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USE OF SILANE MONOMERS FOR MOLECULAR IMPRINTING AND ENZYME ENTRAPMENT IN POLYSILOXANE-COATED POROUS SILICA

MAGNUS GLAD, OLOF NORRLÖW, BÖRJE SELLERGREN, NILS SIEGBAHN and KLAUS MOSBACH*

Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)

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SUMMARY

The use of organic silane monomers in the preparation of substrate-selective polymers by molecular imprinting is described. Silanes are allowed to polymerize on the surface of porous silica particles in aqueous solution. The resulting polysiloxane copolymer becomes covalently anchored to silanol groups of the original silica. Such preparations retain the rigidity of the silica matrix and can therefore be used in high-performance liquid chromatography. Polysiloxane copolymers imprinted with the dyes rhodanile blue or safranine O showed preferential binding of the respective compound. The observed recognition is believed to occur because cavities containing specific binding groups for the dyes at defined positions are developed during the polymerization procedure. In this context the synthesis of a new silane, boronate–silane, was carried out. This compound was included in the monomer mixture used for the preparation of a polysiloxane-coated silica showing affinity for the glycoprotein transferrin. Organic silanes were also used for entrapment of enzymes, resulting in block polymers, which after fragmentation yielded relatively high recoveries of enzyme activity. Alternatively, the entrapment/polymerization was allowed to proceed on the surface of porous silica, in analogy with the imprinting procedure, resulting in entrapped enzyme preparations with high mechanical stability.

INTRODUCTION

Increasing interest has recently been shown in the use of molecular imprinting for preparation of substrate-selective polymers having a memory for a substrate around which the polymer was formed. We have previously reported[1] on the use of acrylic monomers for the preparation of block polymers showing selectivity for different substrates (print molecules) which were present during the polymerization. These preparations were subsequently used in low-pressure chromatographic systems[2,21]. It was also demonstrated that the analysis time could be decreased if an acrylic polymer shell is allowed to form on the surface of macroporous micro-particulate silica, permitting its use as a packing material in high-performance liquid chromatography (HPLC).

Macroporous polymers based on styrene–divinylbenzene as well as acrylates have also been successfully used for molecular imprinting[3,4]. Recently such polymers have proven useful for the separation of racemic amino acid derivatives in batch procedures[5,6] and in HPLC systems[7].

The principle of our strategy is to allow substrate and specific monomers to interact non-covalently in solution, prearrange, prior to polymerization[1]. This considerably simplifies the imprinting procedure by obviating, for instance, the need for derivatization of the substrate with vinyl groups and the subsequent requirement for suitable dissociation conditions. On the other hand, to obtain as good recognition as possible it is preferable to use monomers with different functional groups. This facilitates a more complete prearrangement, in which several domains of the substrate (print molecule) participate.

In this report we present data on a new class of monomers applicable for imprinting, namely organic silanes, allowing the preparation of substrate-selective siloxane copolymers coated on microparticulate porous silica. These compounds were chosen because a number of organic silanes with a variety of properties are commercially available. Furthermore, since silanes spontaneously form siloxane polymers in aqueous solutions[8], they may be potentially useful for molecular imprinting of normally water-soluble biomolecules.

The organic silanes were allowed to prearrange around different dye substrates in an aqueous solution in the presence of porous silica particles. A covalently bound polysiloxane layer was spontaneously formed on the surface (including the surface in the pores) of the rigid porous (1000 Å) silica particles. After removal of substrate by washing, the preparations showed selectivity for the substrate. These preparations were subsequently analyzed by HPLC.

An attempt to use silanes for molecular imprinting of the glycoprotein transferrin was also made. For this purpose we synthesized a boronate–silane that was included in the monomer mixture used for imprinting. The boronate group interacts reversibly via ester bonds with carbohydrates[9] and glycoproteins[10] and a preparation with increased affinity for transferrin compared to polymers with randomly distributed boronate ligands was obtained. In this context it should be mentioned that vinyl derivatives of boronate have been studied in a number of other systems to prepare enantioselective polymers for the resolution of carbohydrates (see e.g. ref. 3 and other reports in that series).

Another potential application is the use of organic silanes for entrapment of biomolecules such as enzymes. By use of an appropriate silane mixture, including the cross-linking agent tetraethoxysilane, a number of enzymes have been successfully immobilized by entrapment in siloxane polymers with retained catalytic activities of 10–40%. Two approaches have been followed: (a) the silanes were mixed with an aqueous solution of the enzyme to be entrapped, resulting in a block polymer showing high enzymatic activity; (b) the entrapment within silanes was allowed to proceed on the surface (and in the pores) of porous (100 Å) silica particles, giving preparations with better mechanical properties but with less retained enzymic activity. These preparations were subsequently packed in small columns and used as small scale bioreactors.

