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The reversible binding of anti-human serum albumin to poly β-cyclodextrin-coated porous silica supports☆

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Abstract

A supramolecular system involving host–guest interactions between immobilized β-cyclodextrin (β-CD) cavities and adamantyl groups was evaluated for the preparation of immunosorbents which can be regenerated after use. First a dextran layer bearing both adamantyl groups and carboxylic functions is immobilized onto β-CD-modified porous silica particles (400 nm) by formation of inclusion complexes. Then, antibody molecules are grafted to the polymer layer. The stationary phases can be prepared in batch or directly in the column. They are stable in aqueous media and are able to trap specifically the corresponding antigen. In case of alteration of the antibody layer, it is possible to remove it by passing a SDS solution through the column. The feasibility of the procedure was evaluated, using the anti-HSA/HSA system.

Keywords: β-Cyclodextrin; Adamantane; Immunoaffinity chromatography; Human serum albumin

1. Introduction

The strength and specificity of interactions between antigens and antibodies commonly allow an effective purification of these biomolecules by immunoaffinity chromatography. Due to the development of performant chromatographic supports and immobilization procedures, this technique has become, during the last 20 years, an invaluable tool in the life sciences for the production of antibodies and antigens [1–6]. However, rather harsh conditions are often necessary to elute these biomolecules from the columns, resulting in a limited life-span of antibody or antigen stationary phases. For this reason, it may be useful to remove the spoiled biomolecules from the columns. Nevertheless, this approach cannot be carried out when antibodies or antigens are bound covalently to the support.

Recently, we described a supramolecular system involving host–guest interactions, which permits the reversible immobilization of molecular layers on planar gold surfaces [7]. The procedure was evaluated for the binding of adamantane-modified macromolecules (polymers or proteins) to β-cyclodextrin-functionalized surfaces. β-Cyclodextrin (β-CD) is a cone-shaped cyclic oligosaccharide ("host" molecule) known to form inclusion complexes with compounds containing hydrophobic groups ("guest" compounds) [8]. In some cases, the resulting "host–guest" complex may be quite stable [9]. More specifically, the interactions between β-cyclodextrin-polymers and adamantane-modified macromolecules allowed recently the formation of polymolecular systems which were very stable in aqueous media [7,10,11]. The binding was carried out under mild conditions and had rapid kinetics. Moreover, the strength of "host–guest" interactions decreased in the presence of organic solvents (e.g. acetonitrile) or surfactants (e.g. SDS) [12]. The procedure was also applied to the immobilization of adamantane-modified antibodies onto flat β-CD-coated gold surfaces. It was demonstrated by surface plasmon resonance (SPR) that the resulting biomolecule layers could be removed after use, by passing a SDS solution in the flow cell of the SPR system [11].

In the present study, the β-CD/adamantane system was evaluated for the preparation of porous immunoaffinity supports. Polyclonal anti-human serum albumin (anti-HSA) was used as a model antibody to test the feasibility of the procedure. The immunosorbents were prepared according a two-step procedure. First, a dextran layer bearing both adamantane groups and

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carboxylic functions was immobilized onto a β-CD-modified porous silica support. Then the antibody was bound to COOH functions and its HSA-binding activity was controlled. Additionnally, we examined the conditions under which the immunosorbents obtained by this procedure could be regenerated.

2. Experimental

2.1. Materials

Diol-bonded silica (Nucleosil 4000-7OH: particle size, 7 μm; porosity, 400 nm; surface area, 10 m^2/g) was from Macherey-Nagel (Düren, Germany). 1,4-Diisocyanatobutane, triethylamine, dibutyltin dilaurate (DBLT), 4-dimethylaminopyridine (DMAP), 1-adamantanecarbonyl chloride, *N*-hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), succinic anhydride, pyridine, *N*,*N*-dimethylformamide (DMF) and sodium dodecylsulfate (SDS) were purchased from Aldrich (St Quentin Fallavier, France). All solvents were obtained from SDS (Peypin, France). Rabbit anti-human serum albumin (total IgG fraction) and human serum albumin (HSA; purity 97–99%) were from Sigma (St. Quentin Fallavier, France). Water was purified with a MilliQ RG system (Millipore, Bedford, MA, USA). Dextran T 10 (M = 10,000 g/mol) was purchased from Amersham Pharmacia Biotech (Orsay, France). The polymer of β-cyclodextrin was synthesized in our laboratory [13].

