Covalent Linkage of Proteins to Surface-Modified Poly(organophosphazenes): Immobilization of Glucose-6-Phosphate Dehydrogenase and Trypsin

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ABSTRACT: Glucose-6-phosphate dehydrogenase (G-6-PDH) and trypsin have been linked covalently to a surface-modified poly[bis(aryloxy)phosphazene] supported on porous alumina particles. Poly(diphenoxyphosphazene) was surface-nitrated and then reduced to the aminophenoxy derivative. The aminophenoxy sites were then activated by reaction with cyanogen bromide, nitrous acid, or glutaric dialdehyde, followed by treatment with the enzymes in aqueous buffer solutions. The activities of the immobilized enzymes were monitored spectrophotometrically with the use of glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (for G-6-PDH) and *N*-α-benzoyl-L-arginine-*p*-nitroanilide (for trypsin). These data were supplemented by an assessment of the total yield of immobilized enzyme by means of a Lowry protein measurement. The maximum immobilization yields (8–10% for G-6-PDH and 50–60% for trypsin) were achieved when the glutaric dialdehyde activated system was used. For both proteins, the immobilized enzyme retained its activity and remained linked to the support through numerous cycles. By contrast, the same enzymes adsorbed on uncoated alumina particles were displaced readily by washing. The storage stabilities of both immobilized enzymes were much greater than those of the free enzymes in solution. A continuous-flow assaying system showed that immobilized trypsin generated a constant activity at three different flow rates. For the G-6-PDH system, enzyme activity and scanning electron microscopy were used to examine the relationship between the substrate topography/surface area and the concentration of immobilized protein molecules.

Introduction

Enzymes and other biologically active agents can be immobilized on a variety of insoluble support materials with retention of the biological activity.[1-5] The immobilization of enzymes has many advantages in analysis, biomedical engineering, and industrial production.[3] For example, immobilization usually brings about an improvement in the stability of an enzyme. In addition, the enzyme–support combination is easily separated from the reaction medium. In practical terms, these two factors allow a single aliquot of immobilized enzyme to be used repeatedly to achieve many more catalytic cycles than could be obtained from the same amount of free enzyme in solution.[4]

Four main options exist for the immobilization of bioactive molecules: (1) entrapment within cross-linked gels or fibers,[5] (2) covalent binding to solid polymeric surfaces,[6] (3) immobilization by physical adsorption onto solid surfaces,[7] and (4) microencapsulation.[8] Detailed reviews and monographs have appeared on these different approaches, particularly with respect to the immobilization of enzymes.[9] In this respect, the most widely used method involves the covalent binding of an enzyme to an activated polymeric support.[10]

The activity of an enzyme after immobilization is affected by the coupling procedure, the enzyme site involved in coupling, the orientation of the enzyme on the support surface, and the type of polymeric support. The nature of the support is often the most important factor because its interaction with the enzyme may influence the stability of the polypeptide and the kinetics.[13] Thus, the capacity of the carrier to bind protein molecules, the surface charge or polarity, hydrophobicity, and the dimensional and chemical stability must all be considered.[14]

Poly(organophosphazenes) are a diverse new class of synthetic polymers that have been investigated in our laboratory as biomaterials and carrier macromolecules for a number of biologically active agents.[15-18] Because of the substitutive mode of synthesis used for these polymers,[19] opportunities exist for the subtle modification of solubilities, surface character, molecular flexibility, resistance to hydrolysis, coordination behavior, and biological activity.[20-25]

In the present work we have explored the use of a poly[bis(aryloxy)phosphazene] as a water-insoluble immobilization substrate for glucose-6-phosphate dehydrogenase (G-6-PDH) and for trypsin. These enzymes provide excellent prototypes for immobilization studies since they are commercially available, inexpensive, and easy to manipulate and monitor for enzymic activity.[26] For practical reasons that involve a need to maximize the surface area of the support polymer,[27] the polyphosphazene was used as a coating on porous alumina particles. In this paper, we discuss the following points: (1) development of a synthetic route to generate coupling sites on the polymer surface, coupling of the polymer to G-6-PDH or trypsin, and the spectrophotometric assay methods employed for this system, (2) assessment of the stabilities of the immobilized enzymes by a kinetic examination of their reactivity as a function of time, (3) examination of the role of alumina pore size and polymer coating efficiency on enzyme immobilization, based on kinetic results and scanning electron microscopy of the surfaces, (4) comparisons of the retention of the enzymes attached to the polymeric support and the same enzymes adsorbed on uncoated alumina, (5) the long-term stabilities of immobilized G-6-PDH and trypsin compared with those of the free enzymes in solution, and (6) a preliminary examination of immobilized trypsin in a continuous-flow reactor system.

Results and Discussion

Synthesis and Immobilization Procedure. Poly(diphenoxyphosphazene) (**3**) was prepared by the thermal polymerization of hexachlorocyclotriphosphazene (**1**) to poly(dichlorophosphazene) (**2**) and treatment of this polymer with sodium phenoxide in the presence of tetra-*n*-butylammonium bromide as a phase-transfer agent.

