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Journal of Chromatography B, 808 (2004) 63–67
JOURNAL OF CHROMATOGRAPHY B
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Immobilization of a (dextran-adamantane-COOH) polymer onto β-cyclodextrin-modified silica

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Available online 20 June 2004

Abstract

Adamantane-modified compounds are known to form stable complexes with β-cyclodextrins (β-CD) by host–guest interactions. In this study, the inclusion complex formed between β-CD cavities and the adamantane group was evaluated for the elaboration of a cation-exchange support. The synthesis of the chromatographic supports involved three steps: (i) a polymer of β-CD was grafted to diol-modified silica, (ii) a dextran polymer was modified by both adamantane groups and ionizable COOH functions, (iii) the dextran derivative (Ad-Dex-COOH) was bound to the chromatographic support by complexation between the adamantane groups of the dextran and β-CD cavities of the support. The polymer immobilization on the β-CD support was successful as the resulting support exhibited weak cation-exchange properties. The stationary phase was easy to prepare under mild conditions (aqueous media, room temperature) and was quite stable when using aqueous mobile phases. The chromatographic behaviour of model proteins was studied in isocratic elution by examining the effect of salt concentration in the buffer on retention. A mixed retention mode was found for lysozyme, revealing both electrostatic and hydrophobic interactions with the stationary phase.

Keywords: Immobilization; (Dextran-adamantane-COOH) polymer; Basic proteins; Poly-β-cyclodextrin

1. Introduction

The cyclodextrin molecules (α-CD, β-CD, γ-CD) consist of six, seven or eight α-1,4-linked D-glucopyrranosic units, respectively. They are cyclic oligosaccharides, well known for their ability to form inclusion complexes with a wide variety of compounds bearing hydrophobic moieties. A strong association was noticed with some guest molecules. For example, adamantane derivatives have β-cyclodextrin association constants between 10^4 and 10^5 M^{-1} [1]. Therefore, the β-CD/adamantane system was used in a number of studies to immobilize adamantane-modified polymers to β-CD-coated surfaces via host–guest interactions [2–4]. This associative system has the same advantages as the avidin/biotin couple, which is often used to bind biotinylated proteins or oligonucleotides to avidin-coated surfaces: (i) the coupling reaction is performed under mild conditions, (ii) the reaction kinetics is rapid, (iii) the resulting multilayered structures are stable in aqueous media. However, it should be emphasized that the dissociation of avidin/biotin complexes occurs only under drastic conditions, while adamantane-modified layers can be completely desorbed from the β-CD-coated surface by using organic solvents [3] or surfactants such as sodium dodecyl sulfate (SDS) [2,3].

In a recent study, the β-CD/adamantane system was used for the elaboration of optical biosensors [2]. The procedure involved a dextran layer bearing adamantyl and COOH groups (Ad-Dex-COOH). This intermediate layer permitted the binding of antibody molecules to β-CD-coated gold surfaces. This approach would be helpful for the preparation of various chromatographic supports by an easy adsorption procedure since the method only requires two steps: (i) synthesis of polymers bearing both adamantyl groups and other functionalities allowing a specific interaction of biomolecules with the stationary phase, and (ii) immobilization of the functionalized polymer onto β-CD supports.

The aim of this study was to test the β-CD/adamantane system for the immobilization of functionalized polymers onto β-CD-modified silica particles via host–guest interactions. A negatively charged polymer was selected for this work since the polyanion cannot be directly adsorbed on

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doi:10.1016/j.jchromb.2004.05.025

the silica matrix. In these conditions, the binding of the adamantane-modified polymer is only due to host–guest interactions The porous silica support was first grafted by a polymer of β-CD (poly-β-CD). Then a dextran layer bearing both adamantyl and COOH groups (Ad-Dex-COOH) was adsorbed onto the support by eluting the polymer solution through the poly-β-CD–silica column. The retention properties of the resulting stationary phase (Ad-Dex-COOH silica) were tested using probe proteins. As the upper adsorbed layer was a negatively charged polymer, retention properties of the chromatographic support were examined by studying the elution behaviour of positively charged proteins.

2. Materials and methods

2.1. Chemicals

Diol-bonded silica (Nucleosil 300-7 OH, particle size: 7 μm; porosity: 30 nm; surface specific area: 100 m^2/g) was purchased from Macherey Nagel (Düren, Germany). Dextran T40 (M = 40,000 g/mol) was from Amersham Pharmacia Biotech (Orsay, France). 1,4-diisocyanatobutane, 1-adamantanecarbonyl chloride, 4-dimethylaminopyridine (DMAP), triethylamine, succinic anhydride, *N*,*N*-dimethylformamide (DMF) pyridine and sodium chloride were obtained from Aldrich (St Quentin Fallavier, France). Sodium dihydrogenophosphate and di-sodium hydrogenophosphate were from Fluka (St Quentin Fallavier, France). The proteins used as chromatographic test materials were lysozyme from chicken egg (MW = 14,500 g/mol; pI = 11.0), cytochrome *c* from horse heart (MW = 13,400 g/mol; pI = 9.3) and ovalbumin (MW = 45,000 g/mol; pI = 4.7). The proteins were purchased from Sigma (St Quentin Fallavier, France).

2.2. Synthesis of poly-β-CD-grafted silica

As described previously [5], poly-β-CD-grafted silica was prepared by a two-step reaction. First, diol-bonded silica was activated in 1,2-dichloroethane by 1,4-diisocyanatobutane. Then, the polymer of β-CD (M_n = 30,000 g/mol) [6] was grafted to the immobilized isocyanate functions in pyridine solvent.