2.2. Chromatographic system

The HPLC system used consisted of two pumps (LC 9A, Shimadzu, Kyoto, Japan), a sample injector with a 0.02 mL loop (model 7125, Rheodyne, Berkley, CA, USA) and a variable wavelength UV detector (Spectra 100, Thermo-Finnigan, San Jose, CA, USA). A 2 mL loop was used for the on-line binding of anti-HSA.

Frontal measurements were performed with a six-port commuting valve placed before the sample injector to switch from one eluent to the other. Chromatograms were recorded with a Kipp and Zonen chart recorder (type BD 41, Delft, The Netherlands).

2.3. Methods

2.3.1. Preparation of poly β-CD-grafted silica (CD-Sil400)

Poly β-CD-grafted silica was prepared using a two-step procedure described in the case of 30 nm silica particles [14]. Due to the lower surface area of 400 nm diol-silica (10 m^2/g), the amounts of reagents were decreased. Briefly, 0.4 g (2.8 mmol) of 1,4-diisocyanatobutane, 25 μL of triethylamine and 25 μL of DBLT were added to a suspension of diol-silica (1 g) in 1,2-dichloroethane (10 mL). After reaction the isocyanate-modified silica (1 g) and 0.21 g (1.7 mmol) of DMAP were added to a solution of poly β-CD (0.5 g) in pyridine (77 mL). The number-average molecular mass of poly β-CD used in this study was 30,000 g/mol. After 48 h at room temperature, the poly β-CD-grafted silica was washed with pyridine, then with ethanol.

2.3.2. Preparation of Ad-Dext-COOH

The dextran T10 (MW = 10,000 g/mol) was first modified by 1-adamantanecarbonyl chloride in DMF at 80 °C, as described previously for the dextran T40 (MW = 40,000 g/mol) [14]. The adamantane-modified dextran (Ad-Dext) was further fonctionalized with carboxylic groups, using succinic anhydride [14]. Ad-Dext and Ad-Dext-COOH polymers were charaterized by ^{1}H NMR.

2.3.3. Immobilization of Ad-Dext-COOH onto the poly β-CD-grafted silica

The immobilization of Ad-Dext-COOH onto poly β-CD-grafted silica was carried out in two different ways, using either a batch or a dynamic procedure. In this study, the dynamic method was only used for the immobilization of Ad-Dext-COOH onto regenerated CD-Sil400 columns but it can be carried out as well on fresh cyclodextrin-modified supports [14]. In the batch procedure, a 30 g/L Ad-Dext-COOH solution was prepared in 1 M NaCl. Then, 470 mg of CD-Sil400 were added to the polymer solution (2.5 mL). The suspension was stirred overnight at room temperature. Thereafter, the slurry was filtered and the stationary phase (Dext-CD-Sil400) washed extensively with water. In the dynamic procedure, a chromatography column (30 mm × 4.6 mm) was filled with the CD-Sil400 support. Then, the Ad-Dext-COOH solution (1 g/L in 1 M NaCl) was passed for 90 min through the column at a flow rate of 1 mL/min. Last, the resulting Dext-CD-Sil400 column was rinsed with water.

2.3.4. Preparation of the anti-HSA-modified support (anti-HSA-Sil400)

Carboxylic groups of the Dext-CD-Sil400 support or column were first activated by reaction with NHS (50 mM in water) in presence of EDC (0.2 M), for 60 min. After activation, the stationary phase was rinsed with water. When the coupling of anti-HSA to the NHS-modified silica was performed in batch, the support (400 mg) was suspended in 2 mL of the antibody solution (9.5 mg/mL of total proteins in 10 mM saline phosphate buffer, pH 7.2; 5.5 mg/mL of specific antibody). The mixture was kept under gentle stirring for 3 h at room temperature. After reaction, the antiHSA-Sil400 support was washed with water before being sucked up under vacuum into a PEEK chromatography column (30 mm × 4.6 mm I.D.). In the dynamic procedure, 1.8 mL of the antibody solution (14 mg/mL of total proteins; 4.9 mg/mL of specific antibody) were injected at a flow rate of 0.05 mL/min into the NHS-activated Dext-CD-Sil400 column, using water as a mobile phase. The absorbance of the column outlet was recorded at 280 nm.