Porous alumina particles were then coated with polymer **3**, and the polymeric coating was surface-nitrated with 90% fuming nitric acid and then reduced to the amino derivative by treatment with sodium dithionite at 100 °C.[28]

For immobilization of G-6-PDH, the amino groups were allowed to react with glutaric dialdehyde in a buffer solution at pH 1.4.[27,29] The activated support was then treated with G-6-PDH in Hepes buffer solution at pH 7.5.[30] For trypsin immobilization, three different coupling

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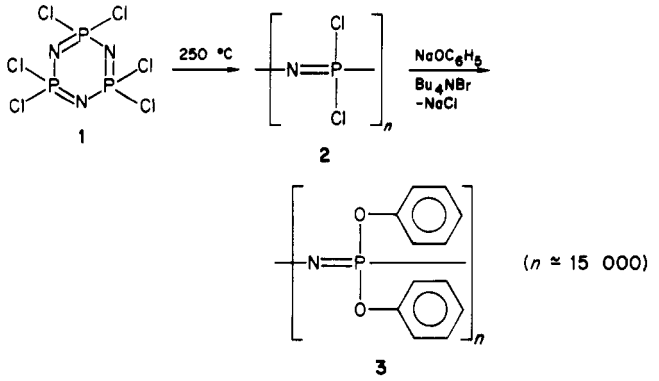

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methods were explored in which the aminophenoxy units were activated with cyanogen bromide, nitrous acid, or glutaric dialdehyde. These pathways are illustrated in Scheme I.

Assessment of Enzyme Activity. The immobilized G-6-PDH enzyme was assayed spectrophotometrically with the use of glucose-6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP).[31] Gluconate-6-phosphate was synthesized from G-6-P with the use of G-6-PDH and NADP as a coenzyme[32] (Scheme II). The reduced form of $NADP^+$ (NADPH) has an absorbance at 340 nm.[33] The activity of G-6-PDH is directly related to the rate of appearance of NADPH. Hence, the absorbance at 340 nm provides a measure of enzymic activity.

Three major concerns about the activity of immobilized enzymes are (a) the retention of long-term activity after linkage to the support, (b) the effect of surface area of the support system on the activity, and (c) the resistance of the linkage site to cleavage.[34-37] In general, covalent linkages are more stable than coordination, dipole–dipole, or van der Waals attachments. Cleavage of the linkage site can be monitored by a decrease in activity as the system is washed with the reaction medium.

If the enzyme-catalyzed reaction rate is defined by the rate of appearance of products,[38] the reaction rate (v) = $d[NADPH]/dt = k_1[enzyme]^x[G\text{-}6\text{-}P]^y[NADP]^z$. When the concentrations of G-6-P and NADP are in large excess, then for a short reaction time, $[G\text{-}6\text{-}P]^y[NADP]^z$ is a constant, and $v = d[NADPH]/dt = k_2[enzyme]^x$, where $k_2 = k_1[G\text{-}6\text{-}P]^y[NADP]^z$. If enzyme molecules are not lost during washing of the system, the influence of the enzyme on reaction rate should be constant, and $v = d[NADPH]/dt = k_3$, where $k_3 = k_2[enzyme]^x$. Thus, $d[NADPH] = k_3\,dt$, $\int d[NADPH] = \int k_3\,dt$, and $[NADPH] = kt + \alpha$. Therefore, the concentration of the product, [NADPH], should show a linear relationship with time. On the basis of the Beer–Lambert law,[39] a stable linkage system between the enzyme and the support should become manifest in a linear relationship between spectrophotometric absorbance and time. As shown in Figure 1, this behavior is found for the G-6-PDH immobilized on this phosphazene/alumina support material.

For trypsin, the activated polyphosphazene support was treated with the enzyme in phosphate buffer solution, and the immobilized enzyme was assayed spectrophotometrically with *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BANA) (**6**) (eq 1).[40] The results of this assay process for the active

$$H_2N(C{=}NH)NH(CH_2)_3CH(NHCOC_6H_5)CONHC_6H_4NO_2$$

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$$+ H_2O \xrightarrow{\text{trypsin}} H_2NC_6H_4NO_2 + H_2N(C{=}NH)NH(CH_2)_3CH(NHCOC_6H_4)COOH \quad (1)$$

enzyme were supplemented by estimates of the total enzyme immobilized by use of a Lowry protein determination

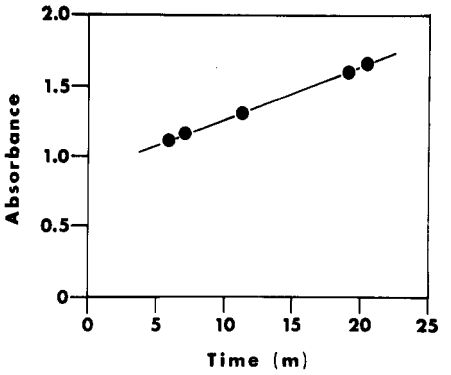


Figure 1. Plot of absorbance of NADPH at 340 nm as a function of reaction time.

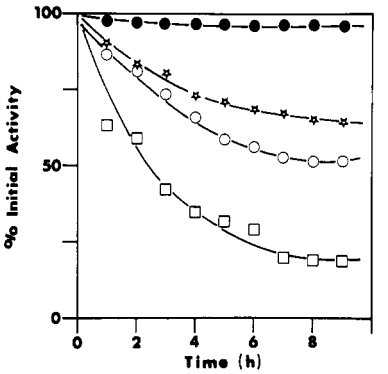


Figure 2. Enzyme activity as a function of time for G-6-PDH immobilized on porous alumina particles coated with different amounts of the activated polymeric support. Polymer to alumina weight ratio of 0.1:1 (○), 0.2:1 (☆), 0.3:1 (●), and 0.4:1 (□).

of the residual protein remaining in solution.[41]

In practical terms, the activity of the immobilized G-6-P was 38.5 U/g of conjugate, and the value for trypsin was 41.7 U/g.

Influence of Particle Topography and Surface Area. The surface area of the support system exposed to the liquid medium affects the amount of enzyme bound to the substrate and the total enzymic activity of the system.

