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Modification of silica with glucose for the separation of proteins by high-performance liquid chromatography

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SUMMARY

The synthesis and testing of a mild stationary phase for protein separation are described. Using either glucose or epoxyglucose coupled to aminopropylsilica it is possible to achieve shielding of the silica surface with preservation of the pore structure. The stability of the coating and the interaction with standard proteins were studied. Basic proteins are not retained on either product. The glucosesilica is unstable in aqueous buffers, but the epoxyglucosesilica is not affected, even at high pH.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a well accepted technique for the purification and separation of proteins (see, *e.g.*, ref. 1). There is a large number of mobile and stationary phases in use[2], which reflects the diversity of proteins, the complexity of protein mixtures and the different applications[3]. It may also be regarded as indicative of the dissatisfaction with present packings.

One of the main concerns when separating proteins is the risk of denaturation. Some general guidelines can be given, *e.g.*, aqueous solutions buffered at a suitable pH and the elimination of strong protein–surface interactions. The latter is achieved with a "hydrophilic" surface. Preservation of the biological activity of a particular protein may require more specific conditions, *e.g.*, a low temperature, a water concentration between certain limits, the presence of special reagents to prevent oxidation and proteolysis[4]. These conditions are especially important with preparative separations.

Polysaccharides have been succesfully used for decades as stationary phases for the liquid chromatography of proteins[5]. Owing to the virtual absence of adsorption effects, size exclusion can be effected with these materials. Interactive chromatography is possible after suitable derivatization, and this derivatization chemistry has been well described[6]. Polysaccharide particles were strengthened for the use in HPLC by cross-linking them intensively[7]. However the chromatographic performance of these materials is poor and the rigidity is still insufficient.

Silica has been the support material of choice for stationary phases in HPLC[8]. It

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can be synthesized as uniform spherical porous particles of small diameter for optimum resolution. The pore diameter can be varied from below 100 to over 1000 Å. Well known disadvantages are the active surface and dissolution at high pH. A polymer coating on the surface can prevent these effects to some extent[9].

In a further effort to synthesize a better phase for mild protein separations, we decided to combine the advantages of polysaccharides and silica. This has been tried before but the results were not completely satisfactory[10–12]. Protein recoveries were low and the stability was poor.

Because significant reductions in the accessible surface area of the silica support can occur after coating procedures owing to narrowing and blocking of the pores[13], we attempted to bind the small molecule glucose onto the surface and to study the size-exclusion behaviour. Such a material would be an ideal base for other separation systems.

EXPERIMENTAL

Chemicals

Silica supports were obtained from Merck (Darmstadt, F.R.G.) (LiChrospher Si 1000, Si 500, Si 300, Si 100, 10 μm) and Shandon (Runcorn, U.K.) (Hypersil, 5 μm; WP300, 10 μm). Triethoxyaminopropylsilane was supplied by Janssen (Beerse, Belgium). The dextrans used in the size-exclusion experiments were from Pharmacia (Uppsala, Sweden). Methyl 2,3-anhydro-4,6-O-benzylidine-α-D-allopyranoside (referred to as epoxyglucose reagent in the following), sodium cyanoborohydride, [^{14}C]glucose and the proteins were obtained from Sigma (St. Louis, MO, U.S.A.) and phenyl mercuriacetate (as an antimicrobial agent) from BDH (Poole, U.K.). Other chemicals and solvents were of analytical-reagent grade and used as received unless stated otherwise.

Equipment

HPLC was performed with constant-flow pumps (Spectroflow 400; Kratos, Ramsey, NJ, U.S.A.), an injection valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.), a UV detector (Spectroflow 757; Kratos) and a recorder (BD 40; Kipp and Zonen, Delft, The Netherlands). Gradients were run with two pumps and a gradient programmer (Spectroflow 450; Kratos).

A liquid scintillation system (Tri-Carb 300 CD; Packard, Downers Grove, IL, U.S.A.) with scintillator cocktail (Scintillator 299; Packard) was used for radioactivity measurements.

Packing procedure

Silicas were slurry packed into stainless-steel columns, using methanol as the slurry and driving liquid, with a maximum packing pressure of 500 bar. Before packing fines were removed by uspending the silica in methanol, allowing it to settle and discarding the top layer of turbid methanol.

