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Immobilization of reactive dyes on several matrices that allow high and very high flow-rates to be used

Application to the purification of a proteinase inhibitor from corn

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ABSTRACT

Several matrices were used for immobilization of the reactive dye Procion Red H3B: TSK HW 40, 65 and 55, Trisacryl GF 2000, Sephacryl S-200, Superose 6, Sepharose CL-4B and laboratory-prepared dextran-coated Spherosil XO15. The amounts of incorporated dye and the flow properties of the various supports were determined, in addition to their capacity for a trypsin inhibitor present in a corn-derived food product. It was shown that dextran-coated silica, TSK HW 55 F, Superose 6 and Sephadex G-50 F allowed immobilized dye chromatographic supports suitable for purification of this protein under high flow-rate conditions to be prepared. Actual results of purification obtained using dyed dextran-coated silica and dyed Sephadex G-50 F (settled in a squat column) are described and shown to compare favourably with those of a more traditional purification procedure.

INTRODUCTION

Immobilized dye chromatography has been shown to be a powerful technique for the purification of a host of different proteins[1]. It has been demonstrated by several workers that it could be beneficial when faced with the problem of purifying a given protein to search systematically among several tens of different dyes for that one which is most efficient for the purification[2–5]. Such approaches have been made using agarose as a matrix for dye immobilization, but it is well known that agarose is not

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a perfect support because it cannot withstand high pressures and flow-rates. It does not seem sound to perform screening experiments with agarose-based supports and subsequently to switch to better performing supports in terms of allowed flow-rates; indeed, it has been demonstrated that the nature of the matrix could be a significant factor in determining the capacity of immobilized dye chromatographic supports[6].

The optimum support for dye immobilization should conform to the following properties and conditions: it must be of good mechanical stability so as to withstand to high flow-rates; obviously full benefit of the eventual use of high flow-rates can be taken only if the capacity of the dyed support does not level off too much with increasing flow-rate; the support must allow the use of the very straightforward chemistry commonly used to graft directly textile reactive dyes onto agarose matrices (hence this implies in particular that the support must be able to withstand to the aggressive alkaline conditions that are commonly used for immobilization of reactive textile dyes); it must be commercially available or at least easily prepared from commercially available materials; and it must be inexpensive.

This paper presents the results of a search for the optimum support and its application to the purification of a trypsin inhibitor present in a corn-derived food product. This inhibitor, named popcorn inhibitor (PCI), is known to inhibit activated Hageman factor but not kallikrein, another serine proteinase implicated in the contact activation of plasma coagulation[7]. An effective purification procedure for PCI has already been described using immobilized dye in the first step[8].

EXPERIMENTAL

Materials

Dextran T-500, Sephadex G-50, Sephacryl S-200, Superose 6 Prep Grade and Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden) and Trisacryl GF 2000 M and Spherosil XO15 M from IBF Biotechnics (Villeneuve la Garenne, France). TSK HW 55 and HW 65 gels were Tosoh products and were either purchased or obtained as a much appreciated gift from Merck Clevenot (Nogent sur Marne, France). Chemicals were bought from Aldrich Chimie (Strassbourg, France) or from Merck. Procion Red H3B was a kind gift from ICI France (Clamart, France).

Preparation of dextran-coated silica

The preparation of dextran-coated silica has been described in detail elsewhere[9]. Briefly, Spherosil XO15 M was impregnated with specially prepared DEAE-dextran (dextran T-500 derivatized with diethylaminoethyl chloride so that 4% of glucose units carried a DEAE substituent). After impregnation, the polymer layer was stabilized using butanediol diglycidyl ether as a reticulating agent.

Dye immobilization onto the various supports

The same method was used for grafting of dyes onto the various chromatographic supports and is similar to those in common use[1]. The chromatographic support was rinsed on a Buchner funnel with water then with 0.2 *M* sodium hydroxide solution containing 2% (w/v) sodium chloride. The support was then transferred to a vessel containing a suitable amount of reactive dye (20 mg per millilitre of support) and the same solution as above was added to the vessel up to a final volume twice that

of the settled support. The vessel was then tumbled at 60°C for 1 h. The support was then quickly rinsed with 2% sodium chloride in 0.2 *M* sodium hydroxide solution and transferred to a new vessel containing fresh reactive dye and treated as above. The experiments described below were done with various supports treated three times. At the end of the last treatment the supports were rinsed exhaustively with 10 m*M* sodium hydroxide solution, water, 6 *M* urea and water again.

