and silanization in distilled water is better than silanization in toluene.

Table 11. Activation Energies for IME Deactivation in Alkaline Buffer

IMEs	Activation Energy (Kcal/mol)	Average Activation Energy (Kcal/mol)
75Å CPG, Glut	2	
500Å CPG, Glut	2	3 ± 1
240Å CPG 10% APTS in toluene, Glut	4	
240Å CPG 30% APTS in toluene, CDI	1	
240Å CPG 1% APTS in dH_2O, CDI	1	1 ± 0.5
75Å CPG, CDI	2	
500Å CPG, CDI	1	

Table 12. Activation Energies for IME Deactivation in Dry Storage

IMEs	Activation Energy, (Kcal/mol)
100-4000Å PS, 10-30% APTS in dH_2O, CDI	7 ± 3
240Å CPG, 1-50% APTS in dH_2O, CDI	7 ± 2
240 Å CPG, 1-40% APTS in toluene, CDI	3 ± 3

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1. Controlled coverage of a porous surface with APTS can best be accomplished by carrying out the silanization in distilled water; XPS and activity measurements show that monolayer surface coverage of 240Å CPG (surface area 130 m^2/g) in distilled water requires a 10-30% solution of the silane. XPS and activity measurements show that silanization in toluene leads to multilayer surface coverage.

2. Maximum silane loading on a porous support appears to depend on the external surface area of the support; it is not achieved by maximizing the surface area of the support.

3. The activities of the IMEs prepared on the 240Å CPG (Pierce) increase with the silane concentration until monolayer surface coverage of the CPG is complete; saturation of the 240Å CPG surface with APTS and, hence, with active enzyme occurs at a 10-30% aqueous silane concentration.

4. Following aqueous silanization, IMEs prepared with carbodiimide derivatization generally have slightly higher activities than those prepared with glutaraldehyde derivatization.

5. The IMEs prepared on the 75Å CPG with diisothiocyanate derivatization retained 100% of their initial activities when kept in cold, dry storage for eight months while the IMEs prepared on the 75 and 500Å CPGs with glutaraldehyde derivatization retained 75% of their activity during the same eight month time period. The IMEs prepared on 500Å CPG with succinic anhydride derivatization and carbodiimide coupling lost 25% of their initial activities after only three months of cold, dry storage.

6. The IME samples prepared with glutaraldehyde derivatization were the most stable in cold, alkaline buffer while the IME samples prepared with diisothiocyanate derivatization were the least stable.

7. By varying the time of the assay for enzyme activity, enzyme deactivation in alkaline buffer was determined not to be caused by the assay itself.

8. The stabilities of the IMEs in cold, alkaline buffer were dependent on the age of the samples and on the silane derivatization method.

9. XPS measurements showed that the instabilities of the IMEs in cold, alkaline buffer did not result primarily from hydrolysis of the siloxane linkage.

10. Activity measurements on the CPG samples and the storage buffer determined that enzyme deactivation was the primary cause of alkaline buffer instability.

11. IME stability is poorer in cold, phosphate (pH 7.8) buffer.

12. The average activation energy for IME deactivation in alkaline buffer is 3 ± 1 Kcal/mol for samples prepared with glutaraldehyde derivation and 1 ± 0.5 Kcal/mol for samples prepared by the carbodiimide method.

13. The average activation energy for IME deactivation in dry storage is 7 ± 3 Kcal/mol for samples prepared with aqueous silanization and carbodiimide derivations. For IME samples prepared with toluene silanization and carbodiimide derivatization the average activation energy for deactivation in dry storage is 3 ± 3 Kcal/mol.

Recommendations

1. It has been established that alkaline phosphatase can be immobilized on CPG or porous silica by either the glutaraldehyde, carbodiimide or diisothiocyanate derivatization methods with a reasonably high level of enzyme activity. It has also been established that the IMEs prepared on CPG by the glutaraldehyde derivatization method retain a high level of their initial activities after eight months of cold, dry storage. An obvious extension of this work would be to immobilize an antibody onto the CPG surface and to monitor its stability under different storage conditions by adding an alkaline phosphatase

labeled second antibody. The alkaline phosphatase activity can be determined by the standard assay procedure used previously.

2. Another important extension of the immunosensor concept is the immobilization of an antibody onto a sensor surface and the subsequent testing of the immunosensor response to an antigen challenge. Two chemical microsensors that are developed adequately for this purpose are the optical waveguide and the acoustic wave device. A brief description of these two chemical microsensors along with recent references to their use as immunosensors is given in the Appendix. The testing of immunosensor response should be carried out with both high and low molecular weight antigens.