MATERIALS AND METHODS

Materials

Porous silica (LiChrospher Si 1000, 10 μm; LiChroprep Si 100, 40–63 μm; LiChroprep Si 60, 40–63 μm; Kieselgel 60, 63–200 μm), thin-layer chromatographic (TLC) plates (Kieselgel 60 F254, precoated), hydrogen peroxide, phenol and D-glucose were obtained from Merck (Darmstadt, F.R.G.). Rhodanile blue and 3-aminobenzeneboronic acid (hemisulphate) were from Aldrich-Europe (Beerse, Belgium), safranine O from BDH (Poole, U.K.). Phenyltriethoxysilane and 4-toluenesulphonic acid were from Fluka (Buchs, Switzerland), and bis(2-hydroxyethyl)aminopropyltriethoxysilane (62% in ethanol), dodecyltriethoxysilane and N-2-aminoethyl-3-aminopropyltrimethoxysilane from Petrarch Systems (Bristol, PA, U.S.A.). 3-Glycidoxypropyltrimethoxysilane (Z-6040) was obtained from Dow Corning (Midland, MA, U.S.A.). Glucose oxidase (Type V, about 200 units per mg), bovine serum albumin (BSA), lactoferrin (human milk) and 4-aminoantipyrine were from Sigma (St. Louis, MO, U.S.A.) and peroxidase (horse radish, grade II, about 100 units per mg) from Boehringer (Mannheim, F.R.G.). Human transferrin was a gift from Kabi (Stockholm, Sweden). All other chemicals were of analytical grade and used as supplied.

The HPLC experiments were performed with a Model 110A pump and a Model 210 injector supplied with a 20-μl loop, all from Altex (Berkeley, CA, U.S.A.), and a Spectromonitor II UV/vis detector (Laboratory Data Control, Riviera Beach FL, U.S.A.).

Enzyme activities were measured with a Zeiss MQ3 spectrophotometer equipped with a magnetic stirring device (Carl Zeiss, Oberkochen, F.R.G.).

Synthesis of boronate–silane, N-{2-hydroxy-3-[3-(tripropoxysilyl)propoxy]propyl}-3-aminobenzeneboronic acid propyl ester

The hemisulphate of 3-aminobenzeneboronic acid (ABBA) was converted into the free base according to the method of Weith *et al.*[11]. An aqueous solution (250 ml) containing 10 g of the salt was brought to pH 7 with 5 *M* sodium hydroxide and then evaporated to dryness. The residue was extracted with dioxane and the extracts were filtered and evaporated to dryness. After recrystallization from water, 4.6 g (62%) of product were obtained.

The boronate moiety of ABBA was first protected with *n*-propanol. A solution of the free base of ABBA (2.0 g, 14.6 mmol) in 250 ml of *n*-propanol was evaporated to near dryness under a stream of nitrogen. The residue was dissolved in dry *n*-propanol (50 ml) and then 3-glycidoxypropyltrimethoxysilane (epoxysilane; 3.5 g, 14.6 mmol) in dry *n*-propanol (50 ml) was slowly added. The reaction was performed under a nitrogen atmosphere with 4-toluenesulphonic acid (PTS; 25 mg, 0.13 mmol) as catalyst for 20 h at 50°C. The product mixture was analyzed by TLC using methanol–chloroform (1:9) as eluent; development was with ninhydrin and iodine and UV detection was employed. One major (R_F 0.56) and one minor (R_F 0.75) product were found together with unreacted ABBA (R_F 0.38) and epoxysilane (R_F 0.92). The ninhydrin test indicated a primary amine only for ABBA. The product mixture was evaporated to dryness and then dissolved, followed by evaporation to dryness, twice in toluene and twice in methylene chloride in order to remove excess of *n*-propanol. Each time one fourth of the oily residue was dissolved in a small

amount of methanol–chloroform (1:9) and applied to a column (22 × 2 cm I.D.) packed with 25 g of silica gel (Kieselgel 60), and then eluted with methanol–chloroform (1:9) at a flow-rate of 0.1 ml/min. Fractions of 1 ml were collected. Those containing the two products were pooled and evaporated to dryness, giving a slightly greenish crystalline product (3.85 g, 49%) showing two spots on TLC (R_F 0.56 and 0.75, the former one being in excess) and showing no reaction with ninhydrin.