This immobilization method will be referred later in the text as the direct immobilization method, as opposed to those other methods from the literature in which dye is first modified by grafting of a spacer arm before immobilization onto the chromatographic support.

Evaluation of amounts of dye grafted onto the supports

Amounts of incorporated dye in most supports were evaluated by hydrolysis methods; basically, one volume of dyed support was hydrolysed in ten volumes of hydrolysis medium for durations sufficient to induce either complete solubilization or at least complete discoloration of the supports (in this case the coloured supernatant was recovered by filtration).

The hydrolysis conditions were 50% acetic acid in water at 110°C for most supports, but for dyed Trisacryl hydrolysis was effected in methanol–sulphuric acid mixture (5:20, v/v) at room temperature for 30 min. The amounts of dye liberated through these hydrolysis methods were determined by spectroscopic measurements.

No satisfactory hydrolysis method could be worked out to evaluate the amount of dye incorporated into the deep red Sephacryl S-200. For determination of the amounts of dye incorporated into the dyed dextran-coated silica samples, an alkaline hydrolysis method taken from the literature[10] was found to be not satisfactory (a large residue of undissolved, uncompletely decolorized silica was constantly obtained even after long incubation times), so another method was adapted from data published elsewhere[11]: the dyed dextran layer was stripped from the coated silica by incubation in fuming nitric acid (2 ml for 50 mg of dyed support) for 15 h at 20°C. and the amount of solubilized dye derivative was then evaluated through spectroscopic measurements.

Evaluation of flow properties of supports

To evaluate the flow properties of the supports they were sedimented in Pharmacia FPLC HR 5/5 columns up to a height of 5 cm. The columns were equilibrated at 0.05 ml/min in 30 m*M* sodium phosphate buffer (pH 6.5) containing 2 *M* sodium chloride (buffer B), the pressure was recorded and thereafter the flow-rate was increased in increments of 0.05 ml/min, the pressure was allowed to come to a stable value and recorded. TSK gels were settled according to recommendations given by the supplier of the gel, *i.e.*, they were sedimented to constant height at a back-pressure of 4 bars.

Evaluation of capacities of dyed supports for popcorn inhibitor

The capacities were evaluated by frontal chromatography. The dyed supports were sedimented to a height of 2.8 cm in Pharmacia FPLC HR 5/2 columns, and equilibrated in 30 m*M* sodium phosphate buffer (pH 6.5) containing 20 m*M* sodium chloride (buffer A). Thereafter polenta extract prepared as indicated below was pumped to the column. The effluent was collected in 1-ml fractions and the amounts of

PCI present in the fractions were evaluated by measurement of its inhibitory activity towards trypsin.

Preparation of polenta extract and buffers used in the purification procedures

The advantage of using polenta, a traditional Italian corn-derived food product, as a source for purification of PCI instead of the more commonly used corn kernels has been described elsewhere[8]. Polenta obtained from a local food store was suspended in buffer A (400 ml of buffer for 100 g of polenta), toluene added (80 ml per 100 g of polenta) and the suspension shaken overnight in a cold room. The aqueous phase was then filtered through a Whatman 1 MM filter-paper and the filtrate used for purification. Buffers used for chromatography of polenta extracts on the immobilized dyes were buffer A, buffer B with the same composition as buffer A but containing 2 *M* sodium chloride) and buffer C (with the same composition as buffer B but containing 6 *M* urea).

RESULTS AND DISCUSSION

Amounts of dye immobilized on the different matrices

A list of the dyed supports and relevant characteristics taken from the manufacturers' data are given in Table I. Also are given the amounts of dye that were grafted on each individual matrix.