The alumina particles used in the present work had a pore diameter of 100–300 Å. Treatment of these particles with increasing amounts of the carrier polymer (followed by derivatization and aldehyde activation) first increased the amount of G-6-PDH immobilized. Heavier coatings of the carrier polymer then brought about a reduction in the amount of bound enzyme as the polymer blocked the pores in the alumina and lowered the overall surface area (Figure 2). This phenomenon is illustrated by the scanning electron micrographs shown in Figure 3. These results allowed the design of an optimized support system. Details are given in the Experimental Section.

Effectiveness of Immobilization Process. In this system, three types of linkage between the enzyme and the solid support are possible: (1) covalent binding to the polyphosphazene, (2) van der Waals association with the polymer surface, and (3) dipolar or hydrogen-bonding attachment to alumina surfaces not covered by the polyphosphazene. Experiments were performed to test the relative importance of these three factors in this system.

First, an equal amount of G-6-PDH was immobilized on the aldehyde-activated aminophosphazene/alumina support and on the nonactivated polymeric support (**3**). As shown in Figure 4, the enzyme activity was essentially unchanged in the Schiff base covalently coupled system.

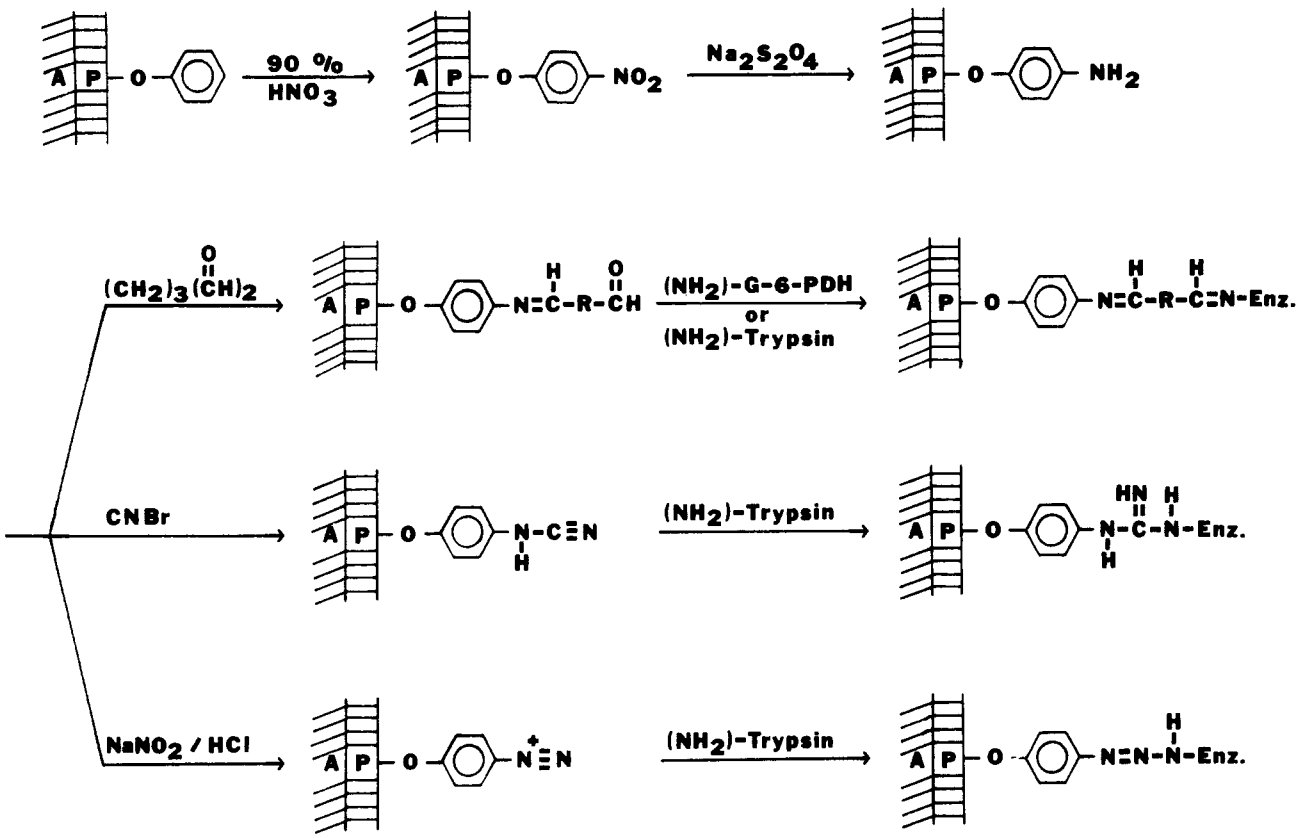
It was also found that the enzyme was leached from an alumina surface only partly covered by the poly-

Table I
Variation in the Yield of Immobilized Trypsin with Changes in Coupling Agent and Reaction Conditions[a]

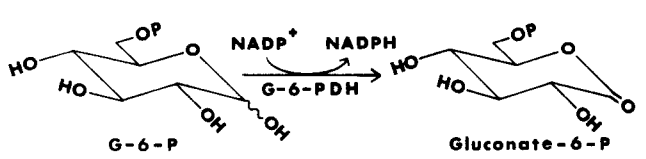
activation agent	activation pH	activation temp, °C	coupling pH	coupling time, h	immobilization yield,[e] %
Ald[b]	1.4	25	4.9	20	27
Ald[b]	1.4	25	6.8	20	34
Ald[b]	1.4	25	7.5	20	51
Ald[b]	1.4	25	8.1	20	29
Ald[b]	1.4	25	7.5	20	51
Ald[b]	3.4	25	7.5	20	35
Ald[b]	5.5	25	7.5	20	26
CNBr[c]	11.0	25	7.5	20	21
CNBr[c]	11.0	25	8.2	20	13
CNBr[c]	11.0	25	9.0	20	13
diazo[d]	0.1	0	7.5	2	21
diazo[d]	0.1	0	8.2	2	22

[a] The amount of trypsin used in each case was 12.5 mg, and the amount of alumina/aminophosphazene polymer was 1.3 g. [b] Glutaric dialdehyde. [c] Cyanogen bromide. [d] Diazonium salt coupling. [e] Determined by [immobilized enzyme activity (12.5 mg)]/[soluble enzyme activity (12.5 mg)].