An aniline–silane analogue used as a reference compound was synthesized by an identical procedure. The boronate–silane was characterized by 60-MHz ^{1}H NMR analysis (20%, w/w in deuterochloroform): δ 0.6 (17H, m); 1.7 (11H, m); 3.5 (19H, m); 7.0 (5H, m).

In order to verify the properties of the boronate–silane it was used for coupling to silica, which was subsequently exposed to a binding study with catechol. The aniline–silane was similarly tested. Silica (100 mg, Kieselgel 60) was added to an aqueous solution (pH 2) of boronate–silane (10 mg). The coupling was allowed to proceed for 17 h at 85°C. After filtration and washing with water and acetone on a glass filter, a pink boronate–silica and a brown aniline–silica were obtained. Catechol (0.1 equivalent of the amount of silane used for coupling) in 5 ml of 10 mM sodium phosphate pH 7.5 was incubated for 15 min with 100 mg of coated silica. After centrifugation, the absorbance of the supernatant was measured at 276 nm.

Molecular imprinting with silanes

A solution of dye substrate (0.1 mmol) dissolved in 5 ml of distilled water was thoroughly mixed with 2.0 g of silica (LiChrospher Si 1000). The silica slurry was then mixed well with 70 μl of phenyltriethoxysilane (0.30 mmol), 100 μl of N-2-aminoethyl-3-aminopropyltrimethoxysilane (0.45 mmol) and 300 μl of bis(2-hydroxyethyl)aminopropyltriethoxysilane (0.60 mmol). The polymerization was performed at 85°C for 4 h and the pH was occasionally adjusted to between 2.5 and 3.0 with 3 M sodium hydroxide. The product was thoroughly washed with distilled water, acetone and finally with water on a glass filter. A blank polymer was prepared in the same way except that no substrate was added.

The boronate–silane was used for molecular imprinting with proteins as substrate. First, the silane was pretreated in the following way to hydrolyze its propyl esters. Boronate–silane (14 mg) in 3.5 ml of methanol containing 100 μl of 3 M hydrochloric acid was heated at 80°C for 30 min and the pH was then adjusted to 7 with 3 M sodium hydroxide. A solution of the protein substrate (25 mg) in 4 ml of distilled water was then thoroughly mixed with 1.0 ml of the above boronate–silane solution (containing 7.0 μmol of silane) followed, after 5 min, by 15 μl of dodecyltriethoxysilane (40 μmol), 15 μl of phenyltriethoxysilane (62 μmol), 50 μl of N-2-aminoethyl-3-aminopropyltrimethoxysilane (227 μmol) and 150 μl of bis(2-hydroxyethyl)aminopropyltriethoxysilane (62% in ethanol, 276 μmol). Finally, 1.0 g of silica (LiChrospher Si 1000) was added, and after thorough mixing the pH was adjusted to 7.0. The imprinting procedure was performed at 4°C for 48 h with occasional adjustment to pH 7.0. The product was washed by mixing in a test-tube on an end-over-end rocking table, followed by sedimentation, with 3 × 30 ml of distilled water, 2 × 30 ml of 0.2 M sodium acetate pH 4.0 containing 10% (v/v) methanol, 30 ml of 0.1 M sodium phosphate pH 7.0 and 3 × 30 ml of distilled water.

The polysiloxane-coated silicas were suspended in a 50% sucrose–water solu-

tion and packed[12] in stainless-steel HPLC columns at 14 MPa (2000 p.s.i.). The dye-printed polymers (column: 5 × 0.5 cm I.D.) were analyzed at 22°C with a flow-rate of 1.0 ml/min. Sulphuric acid (1 mM) adjusted to pH 2.5 with 5 M sodium hydroxide was used as eluent. Samples of 20 μl (1 mg/ml) were injected one at a time, and dye elution was detected from the absorbance at 280 nm. For the protein-printed polymers (column 5 × 0.3 cm I.D., void volume 0.35 ml), 0.1 M sodium phosphate pH 7.0 (22°C) was chosen as the eluent with a flow-rate of 0.5 ml/min. Again the absorbance at 280 nm was used for detection. Samples of 20 μl (1 mg/ml) were injected one at a time.

Enzyme entrapment with silanes

A solution of glucose oxidase (100 μl, 1 mg/ml) in 0.1 M sodium phosphate pH 7.0 (buffer A) was added to a mixture of 300 μl of bis(2-hydroxyethyl)aminopropyltriethoxysilane and 100 μl of tetraethoxysilane at 4°C. After careful mixing, polymerization was allowed to proceed without further mixing for 17 h at 4°C. The polymer was then transferred to a glass filter and fragmented using a glass rod while washing thoroughly with 20 ml each of water, buffer A, 0.5 M sodium chloride in buffer A and finally with buffer A, all at 4°C. The pooled washings were collected for subsequent assay of enzyme activity (see below). The yield of moist polymer was typically 90–100 mg.