TABLE I

CHARACTERISTICS OF THE VARIOUS DYED SUPPORTS PREPARED FOR THIS STUDY

Matrix	*Bead diameter (μm)*	*Chemical nature of matrix*	*Amount of dye incorporated (μmol/ml of settled support)*	*Letter code used for identification of relevant chromatograms in Fig. 1*
TSK HW 40 F	32–63	Poly(vinyl alcohol)	22.6	A
TSK HW 65 F	32–63	Poly(vinyl alcohol)	11.6	B
TSK HW 65 S	25–40	Poly(vinyl alcohol)	11.3	C
TSK HW 55 F	32–63	Poly(vinyl alcohol)	31.0	D
TSK HW 55 S	25–40	Poly(vinyl alcohol)	16.3	E
Superose 6 prep grade	20–40	Cross-linked agarose	3.7	F
Sepharose CL-4B	40–165	Cross-linked agarose	11.2	G
Sephadex G-50 SF	20–50	Cross-linked dextran	18.8	
Sephadex G-50 F	20–80	Cross-linked dextran	5.6	H
Sephadex G-50 C	100–300	Cross-linked dextran	3.8	I
Sephacryl S-200 SF	40–105	Allyldextran cross-linked with N,N'-methylenebisacrylamide	n.d.	
Trisacryl GF 2000 M	40–80	Hydrophilic acrylic polymer	0.4	
Dextran-coated Spherosil XO15 M	40–100	Composite support	24.6	J

The smallest amount of incorporated Procion Red H3B was obtained with Trisacryl GF 2000, and indeed it was very low. The manufacturer of Trisacryl GF 2000 markets Red Trisacryl on which is grafted the related dye Procion Red HE 3B. However, Procion Red HE 3B is incorporated into Trisacryl by the manufacturer in a very different way: the dye is first derivatized with diaminohexane and thereafter aminohexyl dye is grafted onto the gel activated by suitable means. Another procedure was described recently[12] in which Red Trisacryl is prepared by copolymerization of the usual monomers of Trisacryl together with an acrylic derivative of the dye.

The disappointing results obtained with Procion Red H3B and Trisacryl GF 2000 preclude the use of this latter matrix as a first choice for the preparation of a panel of several different immobilized dyes for dye screening procedures, even though with other dyes and the same technique we could incorporate dyes into Trisacryl at a comparatively satisfactory level (data not shown).

It is worth noting that a fairly large amount of dye could be grafted onto dextran-coated silica, even though the grafting technique was very simple; this is an obvious advantage of the dextran coating. Another advantage is that the coating makes the silica resistant to alkaline conditions; this is demonstrated by the fact that the alkaline hydrolysis method allowing dyed silica (prepared by grafting of an aminohexyl derivative of the dye onto epoxy silica[10]) to be dissolved was found to be ineffective with dextran-coated silica.

Flow properties of the dyed supports

Plots of flow-rate *versus* pressure were established for most of the dyed supports listed in Table I. Three different dyed supports followed Darcy's law up to the highest linear flow-rate tested (25 cm/min), *viz.*, dextran-coated silica, Sephadex G-50 coarse and TSK HW 40 F (with the latter gel, the flow-rate *versus* pressure plot showed a slight upward curvature), the back-pressures generated at 25 cm/min being 0.8, 1.9 and 6 bar, respectively.

Several other gels followed Darcy's law only up to limiting values of flow-rate and pressure, higher flow-rates being followed by a very large increase in back-pressure. The limiting flow-rates found for dyed Sephadex G-50 F and SF were 7.5 and 1.25 cm/min, respectively (limiting pressure values 0.75 and 1 bar, respectively). The limiting values of flow-rate and pressure for dyed Sephacryl S-200 were found at 5 cm/min and *ca.* 0.5 bar, respectively, and for both Sepharose CL 4 B Superose 6 prep grade at ca. 12 cm/min and 1.3 bar, respectively. We assume that limiting values of pressure are reached when irreversible gel collapse occurs; in this regard, it should be emphasized that once the limiting pressure had been reached, a second recording of the curve of flow-rate *versus* pressure showed a greatly lowered permeability of the gel columns.

TSK gels HW 65 and 55 showed a different behaviour. With TSK HW 65 F and S the curves of flow-rate *versus* pressure deviated progressively from linearity with an upward curvature, but buffer could be pumped at velocities in excess of 20 and 18 cm/min, respectively, at a back pressure of 8 bar. The permeability of TSK HW 55 seemed to be lower and when the flow-rates were increased above 7.5 and 2 cm/min with F and S grade, respectively, large increases in the generated back-pressure were observed (it must emphasized, however, that TSK gels did not show signs of irreversible collapse of the gel structure; we recorded variations of pressure with

increasing flow-rate twice and the results were identical in both instances. This is in contrast with the results obtained with the polysaccharidic gels the performance of which has been described earlier and which showed a greatly diminished permeability after having been exposed once to elevated pressure.

