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ACRYLIC POLYMER PREPARATIONS CONTAINING RECOGNITION SITES OBTAINED BY IMPRINTING WITH SUBSTRATES

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SUMMARY

Results from the "impriting" of various molecules in highly cross-linked synthetic polymers are described. In the procedure followed, polymerization of different acrylic monomers around a "substrate" was carried out. The bulk polymer so formed was then crushed to particles of size 300–500 μm, and the substrate was eluted. A number of dyes, notably rhodanile blue and safranine O, were used in the impriting procedure, and the polymer particles were tested for their recognition properties in column chromatography. It was found that polymers imprinted with rhodanile blue and safranine O showed preferential binding for rhodanile blue and safranine O, respectively. It is believed that the observed selective recognition occurs because cavities are formed that resemble the original substrate in size and have groups at fixed sites within them that allow non-covalent binding with the respective complementary groups of the original substrate.

An alternative procedure for the preparation of substrate-selective polymers was subsequently developed. In this procedure a thin shell of acrylic polymer was formed under similar impriting conditions onto microparticulate porous silica carrying acrylate groups. Analysis of these composite particles under high-performance liquid chromatographic conditions showed similar recognition patterns but could be accomplished more rapidly.

INTRODUCTION

Recently there has been considerable interest in the preparation of polymers that have a selective memory for a substrate around which a polymeric structure has been formed. Such preparations have a potential use in chromatographic separations. In most of these studies, polymers based on styrene–divinylbenzene have been used: special mention in this context should be made of the numerous reports by Wulff and Gimpel[1], and to Shea and Thompson[2] and Andersson *et al.*[3]. Some other systems based on alternative polymers have also been described. Among them are the early approach by Dickey[4] to make specific holes in silica gel, and the preparation of selective metal ion adsorbents[5,6]. More recently it has been reported that the

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cross-linking of starch in the presence of methylene blue led to a kind of tailor-made cyclodextrin that had a binding capacity for the dye[7].

In previous work[8] and that described here we have concentrated on polymers based on acrylic monomers as they allow the use of water-soluble substrates and offer a great variety of complementary monomers.

These different approaches have been described as "template"[1,2] or "host–guest" polymerization[8]. "Imprinting" is also now used to describe the technique employed[8,9]. Normally the substrate used is covalently bound to a monomer and after polymerization removed through hydrolysis. Examples of such monomers are vinyl boronate[1] or vinyl dicarboxylate[2].

Our strategy is different in that a monomer mixture containing a large proportion of cross-linking units is polymerized in the presence of a free substrate which is to act as the template during the polymer formation process. It involves a simple mixing of the constituents, and no covalent attachment to the monomeric units is required. The monomers are, however, chosen in such a way as to allow non-covalent binding (*i.e.*, ionic, hydrogen bond, hydrophobic, charge transfer, etc.) complementary to the different sites of the substrate. Owing to the complementarity of the binding sites between the polymerizing units and the substrate, an *imprint* of the latter molecules is developed within the polymer matrix. Because of the cross-linking the imprints, or binding centres, are preserved even after the substrate is removed, and the polymer retains sites complementary to the substrate.

In the work described here the previous approach[8] using batch incubation was extended to the use of column chromatography, leading to more efficient separations. In addition, a variety of dyes has been tested including the system fluorescein (fluorescing)–rhodamine (quenching), to allow sensitive analysis. We also describe a procedure leading to the preparation of microparticulate (10 μm) silica particles coated with substrate-selective polymers suitable for fast analysis in high-performance liquid chromatographic (HPLC) systems.

MATERIALS AND METHODS

Materials

Acryloyl chloride, 1,4-diaminobenzene, methyl methacrylate (all synthetic grade), silica (LiChrospher Si 1000, 10 μm), and rhodamine B were obtained from Merck (Darmstadt, F.R.G.). N,N′-Methylenediacrylamide (electrophoresis grade) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Safranine O was from BDH (Poole, U.K.) and all other dyes from Aldrich-Europe (Beerse, Belgium). 3-Methacryloxy-propyltrimethoxysilane was from Magnus Scientific Instrumentation (Sandbach, U.K.), triethylamine (purum) from Aldrich-Europe. All other chemicals were of analytical grade and used as supplied. Distilled water was used throughout.