Enzyme entrapment with silanes on silica

Silica (0.4 g, LiChroprep Si 100, 40–63 μm) was thoroughly mixed with 750 μl of bis(2-hydroxyethyl)aminopropyltriethoxysilane and 250 μl of tetraethoxysilane at 4°C. A solution of glucose oxidase (250 μl, 1 mg/ml) in 0.1 M sodium phosphate pH 7.0 (buffer A) was then added and carefully mixed with the silica. The mixture was allowed to stand at 4°C for 17 h and then transferred to a glass filter and carefully washed with 60 ml each of water, buffer A, 0.5 M sodium chloride in buffer A and finally buffer A. The pooled washings were collected for subsequent assay of enzyme activity (see below). The yield of moist polymer was typically 0.9–1.0 g.

Enzyme activity

Glucose oxidase was assayed photometrically at 510 nm as described by Gierow and Jergil[13]. Glucose oxidase, in solution (about 5 μl) or in immobilized form (5–10 mg), was added to a mixture of 300 μl of 4 mM 4-aminoantipyrine in 0.1 M phenol and 2.5 ml of 0.1 M sodium phosphate pH 6.8 containing an excess of peroxidase (further addition of peroxidase gave no increase in activity). The polymer suspension had to be stirred magnetically for 20–30 min in order to reach an almost constant absorbance at 510 nm, since grinding of the polymer initially caused an apparent increase in absorbance. This grinding effect was more pronounced for the polysiloxane preparations without silica than with silica. The enzyme assay was initiated by the addition of 0.3 ml of 18% (w/w) D-glucose in 0.1 M sodium phosphate pH 6.8, and the increase in absorbance at 510 nm was recorded.

Peroxidase was assayed in the following way. The enzyme, in solution (about 5 μl) or in immobilized form (5–10 mg), was added to 3 ml of a solution containing 7 mM phenol and 0.5 mM 4-aminoantipyrine in 0.1 M sodium phosphate pH 6.8. After stirring magnetically for 20–30 min in order to reach a constant absorbance at

510 nm (see above), 30 μl of 100 mM hydrogen peroxide in 0.1 M sodium phosphate pH 6.8 were added and the increase in absorbance at 510 nm was recorded.

Column experiments with entrapped enzyme

The small scale bioreactors consisted of 0.5 g of silica-immobilized glucose oxidase packed in a disposable polystyrene minicolumn (13 × 8 mm I.D.) from Pierce Chemical (Rockford, IL, U.S.A.). After equilibration with buffer (0.1 M sodium phosphate pH 6.8), 1 ml of the reagent mixture used for glucose oxidase assay was applied.

RESULTS AND DISCUSSION

Synthesis of boronate–silane

The product mixture obtained after treating epoxysilane with propyl-protected 3-aminobenzeneboronic acid consists of two isomers as indicated by TLC analysis, which result from substitution at carbon 8 or 9 (see Scheme I). Substitution at carbon 9 is predominant, but a mixture of the two isomers is always obtained. The isomers could not be separated by the silica column chromatography, neither could they be distinguished by 60-MHz NMR spectroscopy. However, the molecular imprinting properties of the isomers should be very similar. Comparison of the NMR spectra of the product and the reactants leads to the following conclusions: (a) the signals from the protons at carbons 8 (3.0 ppm) and 9 (2.6 ppm) were shifted into the ether proton region at 3.5 ppm in the product, indicating that the epoxy ring had been opened[14]; (b) the presence of the ABBA backbone in the product is demonstrated by the characteristic signals of the aromatic protons (7.0 ppm) at carbons 10–13; (c) the presence of the silane backbone in the product is demonstrated by the slightly shifted signals of the methylene protons (0.6 ppm) at carbon 4; (d) the integrals are consistent with the expected values.
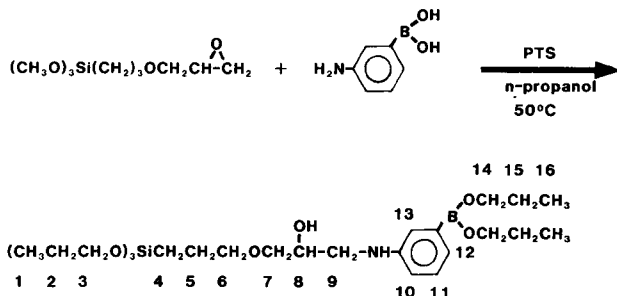
Scheme I. Preparation of boronate–silane. The boronate propyl ester is hydrolyzed prior to use in molecular imprinting.