Capacities of the dyed supports

Results of the measurements of capacity by frontal chromatography of crude polenta extract are shown in Fig. 1. The capacity for PCI was low with dyed TSK HW 40F (curve A). This poor result was not unexpected as this support has an exclusion limit for proteins of 5 kDa according to its manufacturer; hence the interaction of PCI (migrating as a 14.4 kDa protein on SDS gels) is likely to occur only with those dye molecules which are grafted on the outside surface of the gel beads.

TSK HW 55 gels were seen to perform better than TSK HW 65 gels (but less dye was incorporated in the TSK HW 65 gels). Curves B, B′ and C show the results obtained with dyed TSK HW 65 gels. With TSK HW 55 F it was possible at 0.5 cm/min to load more than 55 ml of crude extract before any inhibitory acitivity could be found in the eluate from the 0.56-ml volume column (see curve D). The capacity decreased with an increase in flow-rate but was still *ca.* 40 ml (see curve D′, which was obtained with this gel at 5 cm/min). Dyed TSK HW 55 S gel was less satisfactory than dyed TSK HW 55 F (compare curves E and D, respectively), but this lower performance can be explained by the lower level of substitution which was obtained with this matrix.

Dyed Superose demonstrated an adequate capacity for PCI, as *ca.* 50 ml could be pumped onto the gel at high flow-rate before the inhibitory activity did begin to elute from the column (see curves F and F′).

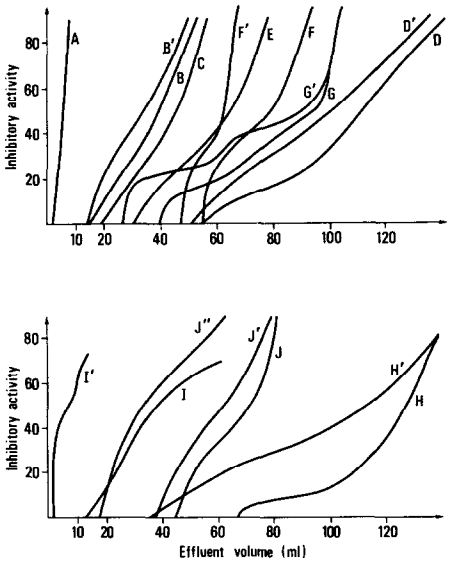

Fig. 1. Frontal chromatography of polenta extract on several different dyed chromatographic supports at high flow-rates. Inhibitory activities (ordinate) are expressed as percentages of the activity present in the starting material. Letter codes used to identify the chromatograms are given in Table I. The letter is followed with a prime when the chromatogram was obtained at a linear flow-rate of 5 cm/min. Chromatogram J″ was obtained at 25 cm/min and all other chromatograms at 0.5 cm/min.

Curves G and G′ show the performance of dyed Sepharose CL-4B at 0.5 and 5 cm/min, respectively. These curves and also data given earlier on the flow properties of this dyed gel demonstrate that Sepharose CL-4B can be used at much higher flow-rates than are commonly used.

Among all the gels tested, dyed Sephadex G-50 fine showed the highest capacity for PCI; *ca.* 70 ml of crude extract could be pumped at 0.5 cm/min onto the column before any inhibitory activity was eluted (see curve H). Indeed, when flow-rate was increased 10-fold the capacity decreased but to the still satisfactory value of *ca.* 40 ml of crude extract for the 0.56-ml column used (see curve H′).

The results obtained with dyed Sephadex G-50 coarse were much less satisfactory; the capacity for PCI at a linear flow-rate of 0.5 cm/min (see curve I) is less than 15 ml and when the linear flow-rate was increased to 5 cm/min the capacity decreased almost to zero (curve I′).

The results obtained with dyed dextran-coated silica demonstrate that the capacity was satisfactory [more than 40 ml of crude extract for a 0.56-ml volume column operated at a linear flow-rate of 0.5 cm/min (see curve J)]. The capacity did decrease slightly when the flow-rate was increased 10-fold (see curve J′), but the column could be operated at flow-rates as high as 25 cm/min and nevertheless retained a significant capacity (see curve J″).