In the low-pressure chromatographic experiments a peristaltic pump Model 2132 from LKB (Bromma, Sweden), a vis-spectrophotometer Model PM2K from Zeiss (Oberkochen, F.R.G.) supplied with a flow cuvette (25 μl) and a modified glass column SR 10/50 from Pharmacia (Uppsala, Sweden) were used. HPLC was performed with an Altex 110A pump and an Altex 210 injector supplied with a 20-μl loop, all from Altex (Berkeley, CA, U.S.A.) and a Spectromonitor II UV–vis detector from Laboratory Data Control (Riviera Beach, FL, U.S.A.). A Hitachi/Perkin-Elmer

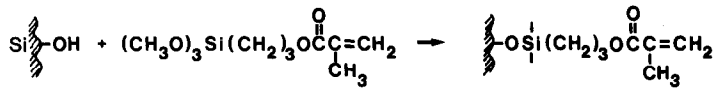
MPF-2A fluorescence spectrophotometer (Tokyo, Japan) was used in the fluorescence experiments, and a bench top centrifuge was employed for centrifugation.

Synthesis of N,N'-1,4-phenylenediacrylamide

A method devised by Arshady and Mosbach[10] was followed in general. 1,4-Diaminobenzene (21.6 g, 0.2 mol) and 60 ml (0.43 mol) of triethylamine were added to 250 ml of dimethylformamide (DMF) and gently warmed until the solid was dissolved. The reaction vessel was cooled in an icebath, to prevent polymerization, and then 36 ml (0.44 mol) of acryloyl chloride, dissolved in 100 ml of DMF, were added dropwise for 1 h. After stirring for 15 h at 22°C the mixture was cooled to 0°C and the precipitate was filtered on paper and washed with small amounts of cold DMF. The collected filtrates were evaporated under reduced pressure without heating and the oily remains were added to 1 l of ice-cold 1 *M* hydrochloric acid under vigorous stirring. After filtration and washing with ice-cold 1 *M* hydrochloric acid the product was dissolved in 1-butanol and extracted three times with 1 *M* sodium hydrogen carbonate. The butanol phase was evaporated and the crystals were finally dried under vacuum to yield 35–40 g (85–90%) pure product (thin-layer chromatography, silica; butanol–acetic acid–water, 75:15:10). Nuclear magnetic resonance data were consistent with the proposed structure.

Preparation of methacrylate–silica

Methacrylate–silica was prepared in analogy with an earlier method[11]. LiChrospher Si 1000 (20 g) was dried in a three-necked flask under vacuum (150–200°C, 4 h). Sodium-dried toluene (250 ml) was then added via a separation funnel, still



under reduced pressure, followed by 5 ml of 3-methacryloxy-propyltrimethoxysilane and 300 μl of triethylamine. The mixture was refluxed under stirring for 15 h and the experimental set-up was continuously flushed with dry nitrogen during the reaction. The methacrylate–silica was washed on a glass filter with 500 ml of toluene followed by 500 ml of acetone and 200 ml of diethyl ether, and finally dried under vacuum overnight, giving 20.1 g of product. The concentration of immobilized methacrylate groups was determined according to the following method. Dried methacrylate–silica (*ca.* 70 mg) was suspended in 10 ml of water. Diluted bromic water was added to the suspension until a faint colour remained. The hydrobromic acid generated was titrated with 0.5 *M* sodium hydroxide to pH 5.0. Two controls, unsubstituted silica treated in the same way, and methacrylate–silica with no bromic water added, gave no consumption of sodium hydroxide. The concentration of double bonds on the methacrylate–silica was 22 μmol/g.