Preparation of aminopropylsilica

A suspension of activated silica (activated in a vacuum oven at 120°C for 16 h) in a 4% (v/v) solution of triethoxyaminopropylsilane in dry, freshly distilled toluene

(dried over molecular sieves in a Soxhlet apparatus) was refluxed for 16 h. The product was isolated by suction filtration, washed with 100 ml each of toluene, acetone and diethyl ether, then dried for 5 h in a vacuum oven at 120°C.

Picric acid assay

The assay for amino content is a modification of a method described by Alpert and Regnier[14]. An 13-g amount of moist picric acid is dissolved in 100 ml of dichloromethane and the aqueous layer is discarded. An accurately weighed amount of aminopropylsilica (50–100 mg) is suspended in 5 ml of this solution. After a few hours the silica is washed on a glass filter with dichloromethane until the washing solution is colourless. Next, the silica is transferred to another glass filter and the bound picric acid is washed off with a solution of 5% triethylamine in dichloromethane and collected. After appropriate dilution the absorbance is measured at 358 nm (ε = 14 500).

Titration of amino groups

Direct titration of amino groups on silica was carried out with hydrochloric acid using an indicator. To 10 ml of an aqueous solution of sodium chloride (4 *M*) a few drops of a 0.1% indicator solution of bromocresol green–methyl red (5:1) in methanol are added. With a small drop of 0.1 *M* hydrochloric acid the indicator is converted to the acid form. Next, a weighed amount of modified silica (*ca.* 400 mg) is added and the suspension is degassed and titrated with 0.1 *M* hydrochloric acid.

Preparation of glucosesilica

To 0.5 g of aminopropylsilica in 25 ml of methanol are added 0.5 g of glucose (a 10-fold excess), 5 mg of ammonium chloride and 200 mg of sodium cyanoborohydride. After refluxing for 16 h the product is washed with 100 ml each of methanol, water, acetone and diethyl ether.

In order to determine the yield, a reaction on a five times smaller scale was carried out and 10 μCi of [^{14}C]glucose were added. After washing, the product was suspended in 50 ml 1.5 *M* sodium hydroxide solution and heated to 50°C to dissolve the silica particles. An aliquot of this solution was counted after addition of scintillator cocktail. With the periodate treatment (see Results and Discussion) the product was suspended in 50 ml 0.1 *M* sodium periodate for 16 h, then washed, dissolved and counted as described.

Preparation of epoxyglucosesilica

To aminopropylsilica (0.5 g) in 25 ml of dichloroethane and 1 ml of triethylamine are added 200 mg (3 equiv.) of methyl 2,3-anhydro-4,6-O-benzylidine-α-D-allopyranoside. The suspension is heated on a water-bath (60°C) for 2 or 16 h, with occasional swirling, then washed with dichloroethane, acetone, water, acetone and diethyl ether. The benzaldehyde protecting group can be removed by heating in dilute sulphuric acid (0.0025 *M*) on a water-bath (60°C) for several hours.

RESULTS AND DISCUSSION

Aminopropylsilica

To attach glucose on the silica surface, the latter has to contain a suitable functional group. This was achieved by silanizing the silica with aminopropylsilane[15]. The product was analysed with a picric acid assay and a titration (Table I). The determination of a functional group was preferred to, *e.g.*, elemental analysis, because the former probably corresponds better to a measurement of the glucose reaction product. Although there is a significant difference in the results of the two methods, these figures are believed to be indicative of the coverage obtained. The coverage of the silica surface can be estimated by relating the values to surface areas as supplied by the manufacturer[16]. These figures correspond well with those reported by others[17].

TABLE I

COVERAGE OF SILICA WITH AMINO GROUPS AFTER SILANIZATION

Surface area data from manufacturer. Picric acid assay and titration as described. NH_2 is the mean of the picric acid assay and titration.