In order to confirm that the boronate–silane could couple to silica via its silane moiety and that it also showed typical boronate interactions with substances containing vicinal cis-diols such as catechols[15], boronate–silica and aniline–silica (as reference) were prepared using the corresponding silane derivatives. These silica preparations were tested in a batch procedure for their capacity to bind catechol. Whereas boronate–silica under the conditions used bound 2.1 μmol catechol per gram silica

(12% of added catechol), aniline–silica did not bind any catechol. In comparison, boronate–silica prepared by coupling ABBA to epoxysilica[9], under the same conditions bound 7.3 μmol catechol per gram. Thus the product couples to silica and the boronate function is intact as it binds catechol.

Molecular imprinting with silanes

Many different types of monomers have been used for the preparation of substrate-selective polymers by molecular imprinting. Among them are methacrylates and styrene together with a suitable cross-linking agent such as phenylenediacrylamide or divinylbenzene respectively. Due to the low solubility of the monomers and in order to obtain macroporous polymers, high proportions of organic solvents have usually been employed in these preparations. In our search for new types of monomers applicable to molecular imprinting of water-soluble substrates such as biomolecules we encountered the organic silanes. These monomers have some advantageous features. First, a plethora of organosilanes with different functional groups is available. This is of great value in molecular imprinting when no covalent bond between the substrate and monomer is to be utilized. Instead, different types of non-covalent interactions, *e.g.*, ionic, hydrophobic and hydrogen bonds, are employed. These interactions are relatively weak and to obtain imprints with high selectivity in the formed polymer it is necessary to use a mixture of different monomers that can interact cooperatively with different parts of the substrate molecule. Secondly, the silanes can be used in aqueous solutions in which they spontaneously hydrolyze to silanols and then polymerize to organic polysiloxanes[8]. This water-catalyzed polymerization has also been utilized for coating of microparticulate silica used in HPLC[16] and affinity HPLC (HPLAC)[17].

We have earlier shown that methacrylate polymers with substrate selectivity can be prepared by molecular imprinting on the surface of porous silica[2,21]. The use of a "soft" polymer in combination with the highly rigid structure of silica makes these preparations well suited for use in HPLC. The advantages of high flow-rates, accurate and low pulse pumping systems and sensitive detectors with small dead volumes can thus be fully utilized. Furthermore, the small particle size of the packing material should result in a considerable decrease in diffusion times. However, we have found these systems to be relatively inefficient (100–300 theoretical plates per m), probably due to slow equilibrium[3]. No increase in back pressure due to compression or breakage of the polymer layer was noted with these composite preparations.

Fig. 1 illustrates schematically the formation of substrate-selective polysiloxane-coated silica particles during molecular imprinting. Different types of monomers (organosilanes) are mixed with the substrate and non-covalent interactions are allowed to occur. The aqueous milieu initiates formation of a polysiloxane copolymer while the interacting functional groups of the monomers are held around the substrate. The polymer obtained will be anchored to the surface of the silica as depicted in Fig. 1. After polymerization the substrate molecules are removed by a simple washing procedure, leaving cavities in the polymer capable of interacting selectively with readded substrate. The polymer layer has been found to have an average thickness of three to five monomers (elemental analysis), thick enough to contain cavities with selectivity for small molecules but thin enough to avoid long diffusion times.
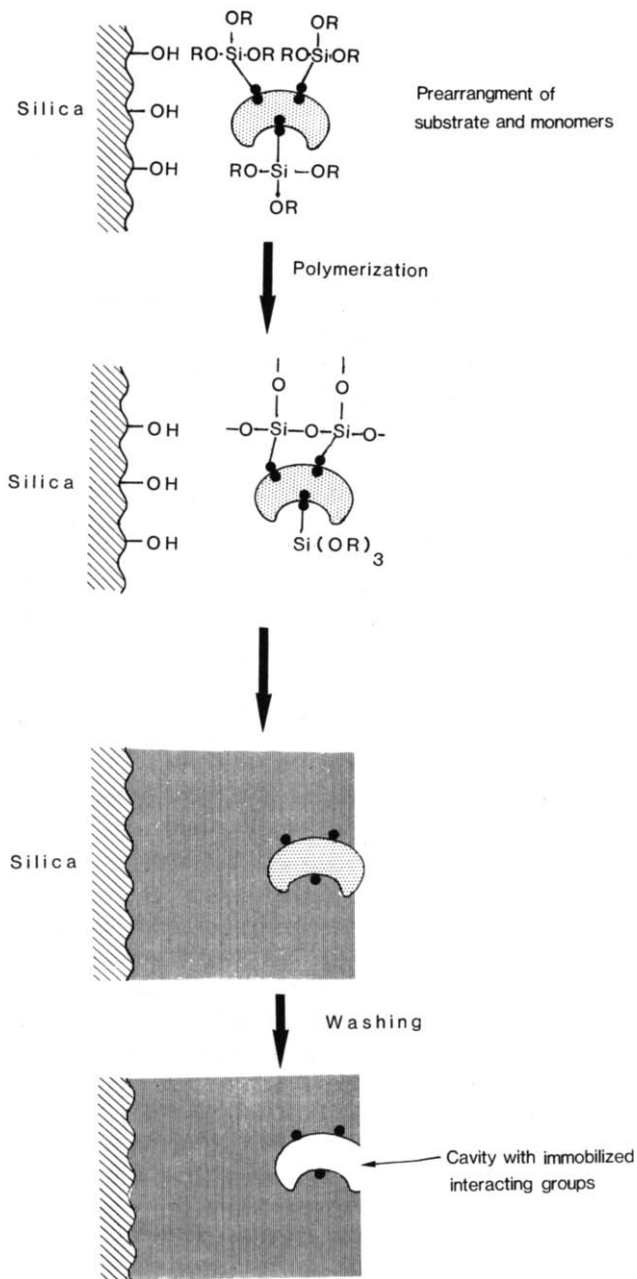
Fig. 1. Preparation of substrate-selective siloxane polymers coated on silica by molecular imprinting with dye substrates. The symbol (●) represents interacting units, *e.g.*, hydrophobic, electrostatic, etc., of monomers and substrate. Silane monomers with phenyl and amino groups were used in this study.