Preparation of polymer particles following bulk polymerization

N,N'-1,4-phenylenediacrylamide (0.65 g, 3.0 mmol) and N,N'-methylenedi-

acrylamide (1.24 g, 8.1 mmol) were added to 6.0 ml of DMF under stirring and warmed (50–60°C) to give a clear solution. Then 5.0 ml of water followed by 1.66 g (16.6 mmol) of methylmethacrylate were added. To the monomer solution was added 0.35 mmol substrate and the mixture was shaken until all the substrate was dissolved. The solution was transferred to a test tube and 0.1 g of ammonium persulphate in 1 ml of water was added and well mixed. The test tube was sealed and kept at 40°C for 48 h. The polymer cake formed was crushed through a 0.5-mm net and the particles were washed on a 300-μm net with water until no more substrate was washed out. The polymer particles were transferred to an E-flask and washed by shaking with 200 ml of DMF–water (50:50, v/v) for 5 h and then shaken with pure DMF for 11 h. This procedure was repeated once. The resulting polymer grains had a particle size of 300–500 μm.

Preparation of polymer-coated silica particles

N,N'-1,4-Phenylenediacrylamide (0.20 g, 0.93 mmol) and N,N'-methylenediacrylamide (0.37 g, 2.4 mmol) were added to 1.8 ml of DMF and carefully warmed (50–60°C) under stirring to give a clear solution. Then 1.5 ml of water, 0.5 g (5.0 mmol) of methyl methacrylate and 0.1 mmol of substrate were added and well mixed. The solution was transferred to a test tube and 20 mg of ammonium persulphate in 0.3 ml of water were added. Methacrylate–silica (2 g) was immediately added and after mechanical mixing the test tube was placed in an ultrasonic bath for 10 min. The tube was well sealed and kept at 40°C for 48 h. The polymer-coated silica was washed on a glass filter with 500 ml of water and 200 ml of DMF and then transferred to a centrifuge tube and thoroughly suspended in 2 × 10 ml each of DMF, water, 0.5 *M* sodium chloride (in water), ethanol, and water. Ethanol (10 ml) was added and the mixture was suspended carefully. The coarse particles were allowed to settle and the fine particles were collected. This procedure was repeated once with the coarse particles. The pooled fines (with at least 80% of total material) were suspended in a 50% sucrose solution and packed in a stainless steel column (10 × 0.5 cm I.D.) at 21 MPa (3000 p.s.i.).

Fluorescence studies

Bulk polymerization was carried out as described above, at half scale with 0.1 mmol of rhodamine B added. The polymer particles (1.0 g) were transferred to a test tube and 5 ml of 1 m*M* fluorescein in DMF–water (50:50, v/v) were added. After mixing on an end-over-end rocking table for 2 h at 22°C the polymer suspension was centrifuged with a bench top centrifuge for 2 min. The clear supernatant was then measured with a fluorometer (excitation: 468 nm; emission: 542 nm). The apparatus was calibrated to 100% fluorescence for a fluorescein reference solution without polymer. Aliquots of a 1 m*M* rhodamine B solution (in DMF–water, 50:50) were then added with a Hamilton syringe, and each time the mixture was equilibrated on an end-over-end rocking table for 2 h, after which the fluorescence was measured (each time the fluorescence of the reference solution was set to 100%).

RESULTS AND DISCUSSION

Preparation of acrylic polymer particles (Scheme I, Route A)