Silica type	*Surface area* (m^2/g)	*Picric acid assay* (*mmol/g*)	*Titration* (*mmol/g*)	NH_2 ($\mu mol/m^2$)
Si 1000	30	0.10	0.13	3.8
Si 500	60	0.20	0.22	3.5
Si 300	250	0.63	0.60	2.5
Si 100	250	0.68	0.82	3.0
Hypersil (5 μm)	170	0.44	0.56	2.9

The absence of water in the reaction mixture should promote the formation of a monolayer. This was checked by varying the concentration of the reagent and measuring the number of amino groups, again with the two methods mentioned (Fig. 1). The relationship suggests a levelling out at about 0.3 mmol/g. A maximum coverage of 4 μmol/m^2, as is often found in such silica surface modifications, corresponds to 0.24 mmol/g for this material of 60 m^2/g. Hence, in the prepared material there is probably some polymerized reagent present. However, the degree of polymerization will be small and the surface probably resembles that of a monolayer.

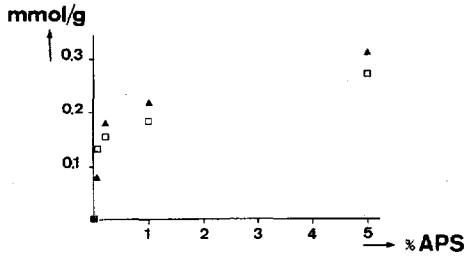

Fig. 1. Load of amino groups on silica using increasing concentrations of the silanizing agent. LiChrospher Si 500 (1 g) in dry toluene (50 ml); vol.-% triethoxyaminopropylsilane. ▲ = Titration; □ = picric acid assay.

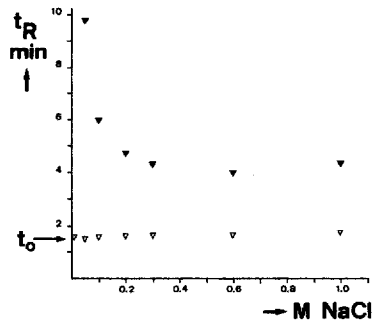

Fig. 2. Retention times (t_R) of lysozyme on aminopropylsilica prepared from Hypersil (WP 300). ▽ = Just after packing; ▼ = after 1.2 l of eluent. Column, 120 × 3 mm I.D.; mobile phase, 0.01 *M* Na_2HPO_4–NaH_2PO_4 (pH 7.0) + NaCl; flow-rate, 0.5 ml/min; lysozyme, 1 mg/ml; injection, 20 µl. Acetone used as t_0 (dead volume) marker.

Using a buffer of pH 7, the retention of lysozyme (p*I* 11) increases rapidly in a column packed with this material, indicating contact between the protein and the native silica surface. The ion-exchange nature of the interaction is demonstrated in Fig. 2. Lysozyme can be eluted easily from freshly prepared aminopropylsilica. After a certain amount of mobile phase, however, addition of salt is necessary for elution. With increasing salt concentration the retention decreases. With the 0.05 *M* buffer in the same column, the retention time of ovalbumin (OVA) decreased from 7 min at 2.6 l to 2 min after 7 l of eluent, while whereas bovine serum albumin (BSA) did not elute. A chromatogram of this used material is shown in Fig. 3.

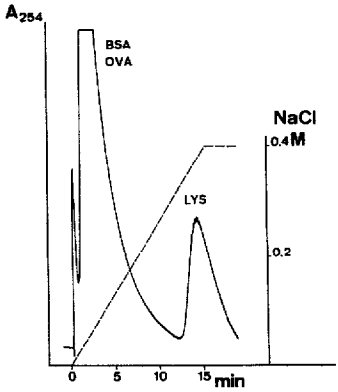

Fig. 3. Chromatogram of standard proteins on aminopropylsilica prepared from LiChrospher Si 300. Column, 150 × 2 mm I.D.; mobile phase, A = 0.05 *M* NaH_2PO_4–Na_2HPO_4 (pH 7.0), B = A + 0.4 *M* NaCl, gradient from A to B in 15 min; flow-rate, 1 ml/min; proteins, BSA 10 mg/ml, ovalbumin 10 mg/ml, lysozyme 1 mg/ml; injection, 20 µl. Absorbance at 254 nm, 0.1 a.u.f.s.