TABLE I

SELECTIVITY FACTORS OBTAINED BY MOLECULAR IMPRINTING WITH SILANES ON POROUS SILICA

Selectivity factor, $R = [r_1/r_1 \text{ (blank)}]/[r_2/r_2 \text{ (blank)}]$ where r_1 = retention volume for applied substrate, r_2 = retention volume for printed substrate and r (blank) = retention volume on blank polymer.

Substrate	Selectivity factor, R	
	Rhodanile blue-printed polymer	Safranine O-printed polymer
Rhodanile blue	1.00	0.81
Safranine O	0.83	1.00

The polysiloxane preparations obtained by molecular imprinting with different dyes as substrates were found to exhibit a memory for their respective dye substrate, as shown by chromatography. Table I shows the selectivity factors, R, for two siloxane polymer preparations obtained using either rhodanile blue or safranine O as substrate (Fig. 2). A blank polymer was also prepared with no substrate present during polymerization. The selectivity factors were determined from the elution volumes according to the equation given in Table I. By definition, $R = 1.0$ for the substrate (print molecule) which was present during polymerization. R values less than 1.0 indicate a lower affinity for the substance tested than for the substrate.

The substrate selectivity found with these polysiloxane preparations is of the same magnitude as obtained with methacrylate polymers[2,21]. The selectivity is suggested to be due to the appropriate orientation of interacting units in the cavity formed during polymerization. The size of the cavity is also likely to play an important rôle. Since the polysiloxane layer on the silica surface is thin it can be assumed that all cavities are accessible to the substrate with a minimum diffusion barrier. Furthermore, as no additional cross-linking agent such as tetraethoxysilane has been used in the imprinting procedure, few cavities with irreversibly bound (entrapped) substrate should remain in the polymer after the washing procedure, as indicated by the high yield of substrate (85–100%) obtained in the washings after polymerization.
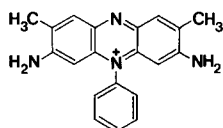
Safranine O
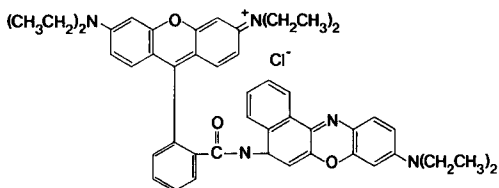
Rhodanile blue

Fig. 2. Structure of the dye substrates used for molecular imprinting.

In our attempts to increase applicability of molecular imprinting to biomolecules such as proteins, the above water-soluble silane monomers were tested. We have chosen as test substance the glycoprotein transferrin, containing two identical carbohydrate moieties each with two branches ending with sialic acid[18]. Since boronate groups are known to interact covalently with glycoproteins containing a high content of silaic acid such as fetuin[10], we thought that prearrangement of the described boronate–silane (in addition to non-covalently interacting silanes) around the transferrin would render a strong complex, and that the subsequently formed polymer would show a high affinity for the print molecule transferrin.