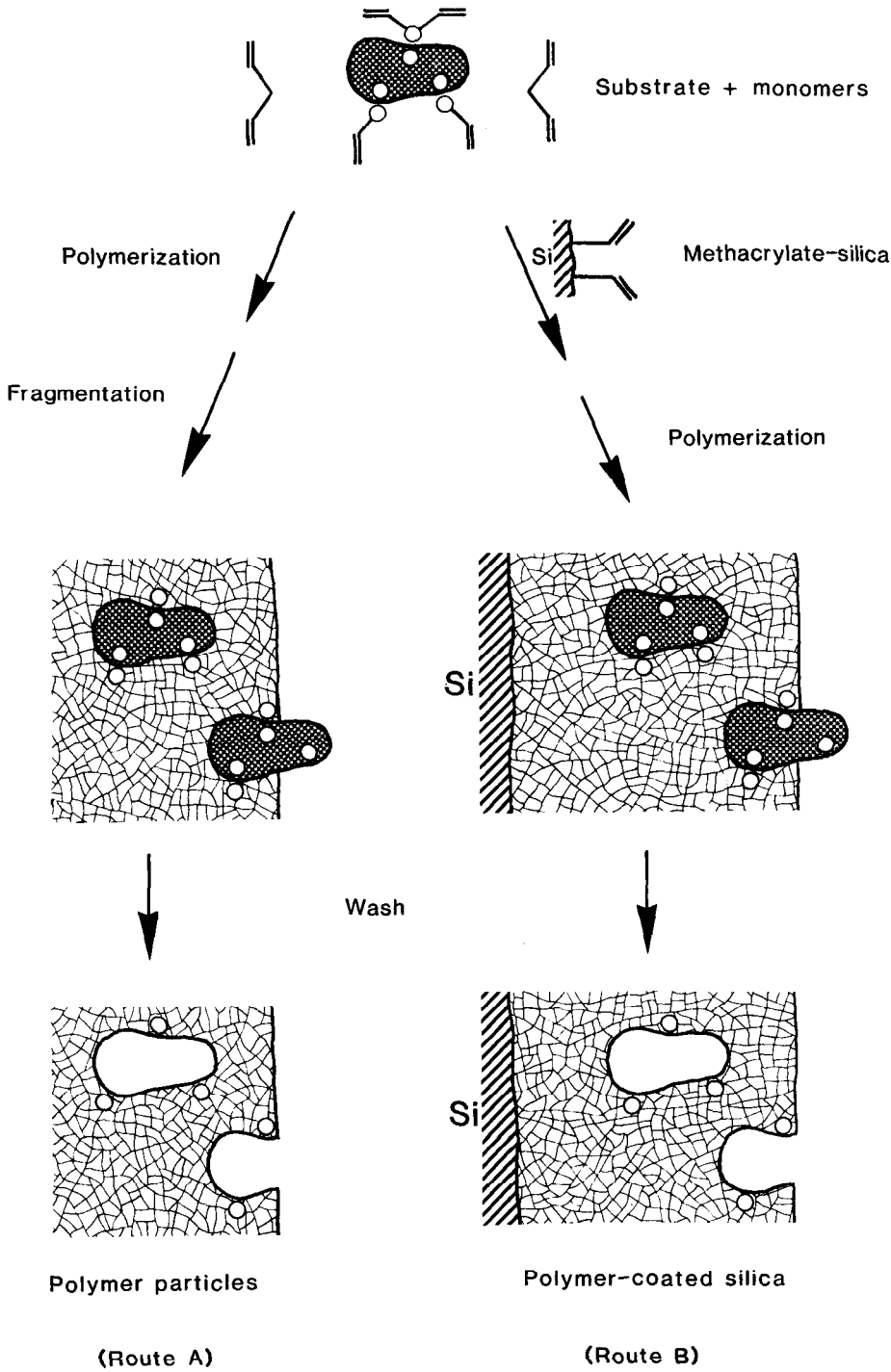
According to our host–guest polymerization strategy[8], monomers and substrate are allowed to interact before the polymerization process is started. No covalent attachment of the monomers to the substrate is necessary. However, the monomers are chosen according to their ability to establish non-covalent interactions with the substrate. The interacting units are subsequently fixed in the substrate-selective compartments so formed in the polymer matrix. The predominant interactions can be hydrophobic, hydrogen bonding or charge transfer bonds, all of a non-covalent and weak nature. These interactions have a universal character and can be used in almost any type of polymer–substrate system. Owing to the weak nature of these bonds multiple interactions between polymer and substrate are necessary to establish sufficiently strong interactions. In Figs. 1 and 2 are shown the dyes selected for the present studies: they were selected because they are easy to detect and allow the establishment of the different types of non-covalent interactions mentioned above.

The monomer mixture was chosen (see Materials and methods) to give the polymers rigid, highly cross-linked domains containing the substrate-selective compartments and interjacent parts with in a macroporous structure with small diffusion barriers[12].