Scheme I



Scheme II



phosphazene. The same result was obtained when no polyphosphazene was present (Figures 4 and 5). Thus, hydrogen-bonding or dipolar attachment to the enzyme is not an effective contributor to the immobilization process.

For trypsin immobilizatin, the glutaric dialdehyde method[42] proved to be more effective than the cyanogen bromide[43] or diazo-coupling techniques,[44] as shown in Table I. Thus, glutaric dialdehyde gave a 51% immobilization activity at pH 7.5, as measured by enzyme activity, and a roughly 60% immobilization yield, as measured by the Lowry assay.

Stability of the Immobilized Enzymes. Comparison samples of free G-6-PDH in solution and G-6-PDH immobilized on the optimized polyphosphazene/alumina support were stored at constant temperature (25 °C) in the dark, and their activities were determined as a function of time. As shown in Figure 6, most of the activity of the free G-6-PDH was lost after 260 h, presumably a consequence of denaturation of the protein. However, the immobilized enzyme retained a high degree of activity for at least 2150 h.

Similar behavior was found for the aldehyde-coupled trypsin system (Figure 7), where most of the activity of the free enzyme was lost after 72-h storage in the dark at

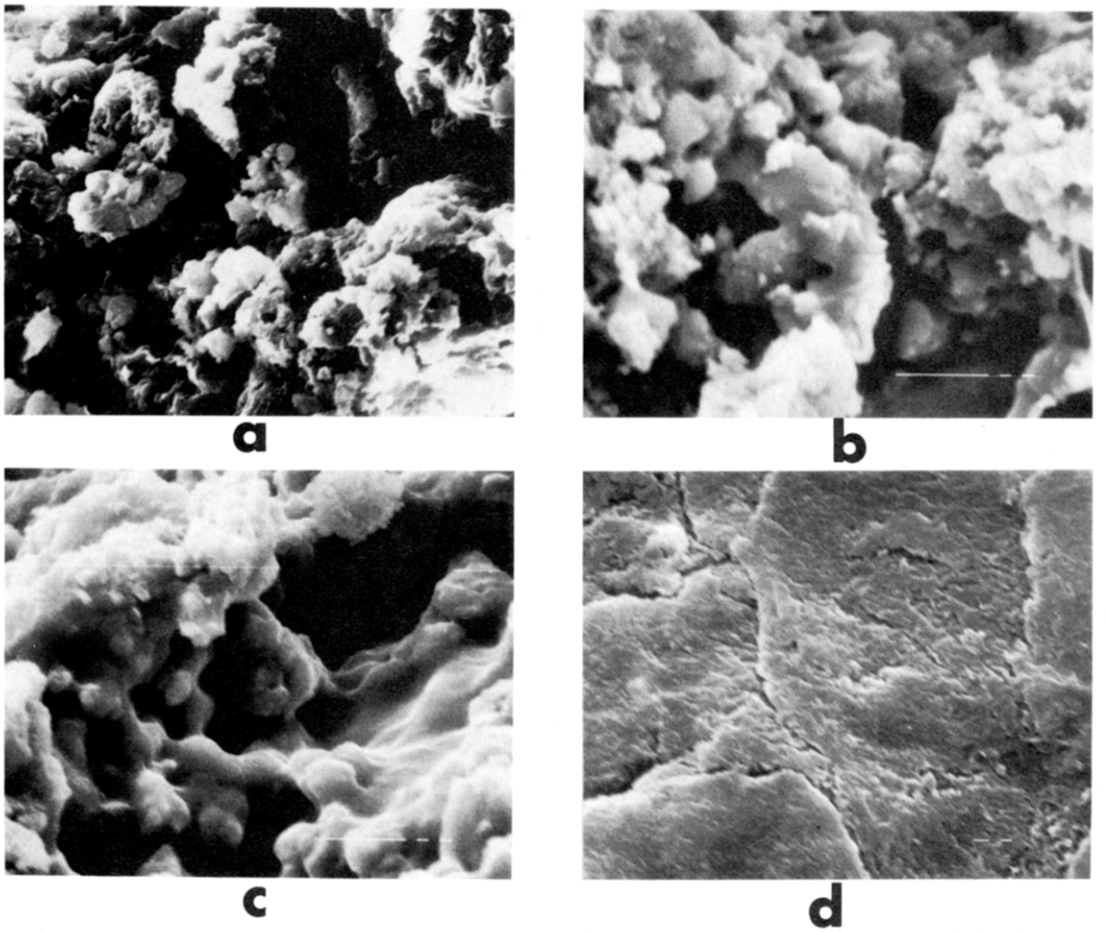


Figure 3. Scanning electron micrographs of porous alumina particles (~0.3-mm diameter) coated with increasing amounts of the carrier polymer. (a) Polymer to alumina weight ratio of 0.1:1 (1020× magnification), (b) 0.2:1 (1200×), (c) 0.3:1 (1200×), and (d) 0.4:1 (1800×). Micrographs a and b showed an incomplete coating of the polymer. Micrograph d revealed that, at this level of polymer coating, the pores were filled with polymer. System c had a complete coating of the alumina surface with retention of the pore structure. When derivatized and coupled to the enzyme, this system showed the highest enzyme activity and retention.