The specificity of the interaction between HSA and anti-HSA-Sil400 immunosorbents was evaluated by injecting in the column 20 μL of various negative proteins (HSA, α-lactalbumin, ovalbumin and β-lactoglobulin) at a concentration of 1 g/L, using a PBS elution buffer (10 mM phosphate buffer, 2.7 mM KCl, 0.137 M NaCl; pH 7.4). Proteins were monitored at 280 nm.

2.3.5. Binding of HSA to (anti-HSA-Sil400) columns

Before use, immunoaffinity columns were washed with a 10 mM phosphate buffer solution (pH 8.0), for 4 h at

1 mL/min, to remove unreacted NHS-ester functions. Then the columns were equilibrated with PBS (pH 7.4). HSA solutions (0.01–0.05 mg/mL in PBS) were pumped through the columns at 0.5 mL/min until equilibrium was reached (plateau at 280 nm). After HSA percolation, the columns were rinsed with the PBS buffer.

2.3.6. Desorption of HSA from the columns

The desorption of HSA from the columns was performed using an acidic 0.1 M glycine buffer (pH 2.1). The solution was passed through the column for 1 h at 1 mL/min. Then the column was washed with PBS.

2.3.7. Restoration of the initial polyCD-Sil400 column

The initial polyCD-Sil400 column was restored, using sodium dodecyl sulfate (10 g/L) in 0.1 M glycine buffer (pH 2.1). The regeneration solution was passed through the column at 1 mL/min for 40 min. Thereafter, the column was extensively washed with PBS.

3. Results and discussion

3.1. Preparation of the immunoaffinity supports

In a recent study, it was shown that the β-CD/adamantane system could be used to immobilize functionalized polymer layers onto β-CD-modified silica particles (30 nm porosity), by formation of inclusion complexes [14]. The feasibility of the procedure and stability of resulting stationary phases (Dext-CD-Sil30) in aqueous media were demonstrated, using Ad-Dext-COOH as a model polymer. In the present study, anti-HSA was grafted to Dext-CD-Sil supports prepared using this method. The properties of the resulting immunosorbents were investigated. The β-CD-modified stationary phase was obtained by grafting a polymer of β-CD (MW = 30,000 g/mol) to diol-silica particles. A porosity of 400 nm was chosen to enable the diffusion of the poly β-CD and proteins into the pores of the support (CD-Sil400). Due to the low surface area of the material, the reaction conditions were adjusted, allowing the binding of 15 mg of poly β-CD per gram of support (determination by elemental analysis).

In order to favour the formation of inclusion complexes between adamantane groups and β-CD cavities, the immobilization of the Ad-Dext-COOH layer was carried out using a saline dextran solution. In the case of similar 30 nm stationary phases, indeed, it was demonstrated that the amount of dextran bound to the support increased with the salt concentration. For example, the ion exchange capacities at pH 7.0 of Dext-CD-Sil30 supports prepared either in the presence of 1 M NaCl or without salt were equal to 0.13 and 0.01 meq/g, respectively. Therefore, Ad-Dext-COOH solutions used herein for the immobilization step onto CD-Sil400 supports contained 1 M NaCl. In these conditions, 21 mg of Ad-Dext-COOH were bound per gram of support (determination by elemental analysis), when using the batch procedure. Interestingly, despite the tenfold lower surface area of the 400 nm material compared to 30 nm silica (10 and 100 m^2/g, respectively), the amount of dextran immobilized on CD-Sil400 herein was higher than the value reported previously for Dext-CD-Sil30 prepared in pure water (7 mg/g) [14], proving the influence of NaCl during the immobilization step.

Just before the protein coupling, the Dext-CD-Sil400 stationary phase was activated by converting the carboxylic groups into reactive NHS-ester functions using a classical procedure [15,16]. Then, the support was exposed to the anti-HSA solution at pH 7.2 during 3 h (batch procedure). The resulting immunoaffinity sorbent (anti-HSA-Sil400) was packed into a short chromatography column (30 mm × 4.6 mm) and its ability to bind HSA was evaluated.