APPENDIX

DESCRIPTION OF CHEMICAL MICROSENSORS

Acoustic Wave Devices

An acoustic wave device is a microgravimetric sensor capable of detecting small mass (10 E-9 - 10 E-12gram) changes. A device consists of a piezoelectric substrate which has a pair of interdigital microelectrodes at each end. It is operated by applying a radio frequency (rf) voltage to one set of microelectrodes. This generates an acoustic wave that moves across the device to the second set of microelectrodes where it is received and converted back into a rf voltage. A rf power amplifier connects the input and output electrodes and causes the device to oscillate at a fixed resonance frequency (determined by the interdigital spacing and the acoustic wave velocity).

Two types of acoustic wave devices are common - bulk acoustic wave (BAW) devices and surface acoustic wave (SAW) devices. In the latter (Fig. A1), the interdigital microelectrodes are designed so that Rayleigh surface waves are generated as well as a variety of bulk acoustic waves and the rf circuitry is designed so that efficient energy transfer between microelectrodes occurs solely via the Rayleigh waves. Although both

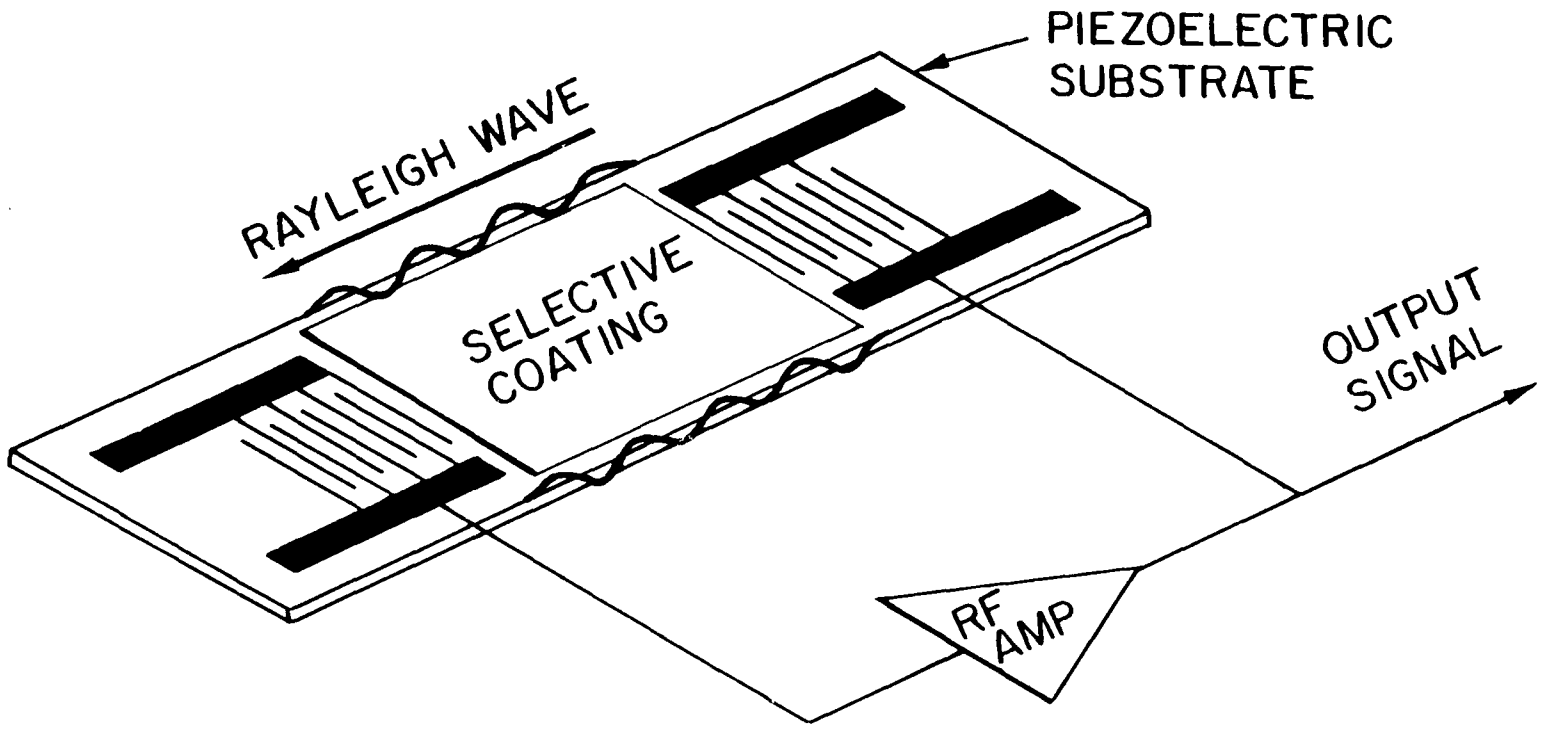


Figure A1. Schematic Diagram of a Surface Acoustic Wave Device

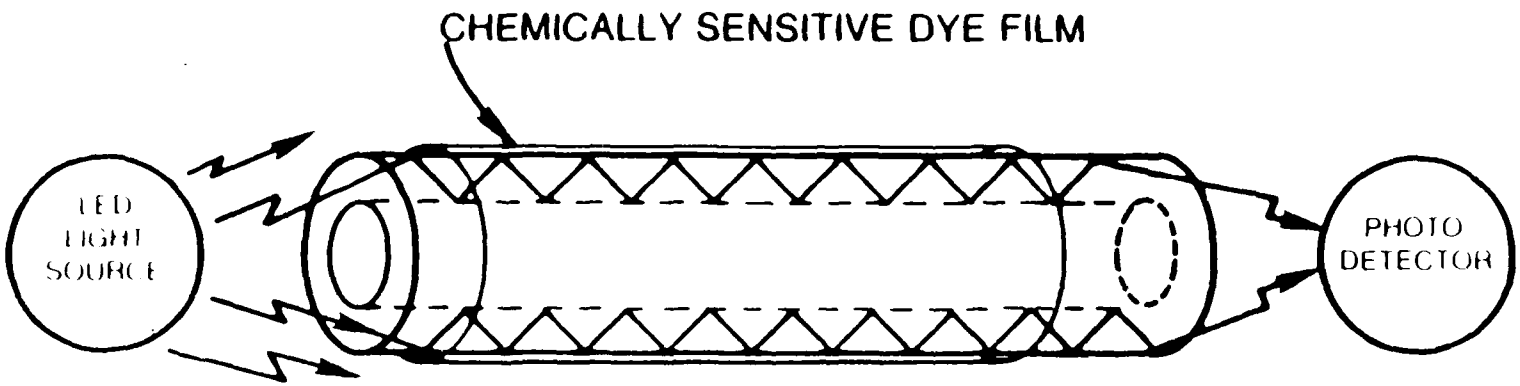
types of acoustic wave devices are being developed as chemical sensors, the SAW device is the more sensitive. This is true since most of the energy associated with the Rayleigh waves is concentrated near the top surface of the device where it is in close contact with any solid film coating or fluid (gas or liquid) medium surrounding the device. In addition, it is easier to fabricate SAW devices that operate at higher frequencies; this also leads to more sensitive devices.