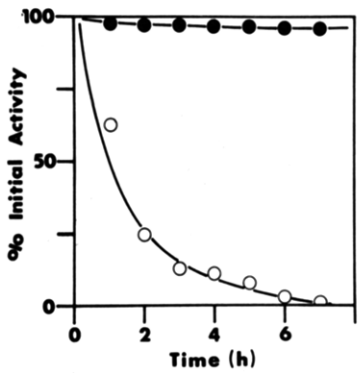


Figure 4. Enzyme activity as a function of time for G-6-PDH immobilized on the aldehyde-activated polymeric support (6) (●) and on the nonactivated support (3) (○).

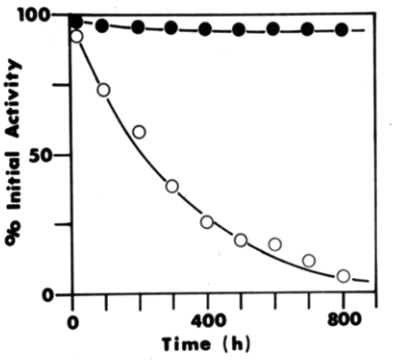


Figure 5. Enzyme activity as a function of time for G-6-PDH immobilized on the aldehyde-activated polymeric support (6) (●) and on the uncoated alumina (○).

25 °C, but the immobilized enzyme had retained more than 75% of its original activity after 960 h.

Behavior of Immobilized Trypsin in a Continuous-Flow Reactor System. One of the main objectives of enzyme immobilization work is to carry out enzyme-mediated reactions under conditions that are appropriate for continuous-flow processes, either in the laboratory or on a larger scale.[45] This prospect was explored with use of an apparatus illustrated schematically in Figure 8. Reactants in solution were passed at a constant flow rate through a column packed with alumina/polyphosphazene particles bearing glutaric dialdehyde linked trypsin. The concentration of product emerging from the catalytic zone was monitored from the electronic absorbance at 406 nm (from *p*-nitroaniline), as shown in Figure 9.

These data are consistent with the view that the immobilized trypsin maintains a constant activity as a function of time. Moreover, as shown in Figure 10, the absorbance at 406 nm, and, therefore, the continuous yield of products, varied in a linear manner with reagent flow rate, at least over the range of flow rates studied.

Experimental Section

Equipment. The ^{31}P NMR spectra were obtained in the Fourier transform mode at 32 MHz with a Varian CFT-20 spectrometer. Infrared spectra were recorded with a Perkin-Elmer Model 283B high-resolution infrared spectrometer. A Hewlett-

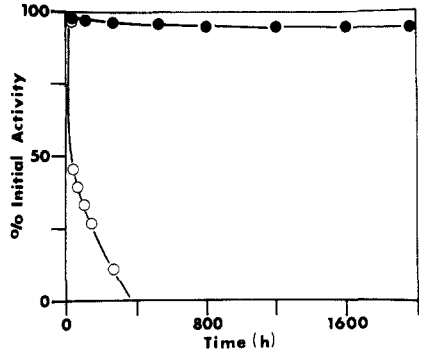


Figure 6. Plot of the activity as a function of time of immobilized G-6-PDH (●) and soluble G-6-PDH (○).

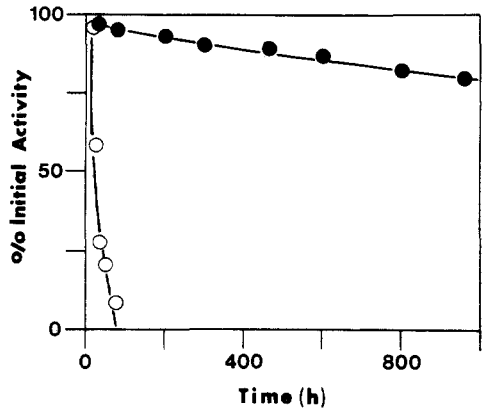


Figure 7. Plot of the activity of immobilized trypsin (●) and free trypsin (○) in solution as a function of time at 25 °C, showing the faster inactivation of the unbound enzyme.

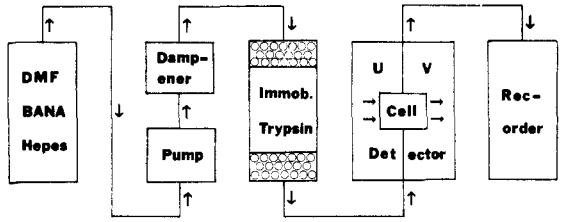


Figure 8. Schematic illustration of the continuous-flow reactor used to monitor the behavior of immobilized trypsin.

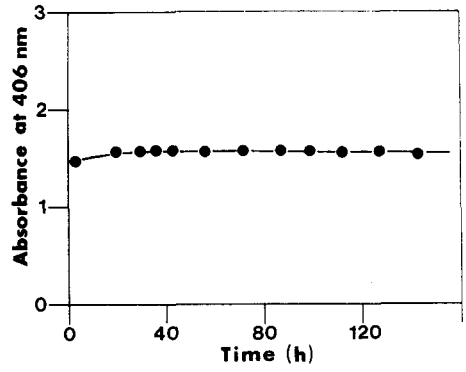


Figure 9. Immobilized trypsin activity in a continuous-flow system, as deduced by spectrophotometric analysis of *p*-nitroaniline released from *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BANA) as a function of time at a flow rate of 3.3 mL/min.