In the imprinting procedure the protein substrate was allowed to equilibrate with the boronate–silane prior to further addition of organic silanes. A number of preparations were made with different proteins and different silane compositions. The results obtained in column experiments with separate injection (20 μl, 1 mg/ml) of protein samples are summarized in Table II. The relative retention was obtained by dividing the elution volume for transferrin by the elution volume for bovine serum albumin (BSA). BSA was also used as a non-glycoprotein reference print molecule. Blank polymers, prepared with no protein present during polymerization, were also tested. It was found that the polymer prepared with transferrin (MW 90 000 dalton), having been allowed to prearrange with a silane mixture including the boronate–silane, showed a higher affinity for transferrin in relation to BSA (MW 68 000 dalton) than the blank polymer prepared with the same monomer mixture but with no protein present. When boronate–silane was omitted from the silane mixture the effect was much less pronounced. On the other hand, the polymers obtained by imprinting with BSA, with or without boronate–silane but without transferrin showed binding properties identical to those of the blank polymer, i.e., no selectivity for BSA was obtained. A minor increase in the affinity for transferrin (relative retention 1.22 compared to 1.16) was noted with all preparations obtained with boronate–silane, which is to be expected since even randomly distributed boronate groups in the polymers should be able to interact with the carbohydrate moiety of transferrin.

Considering the size of the proteins and the thickness of the polymer coating, it is reasonable to suppose, that open cavities with appropriately located boronate ligands are more likely to be formed than deep cavities surrounding the whole print

TABLE II

RELATIVE RETENTION OF TRANSFERRIN/BSA ON POLYMERS OBTAINED WITH PROTEINS AS PRINT MOLECULES

Relative retention = elution volume (transferrin)/elution volume (BSA).

Print molecule	Boronate– silane	Elution volume (ml)		Relative retention
		Transferrin	BSA	
Transferrin	Yes	1.32	0.61	2.16
	No	0.64	0.50	1.28
BSA	Yes	0.71	0.58	1.22
	No	0.59	0.51	1.16
None	Yes	0.73	0.60	1.22
	No	0.51	0.44	1.16

molecule as may be the case when using the smaller dye print molecules (see Fig. 1). The transferrin-selective polymer preparations showed a remarkable stability and could be used at least 50 times over a period of more than 18 months without loss of binding properties.

The results obtained with these preparations could indicate that prearrangement of an affinity ligand (boronate–silane) with an interacting print molecule (transferrin) prior to polymerization contributes to increased affinity for the print molecule by the resulting support in comparison with polymers prepared with randomly distributed affinity ligands. Thus, it should be possible to prepare affinity supports with the incorporation of a minimum of affinity ligand, possibly minimizing side effects such as non-specific adsorption, e.g., hydrophobic interaction with the phenyl group of ABBA. Furthermore, the amount of expensive ligands often used in the preparation of affinity supports could be reduced. It cannot be excluded that the shape of the cavity apart from the boronate interacting group may also contribute to the affinity shown. Attempts have been made to prepare sorbents selective for other glycoproteins such as fetuin, lactoferrin and ceruloplasmin, using the described prearrangement of protein and boronate–silane. So far these attempts have not been succesful due to problems such as protein precipitation. On the other hand, preliminary results with small print molecules, such as catechols, indicate the usefulness of the prearrangement technique. Thus a preparation showing selectivity for 3,4-dihydroxyphenylalanine (DOPA) over tyrosine, with a relative retention of 1.38, was obtained. A polymer prepared with the same amount of boronate–silane but without prearrangement of affinity ligands showed a relative retention of only 1.13.

Enzyme entrapment

Over the past years a number of methods for the immobilization of proteins have been described[19]. Apart from the most widely adopted technique involving covalent attachment to a solid support, physical entrapment in a highly cross-linked polymeric network has also been utilized. However, no immobilization method is ideal for all different applications. Therefore, ideally one should have an array of procedures at one's disposal in order to "tailor-make" an immobilized enzyme preparation. In the course or our studies on molecular imprinting using silanes we envisaged their alternative use for enzyme entrapment as a possibility. A silane monomer mixture containing a cross-linking agent, tetraethoxysilane, will spontaneously polymerize analogously to the described imprinting procedure when mixed with a solution of enzyme in water, obviating the need for an initiator and accelerator. Entrapment of enzyme is ensured by the use of higher amounts of silanes in combination with the cross-linking agent. The polysiloxane-entrapped enzyme preparations showed no detectable leakage of enzyme. During this work a report on the entrapment of antibodies in polysiloxane appeared[20].