Detection of immunological reactions using a microgravimetric immunoassay with a piezoelectric crystal oscillator in contact with a liquid was first reported by Roederer and Bastiaans in 1983.[17] Using a 10.3 MHz SAW device with an IgG antibody immobilized on its surface they showed that the resonance frequency of the device decreased in proportion to the concentration of antigen adsorbed from solution. By this method they were able to detect as little as 13ug (ca. 10 E-6M) IgG in aqueous buffer. In a later publication, Bastiaans and Good reported[18] that as little as 0.2ng (ca. 10 E-10M) human chorionic gonadotropin (hCG) could be detected in buffer solution and in blood serum solutions when anti-hCG monoclonal antibodies were immobilized on SAW surfaces.

Optical Waveguide

The optical waveguide (Fig. A2) is a small, inexpensive sensor that probes the glass-fluid (gas or liquid) interface via a standing wave (termed the "evanescent wave") that develops in the waveguide when light is internally reflected from its surface. The sensor consists of a light source, a detector and a waveguide element enclosed in a sample cell. Typically the waveguide element is a glass slide, a glass capillary tube or an optical fiber. It operates by sensing optical changes (adsorption, refractive index) caused by surface reactions in the region of the evanescent wave; the electric field amplitude associated with this wave extends a small distance (typically a fraction of a wavelength) beyond the waveguide surface into the fluid phase.

OPTICAL WAVEGUIDE



LIGHT TRANSMISSION IS AFFECTED BY AMBIENT VAPORS INTERACTING WITH DYE FILMS.