Packard Model 8450A UV/vis spectrophotometer was used for the UV spectra. An International Clinical Model 4583 centrifuge was used for the separations. The scanning electron micrographs were obtained with an International Scientific Instruments DS 60 electron microscope operated at an accelerating voltage of 30 kV.

Materials. Tetrahydrofuran (THF) and dioxane (VWR) were freshly distilled under nitrogen from sodium benzophenone ketyl.

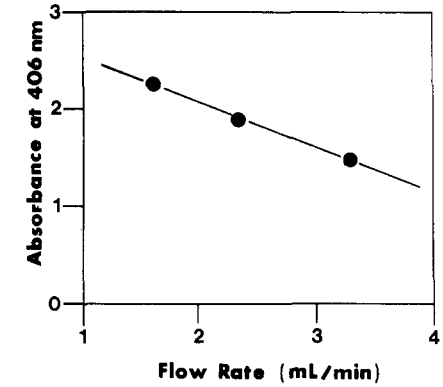


Figure 10. Immobilized trypsin activity at three different flow rates in a continuous-flow reactor, as measured by the release of *p*-nitroaniline.

Hexachlorocyclotriphosphazene (mp 110–112 °C) was obtained from a tetramer–trimer mixture (Ethyl Corp.) and was purified by two fractional vacuum sublimations at 60 °C (0.5 Torr), two recrystallizations from hexane, and two further vacuum sublimations. Poly(dichlorophosphazene) was prepared by the thermal ring-opening polymerization of hexachlorocyclotriphosphazene. δ-Alumina was obtained from Grace Chemical Co. and was dried at 450 °C under nitrogen. The commercial alumina beads ($^1/_8$-in. diameter) were crushed gently with a mortar and pestle, and 40–80 mesh size particles were collected by using sieves. The collected alumina was calcined at 450 °C under nitrogen (flow rate 1.5 ft^3/h) for 2.5 h. G-6-P, NADP, and G-6-PDH were obtained from Sigma, stored at 0 °C, and used after dilution with 0.3 M Hepes buffer (pH 7.5) (Sigma). Trypsin and BANA were obtained from Sigma and stored at 0 °C. Phenol (Aldrich), magnesium chloride (Sigma), sodium hydroxide (Fisher), potassium chloride (Aldrich), sodium hydride (Aldrich), DMF (Fisher), cyanogen bromide (Aldrich), sodium nitrite (Aldrich), potassium phosphate (Sigma), and sodium bicarbonate (Sigma) were used as received.

Preparation of Poly(diphenoxyphosphazene) (3). A solution of poly(dichlorophosphazene) (5 g, 0.04 mol) in dioxane (225 mL) was added during a 1-h period to a stirred solution of sodium phenoxide, prepared from sodium hydride (6.9 g, 0.17 mol) plus phenol (17 g, 0.172 mol), and tetra-*n*-butylammonium bromide (0.5 g, 0.015 mol) in dioxane (300 mL). The reaction mixture was heated for 8 h at 80 °C and then cooled to room temperature. The polymer solution was concentrated to 80 mL under reduced pressure, and the solution was added to water (600 mL) in order to precipitate the polymer. The precipitate was subsequently redissolved in THF (80 mL), precipitated into water a total of three times, and precipitated twice from THF into hexane. The polymer was then thoroughly extracted by Soxhlet techniques for 96 h using pentane. It was soluble in THF, dioxane, and benzene. A ^{31}P NMR spectrum of the polymer consisted of a sharp singlet at −19.33 ppm.

Coating and Derivatization Procedure for Polymer 3 on Alumina for G-6-PDH Binding. Polymer **3** (0.3 g, 0.0013 mol) was dissolved in dry THF. To the polymer solution was added 1 g of dry δ-alumina (40–80 mesh). The suspension was mixed as THF was removed slowly under vacuum. The IR spectrum of the polymer-coated support contained an aromatic stretch at 1500 and 1600 cm^{-1} and a P=N stretch at 1200 cm^{-1}.

Fuming nitric acid (50 mL) was added slowly at 0 °C to the polymer-coated alumina. The reaction mixture was stirred for 2 h. The nitrated support was then removed and washed with water (2 L). The IR spectrum indicated that the polymeric support had a nitro group stretching absorption at 1520 and 1345 cm^{-1} and a P=N stretch at 1200 cm^{-1}. The nitrated polymeric support was suspended in deionized water (100 mL), and sodium dithionite (2.5 g) was added to this mixture. The mixture was heated to boiling and maintained at this temperature for 1 h, after which time the resultant aminophenoxy polymer support was washed with water (2 L). The characteristic nitro group IR absorptions at 1520 and 1345 cm^{-1} had now disappeared, and an N–H bending peak appeared in the spectrum at 1610 cm^{-1}. The aminophenoxy polymer support was added to 50 mL of buffer (pH 1.4). To this solution was added 30 mL of glutaric dialdehyde

(25%, in water). Stirring was continued for a period of 1 h at room temperature. The excess glutaric dialdehyde was then decanted off, and the support material was washed with buffer solution (pH 7.5) and dried under reduced pressure. The IR spectrum of the activated polymeric support contained a C=O absorption at 1700 cm^{-1}, a C=N peak at 1620 cm^{-1}, and a P=N absorption at 1200 cm^{-1}. The activated support was stored under a nitrogen atmosphere.