After polymerization, which is slow and probably does not disturb the interactions between substrate and monomers/polymer, the substrate could be removed by a simple washing procedure. The absorbance of the washings was determined, and normally *ca.* 95% of the substrate could be removed in this way, depending on the strength of the interactions. The remaining "non-extractable" dye is probably the portion completely and "perfectly" encapsulated. The polymer was crushed through a net and, in order to get flow properties suited for chromatography, particles of diameter 300–500 μm were collected. Compared with the batch technique[8], the chromatographic evaluation of the substrate-selective polymers is more convenient. Once packed in a column the polymer is easily washed between consecutive additions of different samples, and the elution volumes are readily measured by the on-line detector.

Chromatographic evaluation of acrylic polymer particles

To achieve the most selective interaction between polymer and substrate it is, in most cases, advantageous to use the same solvent mixture for binding studies (chromatography) as was used for polymerization. Changes in solvent composition can not only change the strength of the interactions between substrate and interacting units in the polymer but obviously also cause swelling or shrinking of the polymer and in that way alter the size and shape of the compartments. Furthermore, the proper localization of the binding units on the polymer could be disturbed. Taken together, these changes may alter the compartmental affinity originally obtained in the polymer. Consequently, we have found that if the polymer is allowed to dry the selectivity for the substrate is lost, indicating an irreversible destruction of the compartments. The swelling of the polymer in different mixtures of DMF–water was investigated: 100% DMF, 2.9 ml/g; 100% water, 2.3 ml/g; DMF–water (50:50), 2.9 ml/g.



Scheme I. Preparation of substrate-selective polymers by molecular impriting with substrates. The symbol (O) represents interacting units (*e.g.*, hydrophobic, electrostatic, etc.) of monomers and substrate.

The polymerization was performed in DMF–water (50:50), and this mixture was also chosen as eluent in most subsequent chromatographic elutions. An increased portion of DMF in the aqueous eluent is likely to result in weaker hydrophobic interactions and stronger hydrogen-bond effects. High ionic strength in the eluent results in the opposite effect. In pure water all substances appeared to be totally adsorbed on the polymer. Particulate bulk polymer (4.5 g) was packed in a glass column fitted with flow adaptors. All chromatographic evaluations were made at 22°C with a flow-rate of 0.5 ml/min, and each time samples of 100 μl (1.28 mM in DMF–water, 50:50) were injected, one at a time. The total amount of accessible compartments was estimated to be 1.6 μmol/g dry polymer (frontal analysis).

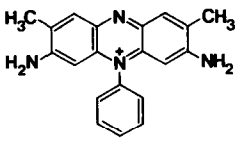
Table I lists some data on the binding of different substances to bulk polymers prepared in the presence of rhodanile blue or safranine O as template molecules. The definition of the selectivity factors (R) is given in Table I. The results clearly show that the two polymers are selective for their two respective substrates. We have also examined the affinity of these polymers for some other chromophoric substances as listed in Table I. It is obvious that the chemical structure, notably its size, of a substance (Fig. 1) decides whether or not strong compartmental binding will occur. Acridine yellow has a chemical structure very similar to those of both rhodanile blue and safranine O, and the selectivity factors for acridine yellow are in both cases almost identical with the printed substrates. Triphenyl tetrazolium chloride, on the other hand, with a completely different structure, gives considerably lower R values in both cases. The two other substances examined, cresyl violet and acriflavine, were also found to bind poorly to the two polymers. A blank polymer, prepared with no substrate present during the polymerization, was exposed to the same substances. Since the retention volumes on the blank polymer vary considerable for different substances (Table I), we have chosen to present these data in the form of R values, which take the background binding into consideration. However, the physical properties of the blank polymer differ markedly from that of a polymer prepared with substrate present during polymerization.

The polymer prepared with rhodanile blue as substrate showed selectivity for rhodamine B (R = 1.00), which consists of just one part of the rhodanile blue molecule (Fig. 2). A polymer prepared with rhodamine B as substrate, on the other hand, exhibited low selectivity for rhodanile blue (R = 0.86). In this case there is probably not room enough in the compartment for the rhodanile blue molecule (MW 778), whereas the smaller rhodamine B (MW 443) easily fits in the rhodanile blue compartments, where properly arranged binding units make strong interactions possible.