Glucosesilica

In a first attempt glucose was coupled to the aminopropylsilica. This reaction has been described by Kiselev *et al.*[10]. Here we used different reaction conditions, with methanol instead of aqueous buffer and addition of cyanoborohydride to reduce the

TABLE II

LOAD OF GLUCOSE ON SILICA WITH RADIOACTIVITY MEASUREMENTS

Amount of glucose in reaction solution, 443 μmol + about 10 μCi of [^{14}C]glucose. Silica: aminopropyl-modified Hypersil (5 μm); NH_2 concentration, 500 μmol/g (with picric acid assay and titration); surface area, 170 m^2/g. Periodate treatment: theoretically only C-1 and C-2 counted, *i.e.*, one third of the glucose load.

Glucose solution (cpm)[a]	*Glucosesilica (cpm)*	*Aminopropyl-silica (mg)*	*Glucose load (μmol/m²)*	*Periodate treatment*		
				Periodate-treated glucosesilica (cpm)	*Aminopropyl-silica (mg)*	*Glucose load (μmol/m²)*
748 350	58 930	73	2.8	8967	64	1.5
	52 034	72	2.5	11 240	80	1.5

[a] cpm = Counts per minute.

Schiff base. Using radioactively labelled glucose we observed a coverage of 2.8 μmol/m^2 glucose (Table II). However, this radioactivity measurement does not discriminate between chemically bound and adsorbed glucose. The adsorbed glucose cannot be eliminated simply by more extensive washing, as this causes hydrolysis of bound material (see above). Therefore, we applied another method for the determination of the glucose moieties, using a periodate treatment preceding the radioactivity counting. The periodate hydrolyses diol functionalities, leaving only C-1 and C-2 of the glucose molecule fixed to the amino group. The degradation products containing the other carbon atoms can be assumed to elute easily in the washing steps. Assuming uniform carbon labelling, a significantly lower bonding of 1.5 μmol/m^2 was found.

Lysozyme, chromatographed on freshly prepared glucose-modified material, merges with the peaks of BSA and OVA (Fig. 4). However, all three proteins still show some retention and peak tailing. These results demonstrate that shielding of the silica surface and aminopropyl indeed occurs, but is not perfect.

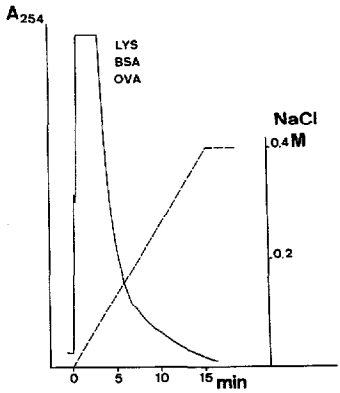

Fig. 4. Chromatogram of standard proteins on glucoseaminopropylsilica prepared from LiChrospher Si 300. Conditions as in Fig. 2.

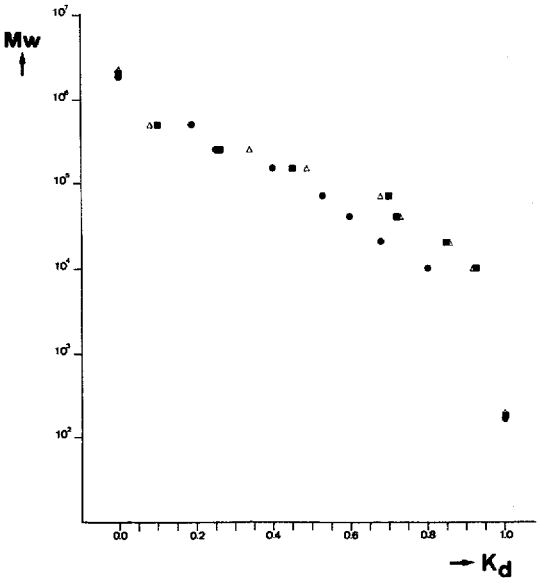

Fig. 5. Size-exclusion behaviour of dextrans. Silica (Hypersil WP 300) (■), glucosesilica (△) and epoxyglucosesilica (●), both prepared from Hypersil WP 300. Column, 450 × 3 mm I.D.; mobile phase, water; flow-rate, 0.2 ml/min. The distribution coefficient K_D is $(V_e - V_0)/(V_m - V_0)$, where V_e is the elution volume of the dextran. The interstitial volume V_0 corresponds to the elution volume of dextran 2 000 000; the total permeation volume V_m corresponds to the elution volume of glucose. Mw = Molecular weight.