Immobilization of Glucose-6-Phosphate Dehydrogenase (G-6-PDH). The final immobilized enzyme conjugate was prepared by treatment of the matrix with 500 units[46] of G-6-PDH. The immobilization medium was maintained at 0 °C by means of an ice bath during a period of 19 h. At the end of this period, the residual, unbound enzyme was removed by washing with Hepes buffer solution (pH 7.5). The immobilized enzyme was packed in a column or centrifuge tube and stored at room temperature in Hepes buffer solution (pH 7.5).

Kinetic Assay of the Immobilized G-6-PDH. Hepes buffer (pH 7.5) (300 mL) was passed through a column of the immobilized enzyme in order to remove noncovalently bound enzyme molecules. After 6 h, the reactants (2 mL of 0.1 M $MgCl_2$, 100 mg of NADP, 100 mg of G-6-P, and 8 mL of Hepes buffer) were passed through the column (flow rate 2.5 mL/5 m 55 s). The first 2.5-mL portion was collected and its absorbance was measured at 340 nm. After the column was washed with Hepes buffer, the same molar ratio of reactants was passed through the column at a different flow rate (2.5 mL/6 m 50 s). The UV spectrum of this product had an absorbance at 340 nm. The intensity of NADPH absorbance was checked at different flow rates (11 m 12 s, 19 m 7 s, 20 m 30 s per 2.5 mL).

Enzyme Assay of Immobilized G-6-PDH with Uncoated Alumina and Polymer-Coated Alumina. Equal amounts of G-6-PDH were immobilized separately with uncoated alumina and the aldehyde-activated, polymer-coated system. Both supports were poured into centrifuge tubes, washed with Hepes buffer (10 mL), and separately mixed and centrifuged. These washings were repeated five times to remove unbound enzyme. The reactants (10 mg of NADP, 10 mg of G-6-P, 1 mL of 0.1 M $MgCl_2$, and 4 mL of Hepes buffer) were added to both tubes. The contents were then mixed at room temperature for 15 s. After centrifugation, the UV spectrum of the solution was obtained. After the solids were washed with another 10 mL of buffer solution, the same reaction was carried out with the same molar ratio of reactants. The absorbance of NADPH at 340 nm was checked after 15 s. The same sequential assays were repeated over a period of 800 h.

Assay of the Immobilized G-6-PDH on the Four Different Coated Supports. A large-pore alumina (40–80 mesh) was used for coating. Four 1-g samples of alumina were treated with solutions of polymer **3**, 0.1 g, 0.2 g, 0.3 g, and 0.4 g in THF. The solvent was removed under reduced pressure and the particles were dried overnight under vacuum. The scanning electron micrographs of the four supports were obtained with the use of an ISI 60 electron microscope. The four polyphosphazene-coated alumina samples were derivatized and activated as described. Equal amounts of G-6-PDH (500 units) were immobilized on the four polymeric supports. These four systems were washed with Hepes buffer solution (pH 7.5) in centrifuge tubes. Then the reactants (10 mg of G-6-P, 10 mg of NADP, 1 mL of 0.1 M $MgCl_2$, and 4 mL of buffer) were added to the four immobilization systems. After 15 s, the absorbance at 340 nm was measured by means of UV spectroscopy. This series of reactions was repeated every hour and the absorbance was checked.

Stability Test of Immobilized and Soluble G-6-PDH Enzymes at Different Storage Times. Soluble G-6-PDH (500 units) was dissolved in 100 mL of Hepes buffer solution (pH 7.5). The G-6-PDH solution and the immobilized G-6-PDH on the polymeric support were stored together at constant temperature (25 °C) maintained by means of a temperature-controlled water bath. Assays of enzymes in solution were performed spectrophotometrically following the standard assay procedures described by Bergmeyer.[31] Immobilized G-6-PDH samples were assayed by similar procedures. The reactants (2 mL of 0.1 M $MgCl_2$, 100 mg of NADP, 100 mg of G-6-P, and 8 mL of Hepes buffer) were added to the immobilized G-6-PDH conjugate. The reaction mixture was shaken for 30 s. After centrifugation, the UV absorbance of the clear solution was measured at 340 nm. The polymeric conjugate was washed three times with Hepes buffer solution to remove remaining reactants. The same amounts of reactants were added and the mixture was shaken for 1 min. The absorbance was recorded again at 340 nm. This assay procedure was repeated for an extended reaction time. Essentially linear assay responses were obtained in all instances.

Trypsin Immobilization: (a) Via Glutaric Dialdehyde Coupling. Polymer **3** (9 g, 0.039 mol) was used to coat 30 g of dry δ-alumina (40–80 mesh) particles, as described earlier. Surface nitration was achieved with fuming nitric acid (1.5 L) and reduction was effected as before with sodium dithionite (75 g). The aminophenoxy polymer support (1.3 g) was added to 50 mL of buffer (pH 1.4, KCl/HCl; pH 3.40, 0.1 M KH_2PO_4/HCl; and pH 5.5, 0.1 M KH_2PO_4/NaOH). To this solution was added 30 mL of glutaric dialdehyde (25%, in water). Stirring was continued for 1 h at room temperature. The excess glutaric dialdehyde was then decanted off and the support material was washed with buffer solution (pH 7.5) and dried under reduced pressure. The IR spectrum of the activated polymeric support contained a C=O absorption at 1700 cm^{-1}, a C=N peak at 1620 cm^{-1}, and a P=N absorption at 1200 cm^{-1}.