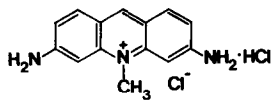
One unexpected observation made during the chromatographic experiments with these substrate-selective polymers was that some fraction (*ca.* 50%) of each substance applied eluted much later, still with the same selectivity factors. This phenomenon is still unexplained.

Binding studies with a fluorescence method

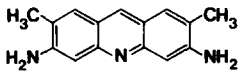
Fluorescence methods have been used to increase the sensitivity of analytical immuno-methods[13]. Energy transfer between two different fluorochromic molecules takes place if the emission spectrum of one molecule overlaps the absorption spectrum of the other. A decrease in fluorescence is then observed, provided that the distance between the fluorochromes is less than 100 Å. This quenching effect is used in an immunoassay system[14] where rhodamine B and fluorescein were covalently



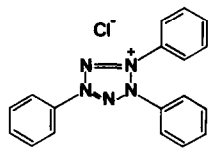
Safranine O



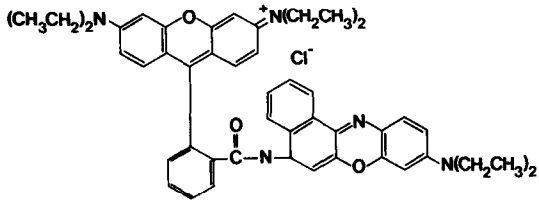
Acriflavine



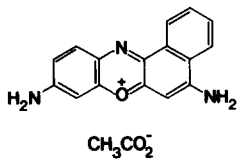
Acridine Yellow G



2,3,5-Triphenyl-2H-tetrazolium chloride

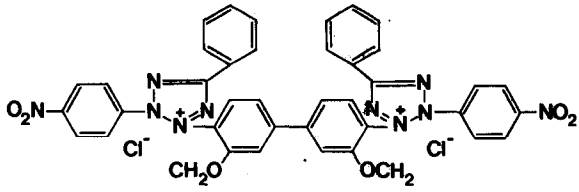


Rhodanile Blue

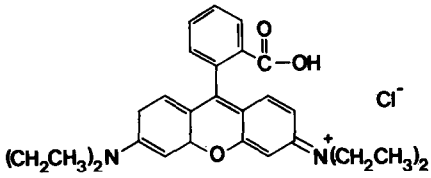


Cresyl Violet acetate

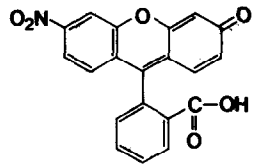
Fig. 1. Various dyes used as substrates.



Nitro Blue Tetrazolium Chloride



Rhodamine B



Fluorescein

Fig. 2. Nitro blue tetrazolium chloride and the fluorescence-quenching couple fluorescein and rhodamine B.

TABLE I

BINDING DATA FOR SUBSTRATE-SELECTIVE ACRYLIC POLYMER PARTICLES

The polymers were prepared according to the bulk polymerization procedure described in Materials and methods. To a column (5 × 1 cm I.D.) containing 4.5 g of wet polymer were applied 100-μl samples of the appropriate substrate (1.28 *M*). Flow-rate, 0.5 ml/min; eluent, DMF–water (50:50).

Substrate applied	*Detection (nm)*	*Retention volume (ml)*			*Selectivity factor, R**	
		Blank polymer	*Safranine O-printed polymer*	*Rhodanile blue-printed polymer*	*Safranine O-printed polymer*	*Rhodanile blue-printed polymer*
Rhodanile blue	550	17.4	12.9	19.8	0.78	1.00
Safranine O	530	23.4	22.2	18.6	1.00	0.70
Cresyl violet	584	14.7	12.6	15.6	0.90	0.93
Acridine yellow	440	11.5	10.8	13.2	0.99	1.01
Acriflavine	430	20.4	13.2	18.0	0.68	0.77
2,3,5-Triphenyl-2H-tetrazolium chloride	350	26.4	16.6	15.6	0.66	0.52

* Selectivity factor $R = \dfrac{\dfrac{r_1}{r_1\ (\text{blank})}}{\dfrac{r_2}{r_2\ (\text{blank})}}$

r_1 = retention volume for applied substrate; r_2 = retention volume for printed substrate.