The preservation of the pore structure was checked with a size-exclusion experiment (Fig. 5). No significant loss of pore volume is apparent.

The same column was used to study the interaction of the surface with standard proteins. As expected, during a 1-day experiment, the retention volumes shifted, indicating the instability of the stationary phase. Therefore, no in-depth study was done.

Epoxyglucosesilica

In another approach we applied a reaction between the amino moiety on the silica and an epoxy group present in a (protected) glucose derivative. Aminopropylsilica was reacted with methyl 2,3-anhydro-4,6-O-benzylidine-allopyranoside[18] (Fig. 6). The protecting benzaldehyde can be easily released by acid hydrolysis, after surface coating. We attempted to determine the surface coverage by means of this hydrolysis and subsequent determination of benzaldehyde (with the DNPH reaction[19]). However, the results corresponded to only 10–20% conversion of the amino groups. We assume that these results are much too low, in view of the observed shielding effect (see below). It is likely that part of the benzaldehyde is already lost in the coupling reaction. Indeed, a strong smell of benzaldehyde is observed on performing the reaction.

A chromatogram is shown in Fig. 7. Lysozyme is nearly unretained whereas BSA and OVA are retained. Compared with Fig. 2, a complete reversal of elution order is observed. It can be assumed that the retentions of the negatively charged OVA and

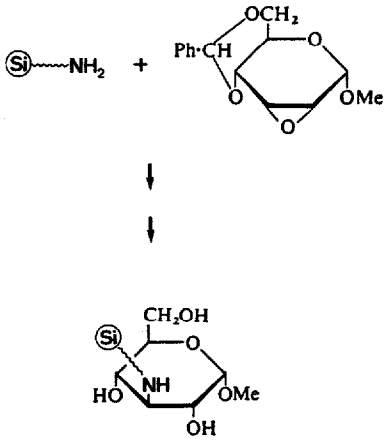

Fig. 6. Reaction of aminopropylsilica with epoxyglucose reagent. Me = Methyl; Ph = phenyl.

BSA are now caused predominantly by anion exchange on the remaining amino groups.

The stability of the layer was tested by monitoring the retention of lysozyme[12]. Although this is less direct a method than, *e.g.*, elemental analysis, it could give an indication of the loss of the glucose layer as bare silica retains lysozyme considerably. It appears that the 2-h reaction product is less stable than the 16-h product (Fig. 8). One explanation for the stability of the 16-h product could be a cross-linking reaction, presumably via the C-4 and C-6 atoms after the migration or release of benzaldehyde[20], or a polymerization reaction.

The size-exclusion behaviour of dextrans shows a slight change when compared with bare silica (Fig. 5). More specific measurements are necessary to study a possible small loss of pore volume.

Owing to the greater stability compared with the glucosesilica, the interaction of this stationary phase with standard proteins could be studied more thoroughly (Fig. 9).

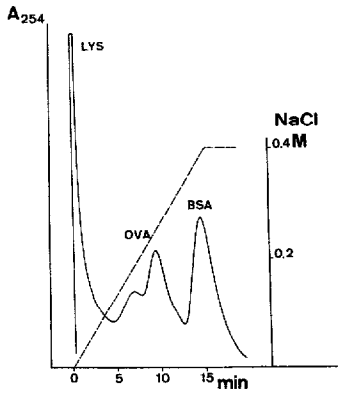

Fig. 7. Chromatogram of standard proteins on epoxyglucosesilica prepared from LiChrospher Si 300. Conditions as in Fig. 2.