Unexpectedly, PCI was not retained at all by dyed Sephacryl S-200 (frontal chromatogram not shown). Indeed, PCI permeates freely in the inside porous volume of the beads, hence it can only be said that dye is not available for interaction with protein, possibly because of some interaction with the matrix. We should emphasize here that our results do not demonstrate that the latter gel cannot be used for dye immobilization; it is possible that, by using a different chemistry for grafting, *e.g.*, by using spacer arms, efficient dyed supports could be obtained. Nevertheles, we stress that Sephacryl S-200 cannot be a good choice as a matrix for preparing by the direct immobilization method a panel of immobilized dyes for screening.

Taken together, these results show that several supports could be used to purify PCI from polenta extract, *i.e.*, they have a satisfactory capacity even at high flow-rates; those which could be considered as good choices are Superose, Sephadex G-50 fine, TSK HW 55 F and dextran-coated silica. Only the last support is sufficiently rigid to allow in practice the pressure strain induced by high flow-rates to be ignored. TSK HW 55 F seems to be fairly resistant (even if TSK HW 65 did show a better flow-rate *versus* pressure curve); with TSK HW 55 F no clue of gel collapse was observed when this support was submitted to a flow-rate and pressure of 22 cm/min and 8 bar, respectively, hence it seems probable that this gel would be easy to use for protein purification under high flow-rate conditions. The data presented show that the other gels (Sephadex G-50 F and Superose) can be used at fairly high flow-rates but the applied pressure must be kept below *ca.* 0.75 bar for dyed Sephadex G-50 F and 1.3 bar for dyed Superoseses in order not to induce irreversible collapse of the gel structure; this obviously sets limits to the column geometry as high flow-rates will be possible only in short columns.

Purification of PCI using dyed dextran-coated silica

Dextran-coated silica was used for the large-scale purification of PCI from polenta extract. A column of small volume (10 cm × 1 cm I.D.) has been used but

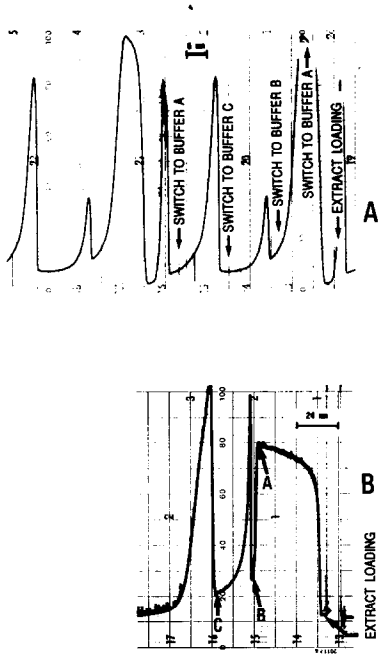

Fig. 2. (A) Recorder trace of automated cyclic chromatography of polenta extract on a Procion Red H3B–dextran-coated silica column. Flow-rate, 5 cm/min. PCI, eluted by buffer C, was adequately automatically collected. (B) Recorder trace of chromatography of polenta extract on a Procion Red H3B–Sephadex G-50 fine column under high flow-rate conditions: the linear flow-rate was 2.5 cm/min during extract loading and column rinsing with buffer A and B and was lowered to 1.25 cm/min during elution of partially purified PCI by buffer C. Arrows A, B and C indicate when buffers A, B and C, respectively, were pumped to the column.

significant amounts were nevertheless treated by repeating purification cyclically under full automatic control. The column was operated at a linear flow-rate of 5 cm/min. To date the column has been used for more than 600 cycles. Fig. 2 shows the recorder trace for some cycles. The yields, and purification factors are given on Table II.

Even if dyed dextran-coated silica seems to fulfil most of the criteria which have given in the Introduction, it is not yet commercially available and even though the technique for coating is relatively straightforward, to date we have not prepared dextran-coated silica on a large scale. It must be said also that less favourable results

TABLE II

COMPARISON OF THREE DIFFERENT PROCEDURES FOR PURIFICATION OF PCI FROM POLENTA BY IMMOBILIZED DYE CHROMATOGRAPHY

	Yield (%)	*Purification factor*
Procion Red H3B–dextran-coated silica	86	6.4
Procion Red H3B–Sephadex G-50 F	89	4.1
Procion Red HE3B–agarose[8]	84	6.7

have been obtained with another dye, another protein and another batch of dextran-coated silica[13].