We have chosen glucose oxidase as a model enzyme for our studies. It could be entrapped in a polysiloxane co-polymer with a retained activity of 40–50%. It was, however, difficult to obtain particles of homogeneous size from this polymer. In order to make the entrapped enzyme preparation more easy to handle and apply, especially in column experiments, the entrapment was alternatively allowed to proceed on the surface of porous silica. In this case the procedure included a thorough mixing of silanes and silica particles before the enzyme solution was added.

An investigation was performed to find the most suitable pore size of the silica to be used for these preparations. Silica of medium pore size (100 Å, 300 m²/g) gave the highest yield of retained glucose oxidase activity (5.0%). This could indicate that the polymerization preferentially takes place on the silica surface inside the pores. With wide pore silica (1000 Å) the enzyme can freely penetrate the pores where the entrapment can take place, but since the surface area is small (20 m²/g) a low enzyme yield (1.1%) results. The silica with the smallest pores (60 Å) has a large surface area (500 m²/g), but the pores are probably too narrow to allow glucose oxidase (MW 150 000 dalton) to penetrate completely, as indicated by an enzyme activity yield of only 2.5%. In a control experiment it was found that glucose oxidase is not irreversibly adsorbed on silica.

Enzyme entrapment in polysiloxane on silica was found to give lower glucose oxidase activity yields than entrapment without silica. The activity yield was typically 5% with silica compared to 40% with cross-linked siloxane polymer. After the entrapment procedure the pooled washings were also assayed for enzyme activity. In the case of entrapment on silica particles the total yield of enzyme activity found, entrapped and in the washings, was 90–95%, indicating that the entrapment procedure *per se* is non-destructive for the enzyme. When glucose oxidase was entrapped solely in silanes (with no silica) the total enzyme activity yield was, however, only 50–60%, which could indicate that the total amount of entrapped enzyme is higher than the apparent activity yield of 40%. One reason for this discrepancy could be that not all of the entrapped enzyme molecules are accessible to substrate. It is reasonable to assume that such diffusion limitations are of lesser importance for entrapment in polysiloxane-coated silica.

Since we used a coupled reaction with peroxidase for the assay of glucose oxidase, we attempted to co-immobilize the two enzymes, allowing the complete assay reaction to take place within the polymeric matrix. To a mixture of silanes and silica particles was added a protein solution containing 5 mg of horse radish peroxidase in addition to the same amount of glucose oxidase (0.25 mg) used above. The yield of entrapped peroxidase activity was only 1%, which could be due to less effective entrapment of the small peroxidase (MW 40 000 dalton) than of the larger glucose oxidase (MW 150 000 dalton). The total yield of peroxidase activity found was 90%, which indicates that the immobilization method is non-destructive for peroxidase as well. The preparation with co-immobilized glucose oxidase and peroxidase was subsequently used in column experiments.

The polysiloxane-coated silica particles containing entrapped enzyme showed good mechanical stability. This feature is valuable, especially in column experiments, since it avoids compression of the bed. Entrapped glucose oxidase packed in a column converted the reagent used for glucose oxidase assay, when applied to the column, into the photometrically detectable product. Practically no leakage of enzyme was observed for repeated, *i.e.*, seven consecutive, analyses. Co-immobilized glucose oxidase and peroxidase was also used in column experiments, obviating the need for peroxidase in the assay solution. Obviously, coentrapment of enzymes used in a coupled analysis of substrates, *e.g.*, for D-glucose, simplifies the analytical procedure.

Apart from glucose oxidase and peroxidase we have used organic silanes (without silica) for entrapment of other enzymes such as trypsin and alkaline phosphatase, obtaining activity yields of 10–40%. Yeast cells could be efficiently entrapped in the

polysiloxane network in high yield, as observed under the microscope. Viability tests remain to be carried out however. More work is needed to assess fully the usefulness of the silane-entrapment procedure for proteins and cells.

CONCLUSIONS

It is likely that organic silanes will find increased use in solid phase biochemistry in the future. The variety of interacting units available makes them well suited as monomers in molecular imprinting. Aqueous solutions can be used in the imprinting procedure which should allow biomolecules to be used as substrates (print molecules) in the preparation of new affinity sorbents. In this context, it should be mentioned that the general approach of coating a rigid silica matrix with a "soft" polymer of desired properties, whether as carrier of ligands or with imprinted cavities, should also be useful in the preparation of new supports for HPLC and HPLAC, in particular due to the better steric accessibility that may be obtained and the lower amounts of ligands required.

In addition, although considerable optimization is required to evaluate fully its general potential, direct entrapment of enzymes with silanes seems to be a straightforward alternative to other methods.

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