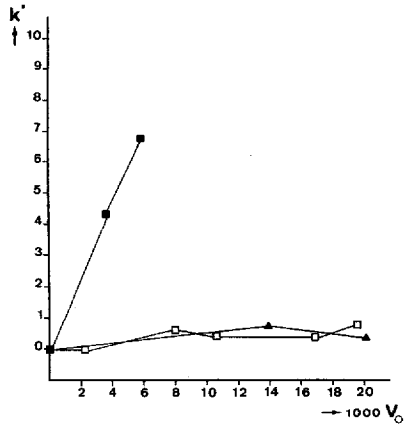

Fig. 8. Stability of epoxyglucosesilica, prepared from Hypersil WP 300, as estimated via the retention of lysozyme. (■) 2-h reaction product; (□, ▲) 16-h reaction product. Column, 60 × 3 mm I.D.; mobile phase, (■, □) 0.05 *M* NaH_2PO_4–Na_2HPO_4 (pH 7.0) + 0.001% phenylmercuriacetate (antimicrobial agent); (▲) same except pH 9.0; flow-rate, 1 ml/min. 20 000 V_0 (= 10 l) corresponding to 1 week of continuous use. Lysozyme, 1 mg/ml; injection, 20 µl. Uracil used as t_0 marker.

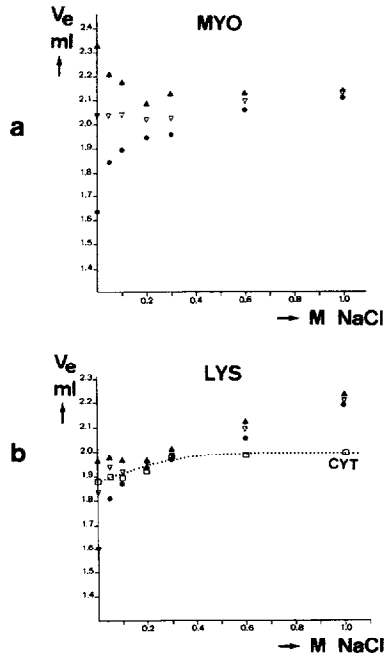

Fig. 9. Retention volumes of standard proteins on epoxyglucosesilica prepared from Hypersil (WP 300). Column, 450 × 3 mm I.D.; mobile phase, 0.01 *M* Na_2HPO_4–NaH_2PO_4, (●) pH 5, (▽) pH 7, (▲) pH 8, + NaCl (for cytochrome *c* only pH 7); flow-rate, 0.2 ml/min. Proteins: myoglobin, lysozyme, cytochrome *c*, 2 mg/ml. Injection, 20 µl.

The influence of increasing ionic strength on the retention of myoglobin (p*I* 7.3) is different for the three pH values: at pH 8 it decreases, at pH 7 it is nearly constant and at pH 5 it increases. This behaviour is opposite to that commonly observed with diol stationary phases[21]. It can be explained by assuming an anion-exchange interaction in this pH range. The behaviour of lysozyme, which is positively charged at all three pH values, also measured as a function of pH and ionic strength (Fig. 9b), indeed precludes the presence of any cation-exchange interaction. This would lead to stronger retention at low ionic strength, which is the opposite of what is observed. At high sodium chloride concentration the retention volume is larger than the estimated void volume of 2.0 ml. The hydrophobic nature of this retention, virtually independent of pH, is confirmed by the results obtained with cytochrome *c*. This small protein has a low hydrophobicity[22] and a constant retention volume at high ionic strength is indeed observed.

The shielding of the silica surface and the remarkable stability of the epoxyglucose silica identify this method as an interesting chemical approach; a small difunctional molecule reacted with aminopropylsilica can cover the acidic surface and give long-term stability at high pH (Fig. 8).

CONCLUSION

There are many chemical approaches to the coating of silica with a hydrophilic layer, two of which have been described here. In both instances a glucose molecule was bound to aminopropylsilica. The main objectives were good shielding of the silica surface and stability in aqueous mobile phases. Although the glucosesilica has a reasonable degree of surface load, it proved to be very unstable. With the epoxyglucosesilica there is good shielding of the acidic silica and very good stability even at high pH. However, the layer is not inert, and ionic and hydrophobic interactions remain. Pore volumes seem to be preserved in both instances.

Although these products on their own do not represent the ideal inert stationary phase, they demonstrate that with the right chemistry stable hydrophilic glucose layers should be attainable. Further attempts in this direction are now being made in our laboratory.

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