From the data in Fig. 1 and current prices, it could be calculated that to treat the same amount of polenta extract in a single pass at high flow-rate, the cost of the purchase of Superose 6 would be 40 times that of Sephadex G-50 F (and those of TSK HW 55 F and Spherosil XO15 would be five times those of Sephadex G-50 F). Clearly dyed Sephadex G-50 F, which has already been shown to be the gel with the highest capacity, also has the highest capacity/cost ratio. This provided the impetus to attempt to use this latter support for the large-scale preparation of PCI from polenta extract.

Purification of PCI using dyed Sephadex G-50 F

Obviously, because of pressure limitations with this matrix, we had to use a squat column (1.8 cm × 5 cm I.D.). The flow-rate was 2.5 cm/min (50 ml/min), except during elution with buffer C (it was observed that during elution of proteins retained on the column by this urea-containing buffer the back-pressure increased, so, for safety, we halved the flow-rate). A recorder trace is shown on Fig. 2. Partial purification of PCI from 1800 ml of polenta extract could be completed in 100 min. The yield was satisfactory (see Table II), but the purification factor was lower than that one obtained using immobilized dye chromatography in a more conventional way, and with a lower througput (1300 ml of extract were treated per day in an immobilized dye column five times larger than that one described here and filled with Procion Red HE 3B–agarose[8]). PCI obtained after a second and anyway necessary purification step (reversed-phase chromatography) was in fact of the same purity with both methods.

General comments and conclusions

We are well aware that our statement that Sephadex G-50 can be chosen as a matrix for dye immobilization for the chromatography of proteins at high flow-rates could seem in some ways provocative. Many efforts are being devoted to the development and marketing of efficient supports for protein purification based on mineral matrices or new resistant polymers but nevertheless our results demonstrate that Sephadex G-50, the development of which began *ca.* 30 years ago[14], can be used advantageously under high flow-rate conditions provided that care is taken to use it in short columns.

Indeed Sephadex G-50 F, which is cheap and commercially available, could be used for preparing a panel of different immobilized dyes in order to establish those most suitable for protein purification. We obviously bear in mind that this gel is of limited porosity and therefore it cannot be envisioned that one would use it to purify proteins of high molecular weight. However, proteinase inhibitors from plants in which we are interested are usually low-molecular-weight proteins and therefore in this instance Sephadex G-50 F could be proposed as an advantageous substitute to agarose, commonly used for making panels of immobilized dyes. Indeed, Sephadex G-50 F, because of its pressure limitations, can be used under high flow-rate conditions only in short columns, hence it sets practical limits to column volume, and therefore probably use of Sephadex G 50 F under large flow-rate conditions will be limited to laboratory use.

In contrast, dextran-coated silica, because of the mechanical strength of the silica matrix, can be settled in columns of almost any volume[15]. Our results show that

dextran coating imparts great chemical resistance to the matrix and also allows very convenient grafting of dyes onto the support, this enabled us to prepare easily an efficient support for protein purification.

Several papers have already demonstrated that dyes could be immobilized onto silica and used advantageously for protein purification under high flow-rate conditions[10,16–18] and even on a process scale[19]. However, from reported data it appears that in order to obtain dyed supports with sufficiently high levels of incorporated dye it was necessary either to prepare aminohexyl derivatives of the dyes prior to grafting them onto epoxy-activated silica[10,16,17] or to use only the more reactive dichlorotriazinyl reactive dyes for direct grafting onto diol silica[17,19]. Direct grafting of monochlorotriazinyl dyes onto aminoalkylated silica was also shown to be feasible, but a large proportion of amino alkyl chains remained unmodified at the end of the derivatization procedure, which might be troublesome for protein purification even though satisfactory results have been obtained with one model protein[18].

In contrast, from our data it is clear that dye immobilization on dextran-coated silica is as straightforward as it is on agarose, moreover dextran-coated silica prepared as described above has been used for size-exclusion chromatography of proteins, making it clear that such coated silica is devoid of unwanted interactions with proteins[9].

Finally, we are aware that the satisfactory results which we have presented in this paper must be backed up by further studies with other dyes and other proteins, but nevertheless it seems plausible that dextran-coated silica could be a matrix of choice for immobilization of dyes and the industrial use of immobilized dye chromatography. Probably such developments will have to await the commercial availability of coated silica.

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