3.2. Properties of anti-HSA-Sil400 columns

After deactivation of residual NHS-ester groups at pH 8.0, the performances of anti-HSA-Sil400 columns in terms of specificity and binding capacity were studied.

3.2.1. Specificity of the interaction between HSA and anti-HSA-Sil400 columns

In order to test the specificity of the interaction between HSA and anti-HSA-Sil400 immunosorbents, various negative proteins (HSA, α-lactalbumin, ovalbumin and β-lactoglobulin) were injected in the column at a concentration of 1 g/L, using a PBS elution buffer (pH 7.4). In these conditions, HSA was irreversibly adsorbed on the support whereas other proteins were eluted with retention factors between 0.1 and 0.5. The protein recovery was calculated as the ratio between the peak area obtained on the anti-HSA-Sil400 column and that obtained on a CD-Sil400 reference column. Values near 100% were determined for α-lactalbumin, ovalbumin and β-lactoglobulin. These results suggest that the interaction between HSA and anti-HSA-Sil400 supports can be considered as specific.

3.2.2. Adsorption and desorption of HSA

The adsorption of HSA onto anti-HSA-Sil400 columns was studied by frontal analysis, by pumping through the column various HSA solutions. An experimental breakthrough curve is illustrated in Fig. 1, indicating a sharp increase of the elution front with a V_{50} volume much higher than the void volume of the column (V_{50} = 45 mL; V_m = 0.32 mL). Moreover, it appears that the amount of protein desorbed during the washing step is negligible. This curve clearly reveals that protein was adsorbed onto the column during the HSA percolation. The sharp profile at the initial stage of adsorption was followed by a slower increase in the second part of the breakthrough curve (Fig. 1). Such adsorption profiles are the result of a complex kinetic adsorption process on polyclonal antibodies, as demonstrated by Renard et al. who observed similar breakthrough curves for the adsorption of HSA onto polyclonal anti-HSA-coated silica supports [17]. It should be noted that the profile obtained by pumping ovalbumin through the anti-HSA-Sil400 column in similar conditions (Fig. 2) was different since, for ovalbumin, the increase of the elution front was observed at the void volume. This demonstrates again the specificity of the interaction between HSA and the immunosorbent.

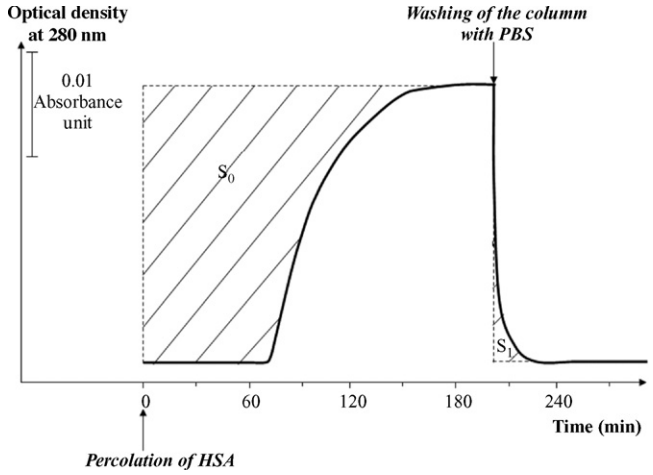

Fig. 1. Breakthrough curve for the adsorption of HSA to the antiHSA-Sil400 column (30 mm × 4.6 mm I.D.). HSA solution: 10 μg/mL in 10 mM phosphate buffer, 2.7 mM KCl, 0.137 M NaCl, pH 7.4. Flow rate: 0.5 mL/min.

In order to confirm the binding of HSA to the immunosorbent, a small volume of an anti-HSA solution (0.28 mg/mL of total IgG, 0.1 mg/mL of anti-HSA) was injected in the column after percolation of HSA through the anti-HSA-Sil400 column. A signal was observed near the void volume. However, the peak area was equal to 58% of the value obtained by injecting the same anti-HSA solution on the initial anti-HSA-Sil400 column (Table 1, lines 2 and 1, respectively). When comparing this value to the massic composition of the anti-HSA solution used for the determination (65% of various IgGs and 35% of anti-HSA), it can be assumed that the peak observed after percolation of HSA is only due to the elution of non specific IgGs, the specific antibody being irreversibly adsorbed on the column.