(b) Via Cyanogen Bromide Activation. Surface-derivatized alumina/(aminophenoxy)phosphazene polymer **5** was added to 50 mL of 0.1 M phosphate buffer (pH 11). To this solution was added 1 g (9.44 mmol) of cyanogen bromide. The reaction mixture was stirred at room temperature for 30 min. The polymeric support was filtered off with a glass-sintered filter, washed with buffer solution (pH 11), and dried under vacuum. The IR spectrum of the activated polymeric support contained a C≡N stretch at 2210 cm^{-1}.

(c) Via Diazo Coupling. Hydrochloric acid (1 N, 50 mL) was cooled to 0 °C with mechanical stirring. To this solution was added the particulate aminophosphazene. Sodium nitrite (0.4 g, 0.05 M) was slowly added at 0 °C by means of an ice bath. After 30 min, the polymeric support was collected on a filter and washed with 400 mL of deionized water.

Immobilization of Trypsin. The final immobilized enzyme conjugate was prepared by treatment of the matrix with 12.5 mg of trypsin. The immobilization reaction with the aldehyde or cyanogen bromide activated support was maintained at 0 °C by means of an ice bath during a period of 20 h. Immobilization of trypsin on the diazo activated support was carried out at 0 °C for 2 h. After the immobilization reaction, the residual, unbound enzyme was removed by washing with 0.1 M phosphate buffer solution (pH 7.5). The immobilized enzyme systems were packed in a column or centrifuge tube and stored at 25 °C in phosphate buffer solution (pH 7.5).

Lowry Protein Measurements. Sodium carbonate (2% in 50 mL of 0.1 N NaOH) and cupric sulfate pentahydrate (0.5% in 1 mL of 1% sodium tartrate) were mixed well. To 2 mL of this reagent were added various amounts of trypsin (0.1, 0.02, 0.03, 0.05, and 0.08 mg) dissolved in 0.1 M phosphate buffer (pH 7.5). After 10 min, 0.2 mL of 1 N Folin phenol reagent solution was added, and the system was allowed to stand for 30 min. The absorbances at 500 nm were followed by means of a UV/vis spectrometer. An almost linear plot was obtained. From this standard line, it was possible to estimate the amount of trypsin removed by washing with the buffer. These washings and measurements were repeated several times. Finally, the amount of immobilized trypsin remaining on the polymeric support could be estimated by difference.

Kinetic Assay through Continuous-Flow System. The immobilized trypsin on the polymeric support (0.13 g) was packed on an Adjusta-Chrom column (Ace, length 300 mm, inside diameter 10 mm). Phosphate buffer (pH 7.5) (200 mL) was passed through the column in order to remove unbound enzyme molecules. The reactants (250 mL of 0.1 M phosphate buffer, 75 mL of DMA, and 575 mg of BANA) were passed through the column (flow rate: 1.7, 2.3, and 3.3 mL/min). The absorbance of *p*-nitroaniline at 406 nm was measured in the effluent from the column.

Stability Test of Immobilized and Soluble Trypsin. Free trypsin (250 mg) was dissolved in 100 mL of 0.1 M phosphate buffer solution (pH 7.5). The trypsin solution and the immobilized trypsin on the polymeric support were stored at constant tem-

perature (25 °C) by means of a temperature-controlled water bath. The assay of trypsin was performed spectrophotometrically following the standard assay procedure described by Glassmeyer.[40] The reactants (15.4 mL of 0.1 M phosphate buffer, 4.6 mL of DMF, and 38.5 mg of BANA) were added to the trypsin. The reaction mixture was shaken for 30 s, and the UV absorbance of the clear solution was measured at 406 nm. The polymeric conjugate was washed three times with phosphate buffer solution to remove remaining reactants. The same amount of reactants was added and the mixture was shaken for 1 min. The absorbance was recorded again at 406 nm. This assay procedure was repeated for an extended reaction time. An almost linear assay response was obtained in all instances.

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Registry No. G-6-PDH, 9001-40-5; BrCN, 506-68-3; HNO_2, 7782-77-6; NaOPh, 139-02-6; NaH, 7646-69-7; PhOH, 108-95-2; NBu_4Br, 1643-19-2; poly[bis(*p*-aminophenoxy)phosphazene], 101760-93-4; poly(diphenoxyphosphazene), 28212-48-8; glutaric dialdehyde, 111-30-8; trypsin, 9002-07-7; poly(dichlorophosphazene), 26085-02-9.

Supplementary Material Available: Spectra of glucose-6-phosphate plus NADP (Figure 11a), gluconate-6-phosphate plus NADPH (Figure 11b), BANA (Figure 12a), and *p*-nitroaniline (Figure 12b) (2 pages). Ordering information is given on any current masthead page.

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(46) One unit will oxidize 1.0 μmol of G-6-P to 6-phosphogluconate per min at pH 7.8 at 30 °C, using NADP as a coenzyme.

Polyphosphazenes with Etheric Side Groups: Prospective Biomedical and Solid Electrolyte Polymers

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ABSTRACT: Poly(organophosphazenes) have been synthesized with alkyl ether alkoxy side groups attached to the phosphorus atoms of the skeleton. These species are water-stable and either water-soluble or hydrophilic polymers. Specific members of this series form complexes with metal salts, which are excellent solid electrolyte materials. Mixed substituent polymers with hydrophobic trifluoroethoxy and alkyl ether alkoxy side groups have also been prepared, and these are of interest as membranes and biomedical materials.

One of the main characteristics of the poly(organophosphazene) system is the ease with which different organic side groups can be incorporated into the macromolecular structure. This is a consequence of the substitutive mode of synthesis used for these polymers, as described in a number of earlier publications.[1-17] The method involves the prior synthesis of a reactive, high polymeric

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