attached to antibody and antigen, respectively. Binding of antigen to antibody results in close contact between the two fluorochromes and consequently to decreased fluorescence. In a competitive assay less than 10^{-10} mol/l antigen could be detected. The high sensitivity of this method prompted us to adapt this technique for the evaluation of substrate-selective polymers. To rhodamine B-selective polymer particles suspended in a solution of fluorescein were added aliquots of rhodamine B. A continuous decrease in fluorescence, caused by the close contact between rhodamine B and fluorescein, was observed (Fig. 3). As expected, the decrease was less pronounced with rhodamine B-polymer present than in the control experiments (with no polymer, with safranine-printed polymer, or with fluorescein-printed polymer). This effect was caused by withdrawal of rhodamine B from the solution, owing to selective binding in the polymer. Equilibrium in the system was reached within 15 min. Some effect on the fluorescence could also be seen for the control polymers, indicating some degree of non-specific interactions between rhodamine B and the polymeric structure itself.

Preparation of acrylic polymer-coated silica (Scheme I, Route B)

The imprinting polymerization technique was also adapted for the preparation of an acrylic polymer-coated silica material to be used in efficient HPLC systems. Highly porous silica (1000 Å mean pore diameter) was allowed to react with methacryl-silane to achieve covalently bound methacrylic groups suitable for anchoring of the acrylic polymer on the silica surface. The high porosity of the silica was chosen to ensure good accessibility of the substrate-selective compartments formed in the acrylic polymer layer. Subsequent derivatization–deactivation of the negatively charged silanol groups was performed in dry toluene to avoid polymerization of the silane and to achieve complete coverage of the silica[11]. To avoid poor flow properties a thin layer of acrylic polymer around the silica particles is desirable. This was achieved by the use of relatively small amounts of acrylic monomers. Otherwise the polymerization of acrylic monomers onto silica particles was performed similarly to the bulk polymerization. In order to distribute the monomers evenly around and in the pores of the methacrylate-silica particles, thorough mechanical stirring was necessary in addition to ultrasonic treatment of the reaction mixture. The main part of the polymeric product spontaneously separated, during the washing procedure, into particles with the same size as the original silica. The remaining agglutinated particles were easily sedimented and discarded after thorough suspension in ethanol. Scheme I (route B) shows how the monomers prearrange around the substrate before polymerization and how the polymer layer is anchored to the silica surface. This layer has an average thickness of 50 Å (determined by elemental analysis).

Irrespective of which approach is used, bulk polymerization or polymerization on silica particles, some of the compartments formed are likely to be on the surface of the polymer. This heterogeneity may contribute to the band broadening observed in the chromatographic evaluation of the polymers. The band broadening is less pronounced in the HPLC system, which may be due to decreased mass transfer limitations in the relatively thin polymer layer on the silica surface.