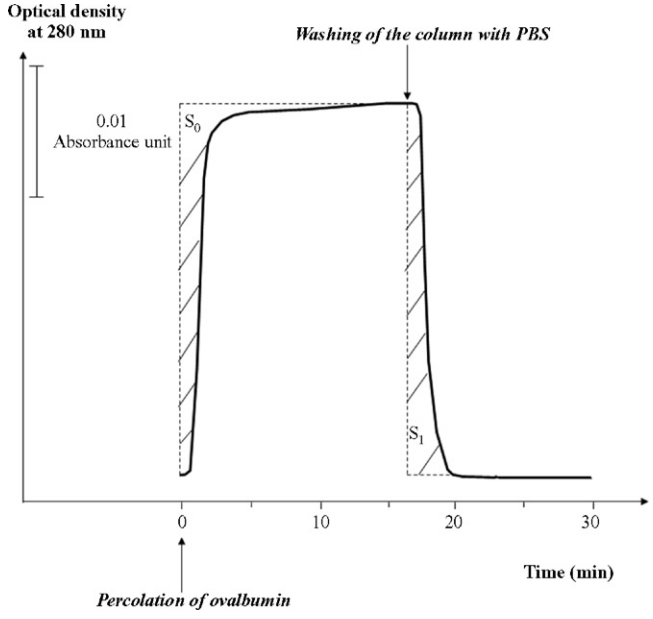

Fig. 2. Breakthrough curve for the adsorption of ovalbumin to the antiHSA-Sil400 column (30 mm × 4.6 mm I.D.). Ovalbumin solution: 10 μg/mL in 10 mM phosphate buffer, 2.7 mM KCl, 0.137 M NaCl, pH 7.4. Flow rate: 0.5 mL/min.

Table 1
Comparison of peak areas obtained after injection of 20 μL of anti-HSA (0.1 mg/mL of anti-HSA; 0.18 mg/mL of other IgGs) on the immunosorbents

Column	k	Peak area (arbitrary unit)
Anti-HSA-Sil400	0.54	16.7 ± 0.8
HSA-anti-HSA-Sil400	0.54	9.7 ± 0.5
After desorption of HSA from HSA-anti-HSA-Sil400	0.54	17.0 ± 0.8

Line 1: before adsorption of HSA (anti-HSA-Sil400 column), line 2: after adsorption of HSA (HSA-anti-HSA-Sil400 column), line 3: after desorption of HSA. Eluent: PBS pH 7.4. Flow rate: 1 mL/min. Detection at 280 nm.

After the binding of HSA to the immunosorbent, the immobilized protein was removed from the column, using a 0.1 M glycine buffer (pH 2.1). The complete desorption of HSA was controlled by injecting the anti-HSA solution. As reported in Table 1 (lines 1 and 3), peak areas measured on the initial anti-HSA-Sil400 column and after desorption of HSA were similar, indicating the absence of HSA on the support after washing with the glycine buffer. Moreover, by passing a fresh HSA solution in the column, it was demonstrated that the recognition ability of the immobilized antibody was intact after percolation of the glycine buffer, since the breaktrough curves were similar to those observed on the initial anti-HSA-Sil400 column.

3.2.3. Binding capacity of anti-HSA-Sil400 sorbents

The dynamic binding capacity of anti-HSA-Sil400 sorbents was determined by passing HSA solutions at 10 and 50 μg/mL through the column. The amounts of HSA bound to the immunoaffinity columns were obtained from the breakthrough curves, after rinsing the column with PBS (Fig. 1). The amount of protein desorbed (area S_1 under the desorption signal) was substracted from the amount adsorbed (area S_0 above the adsorption signal). As indicated in Table 2, the values determined for HSA were between 0.41 and 0.45 mg, corresponding to a binding capacity close to 1.5 mg/g of silica. This value is tenfold higher than binding capacities reported by Renard et al. on 6 nm silica supports (650 m^2/g) [17]. This result clearly confirms the necessity of using materials with good flow properties for the elaboration of immunosorbents, as recommended by Muronetz and Korpela [1], despite the low surface area of such supports. It must be noted that silica materials with a porosity between 30 and 400 nm could be an interesting compromise between flow properties and binding capacity.