Figure A2. Schematic Diagram of an Optical Waveguide

The earliest attempt to take advantage of evanescent wave interactions in an optical waveguide to detect immunological reactions was made in 1975 by Kronick and Little.[19] These researchers physically adsorbed morphine-bovine serum albumin conjugate onto the surface of a quartz slide which served as their waveguide. With the subsequent addition of morphine antibody labelled with fluorescein isothiocyanate (FITC) as little as 200nM morphine (MW 285) could readily be detected. This was accomplished by monitoring the increase in fluorescence that occurred as the totally internal reflected light in the waveguide excited fluorescence in the labelled antibody as it bound to the drug on the surface of the waveguide. Similarly, in 1984 Sutherland et al. reported [20] that as little as 10-20nM human IgG (MW 150,000) could be detected using a sandwich fluoroimmunoassay. In this procedure, the first antibody was covalently bound to a waveguide (either slide or fiber) surface and detection was accomplished by monitoring the fluorescence change that resulted from the binding of FITC-labelled second antibody to the test antigen.

Optical waveguide techniques other than total internal reflection fluorescence (TIRF) have been demonstrated as sensitive chemical sensors. Sutherland et al. showed[21] that 270nM methotrexate (MW 454) could be detected by measuring the change in transmission that occurred when unlabelled drug was bound to an antibody immobilized on a waveguide surface. In addition, they showed that as little as 10nM IgG could be detected by measuring the increase in scattered light that resulted from antigen-antibody complex formation on the waveguide surface. Recently, Seifert et al. have shown[22] that a planar waveguide with a surface relief grating is extremely sensitive to refractive index changes in solutions and to adsorbed layer thickness changes.

REFERENCES

1. (a) Y. Ueno in "Mycotoxins In Human and Animal Health," Rodricks, Hesseltine and Mehlman, eds., Pathotox Publishers, Inc., 1977, p. 189.

   (b) S.V. Pathre and C.J. Mirocha, ibid, p. 229.

2. H.A. Bates and H. Rapoport, J. Agr. Food Chem., 23, 237 (1975).

3. M.T. Nunez, S. Fischer and E. Jaimovich, Anal. Biochem., 72, 320 (1976).

4. S. Notermans, J. Dufrenne and M.V. Schothorst, Japan J. Med. Sci. Biol., 31, 81 (1978).

5. G. Stiffler-Rosenberg and H. Fey, J. Clin. Microbiol., 8, 473 (1978).

6. H. Peters, M.P. Dierich and K. Dose, Hoppe-Seyler's Z. Physiol. Chem., 363, 1437 (1982).

7. F.S. Chu and T.S.L. Fan, J. Assoc. Off. Anal. Chem., 68, 13 (1985).

8. W.F. Line, A. Kwong, and H.H. Weetall, Biochim. Bioophys. Acta, 242, 194 (1971).

9. P.J. Robinson, P. Dunnill, and M.D. Lilly, ibid, p. 659.

10. R.D. Mason, C.C. Detar, and H.H. Weetall, Biotechnol. Bioeng., 17, 1019 (1975).

11. C. Yuan, G. Guilbault, and S.S. Kuan, Anal. Chem., 53, 190 (1981).

12. M.Y. Yeating and G.A. Rechnitz, ibid, 56, 801 (1984).

13. J.R. North, Trends in Biotechnol., 3, 180 (1985).

14. H.H. Weetall in "Method in Enzymology," Vol. XLIV, K. Mosbach, ed., Academic Press, 1976, p. 134.

15. H. Wyckoff, personal communication.

16. M. Lynn in "Immobilized Enzymes, Antigens, Antibodies and Peptides," Volume 1, H.H. Weetall, ed., Marcel Dekker, Inc., New York, 1975.

17. J.E. Roderer and G.J. Bastiaans, Anal. Chem., 55, 2333 (1983).

18. G.J. Bastiaans and C.M. Good, Proc. of the 2nd Intern. Meeting on Chemical Sensors, Bordeaux, 1986, p. 618-621.

19. M.N. Kronick and W.A. Little, J. Immun. Methods, 8, 235 (1975).

20. R.M. Sutherland, C. Dahne, J.F. Place and A.S. Ringrose, Clin. Chem., 30, 1533 (1984).

21. R Sutherland, C. Dahne and J. Place, Anal. Lett., 17, 43 (1984).

22. M. Seifert, K. Tiefenthaler, K. Heuberger, W. Lukosz and K. Mosbach, ibid, 19, 205 (1986).