Chromatographic evaluation of acrylic polymer-coated silica

To make the chromatographic evaluation of the substrate-selective polymers

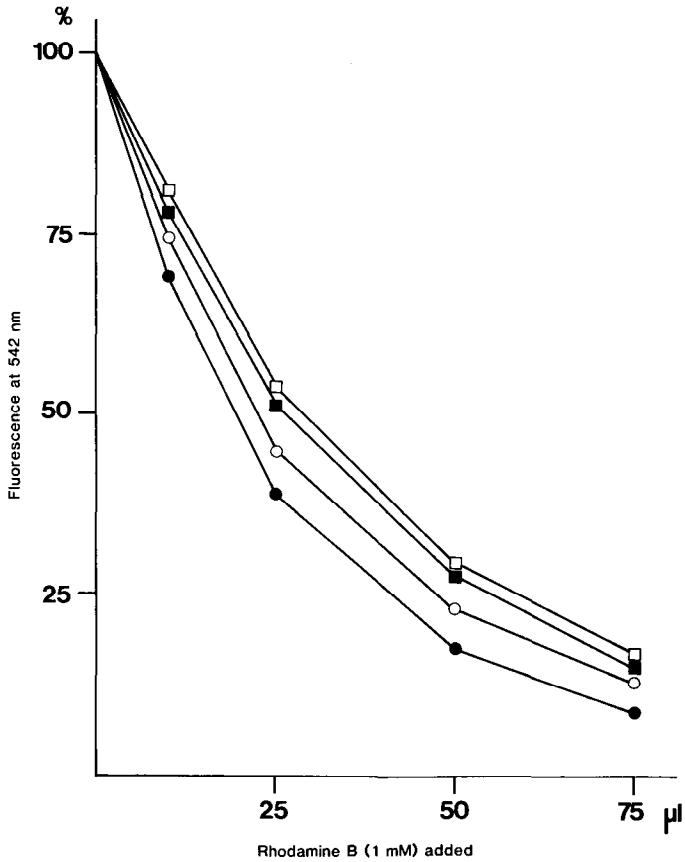


Fig. 3. The quenching effect of rhodamine B on the fluorescence from a fluorescein-containing solution in the presence of different polymer particles. Polymer printed with rhodamine B (□); safranine O (■); fluorescein (○); and without polymer added (●).

more convenient, a silica-based HPLC material was developed. The main benefits of using HPLC are shorter time of analysis, higher resolution owing to shorter diffusion times and more sensitive detection systems. Rigid, microparticulate silica particles, suitable for fast separation under pressure, were coated with a thin layer of "soft" acrylic polymers containing the substrate-selective compartments. No substantial increase in back pressure, owing to compression or breakage of the polymeric layer, was noticed. As can be seen in Table II, the selectivity factors for these composite materials are comparable with those obtained with the fragmented bulk polymers. HPLC was normally performed at 22°C with a flow-rate of 1 ml/min and with DMF–water (50:50) as eluent. Each time 20-µl samples (1.28 m*M* in DMF–water, 50:50) were injected one at a time. Although the efficiency of the system was low (*ca.* 100–200 theoretical plates/m) the chromatographic evaluations of the polymers coated on silica were at least 5–10 times faster than those of the bulk polymer.

The chemical structure of another substrate used for imprinting, nitro blue tetrazolium chloride, is shown in Fig. 2. The main difference between this substrate

TABLE II

SELECTIVITY FACTORS: COMPARISON OF POLYMER PARTICLES AND POLYMER-COATED SILICA

Substrate applied	*Selectivity factor, R**			
	Acrylic polymer		*Acrylic polymer-coated silica*	
	Safranine O-printed polymer	*Rhodanile blue-printed polymer*	*Safranine O-printed polymer*	*Rhodanile blue-printed polymer*
Safranine O	1.00	0.70	1.00	0.79
Rhodanile blue	0.78	1.00	0.74	1.00

* See Table I.

and those previously used are the two nitrated aromatic ring structures, capable of developing charge transfer interactions. A polymer-coated silica, prepared with nitro blue tetrazolium chloride as substrate, was tested against the silica-based rhodanile blue-selective polymer. In this case we used a simplified selectivity factor (R' = retention volume for applied substrate divided by retention volume for printed substrate; *i.e.*, R' for printed substrate = 1.00), which just compares the binding properties of two polymers prepared with imprinting of two different substrates. The R' values for rhodanile blue applied to the nitro blue tetrazolium chloride-printed polymer was 0.75 and for nitro blue tetrazolium chloride applied to rhodanile blue-printed polymer 0.72. The high selectivities for printed substrate (low R' values for non-printed substrate) were obtained with an eluent containing 50% DMF–0.05 M sodium chloride in water. This increase of ionic strength was necessary to suppress non-specific ionic interactions between nitro blue tetrazolium chloride and the support. Without this modification of the eluent, the extremely large elution volumes obtained resulted in severe detection problems owing to dilution of the sample. It should be pointed out that of all the dyes tested the latter has the highest net-charge, which may account for the pronounced non-specific ionic interaction observed.

In conclusion, apart from its potential for chromatography through preparation of column materials tailor-made for a particular separation, we feel that the gel-printing method might be useful in designing active site-like cavities that when substituted with proper catalytically active ligands may mimic enzymic functions.

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