3.2.4. Regeneration of the immunosorbents

In previous studies, it was observed that host–guest interactions between β-CD cavities and adamantyl-modified polymers

Table 2
Amounts of protein (q) bound to the anti-HSA-Sil column (30 mm × 4.6 mm), determined from frontal analysis elution profiles (Figs. 1 and 2)

Protein	C (μg/mL)	q (mg)
Ovalbumin	10	0
HSA	10	0.45
	10	0.44
	50	0.41

were decreased in the presence of organic solvents (e.g. acetonitrile) or surfactants (e.g. SDS) [7,12]. Moreover, it was shown by surface plasmon resonance (SPR) that sensing layers resulting from the adsorption of adamantyl-modified antibodies onto flat β-CD-coated gold surfaces could be regenerated after use, by passing a SDS solution in the flow cell of the SPR system [11]. The question as to whether this procedure could be applied to the regeneration of porous immunosorbents was examined herein. To this end, an acidic SDS solution was passed through the HSA-anti-HSA-Sil400 column to induce both the elution of the antigen and cleavage of interactions between adamantane groups of the dextran-anti-HSA layer and β-cyclodextrin cavities. After a short time, a large signal was observed at 280 nm, corresponding to the elution of proteins from the column. The percolation of SDS was continued until a stable baseline was observed. To test the efficiency of the regeneration procedure, an HSA solution (1 mg/mL) was injected in the column after percolation of the SDS solution and extensive washing with PBS. The mean peak area (89 a.u. ± 5%) was similar to the value obtained on the CD-Sil400 reference column (95 a.u. ± 5%), suggesting that regeneration of the column could be considered as complete, since the protein was not trapped by the stationary phase.

After regeneration, a fresh immunosorbent was prepared in situ, by a three-step procedure involving consecutively (i) adsorption of Ad-Dext-COOH onto the regenerated CD-Sil400 support; (ii) activation of carboxylic groups with NHS and EDC; (iii) binding of anti-HSA. The first step was described previously for CD-Sil30 columns, using an aqueous solution of Ad-Dext-COOH [14]. In that case, it was possible to determine the amount of dextran bound to the column from the breakthrough curve recorded with a refractometric detector. However, in the present study, the presence of a high salt concentration (1 M NaCl) during the percolation of Ad-Dext-COOH through the CD-Dext400 column made this determination impossible. So the amount of polymer bound to the column using the dynamic procedure was unknown. After immobilization of the antibody, the ability of the regenerated anti-HSA-Sil400 column to bind HSA was evaluated and compared to the performances of the initial anti-HSA-Sil400 support (before regeneration). A 30% lower binding capacity (close to 1 mg/g) was determined for the regenerated immunosorbent. However, it should be emphasized that the anti-HSA content of solutions used for the coupling of the antibody to the activated Ad-Dext-COOH supports, was not the same in both experiments (4.9 mg/mL after regeneration and 5.5 mg/mL before regeneration). Moreover, an higher amount of non specific immunoglobulins was present in the solution used after regeneration (65% versus 42% before regeneration). These proteins act as a competitor towards NHS-ester reactive groups during the binding of anti-HSA to activated CD-Dext400 supports. For these reasons, the amount of anti-HSA in the column was probably lower after regeneration than before, explaining its lower HSA binding capacity. So, as shown previously in the case of flat gold surfaces, the regeneration procedure can be considered as efficient since it permits the elution of altered proteins from the column and the preparation of fresh immunoaffinity columns.

4. Conclusion

This study evaluates the feasibility of regenerable immunosorbents using the supramolecular β-CD/adamantane system for the immobilization of biomolecules to porous silica. The procedure involves the formation of inclusion complexes between β-CD silica particles and a reactive dextran layer which permits the subsequent binding of antibody molecules. The preparation of immunosorbents can be carried out either in batch or in the column. The resulting immunoaffinity supports are stable in aqueous media and their recognition properties were demonstrated. Interestingly, the initial β-CD support can be easily restored using a SDS solution, in case of alteration of the immobilized biomolecules. It should be underscored that the feasibility of the procedure was evaluated with the anti-HSA/HSA system. However, the method could be applied to any antibody/antigen or antigen/antibody system. Moreover, experiments which were carried out in this study using 400 nm porous silica particles with a small surface area could be applied to any porous silica material or to monoliths.

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