2.3. Synthesis of Ad-Dex-COOH

The dextran T40 was first esterified by 1-adamantanecarbonyl chloride before being modified by succinic anhydride. One gram of dextran was dissolved at 80 °C in 30 ml of freshly distilled DMF in the presence of lithium chloride. Then, 1.2 mmol of 1-adamantanecarbonyl chloride, DMAP and pyridine were added to the dextran solution. The reaction mixture was stirred for 3 h at 80 °C, then kept overnight at room temperature. After precipitation in 2-propanol and drying, the modified polymer (Ad-Dex) was dialyzed against water and lyophilized. Its modification ratio was determined by ^{1}H NMR. It was equal to 7% (7 adamantyl groups per 100 glucosic units). Thereafter, the Ad-Dex polymer (0.5 g) was dissolved in 15 ml of DMF; then 1.8 mmol of succinic anhydride, 0.16 mmol of DMAP and 20 μl of triethylamine were added to the Ad-Dex solution. The reaction was carried out overnight at 70 °C. After evaporation of the solvent, the polymer was dissolved in water, dialyzed against water and lyophilized. The COOH content of Ad-Dex-COOH polymer was determined by titration of the basic form (Ad-Dex-COO^-) using 0.1N hydrochloric acid.

2.4. HPLC experiments

The HPLC system used consisted of two pumps (LC 9A, Shimadzu, Kyoto, Japan) and a sample injector with a 0.02 ml loop (Model 7125, Rheodyne, Berkley, CA, USA). The proteins injected onto Ad-Dex-COOH silica columns were monitored at 280 nm with a variable wavelength UV detector (Spectra 100, Thermo-Finnigan, San Jose, CA, USA). The buffers used for the isocratic elution of proteins were 20 mM Tris–HCl buffer at pH = 7.0 and 20 mM phosphate buffer at pH = 6.0. The ionic strength was adjusted by adding sodium chloride to the buffer. The eluent was used for preparing protein solutions (1 g/l). The column void volume V_m was determined from the retention time of a solvent perturbation. All experiments were carried out at 1 ml/min.

Frontal measurements were performed with a six-port commuting valve placed before the sample injector to switch from one eluent to the other, and a differential refractometer (R 401, Waters, St Quentin en Yvelines, France) was used to record the signals. The method was applied to determine the amount of (Ad-Dex-COOH) immobilized onto poly-β-CD-coated silica columns and the ion-exchange capacity of the Ad-Dex-COOH supports.

2.5. On-line preparation and characterization of the Ad-Dex-COOH silica columns

Poly-β-CD-grafted silica was slurry-packed at 30 MPa into stainless steel columns (100 mm × 4.6 mm) using dichloromethane. The columns were rinsed with methanol and water. Thereafter, the Ad-Dex-COOH solution (1 g/l in water) was flown through the columns at 1 ml/min until equilibrium was reached. The columns were rinsed with water. The amount of Ad-Dex-COOH bound per gram of silica was determined by frontal analysis. The ion-exchange capacity of the Ad-Dex-COOH support was measured from breakthrough curves obtained by applying onto the columns a 20 mM buffer containing tetramethylammonium chloride (0.15 M at pH = 7.0 or 0.09 M at pH = 6.0). After performing an adsorption front, the initial Na^+ form of the cation exchanger was recovered by rinsing with 0.3 M NaCl in the buffer.

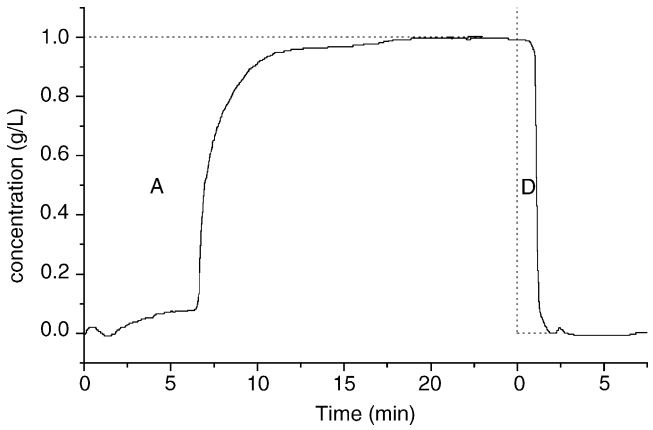

Fig. 1. In situ adsorption of Ad-Dex-COOH on poly-β-CD-grafted silica. Flow rate: 1 ml/min; 1 g/l solution of Ad-Dex-COOH in water; column (100 mm × 4.6 mm).

3. Results

3.1. Preparation of the Ad-Dex-COOH silica support

The stationary phases were produced by adsorbing Ad-Dex-COOH onto poly-β-CD-grafted silica particles. In order to prepare dextran derivatives with a high degree of COOH substitution, a protocol using succinic anhydride [7] was preferred to the carboxymethylation method described by Gekko and Noguchi. [8]. The COOH content of Ad-Dex-COOH polymers obtained by reaction with succinic anhydride was close to 60%.

The adsorption of Ad-Dex-COOH onto the poly-β-CD-coated silica was carried out *on line* by pumping a polymer solution through the β-CD column until a refractive index plateau value was reached. The amount of Ad-Dex-COOH bound to the support was determined after rinsing the column with water. The amount desorbed (area under the desorption signal *D*) was subtracted from the amount adsorbed (area above the adsorption signal *A*) (Fig. 1). The mass of polymer adsorbed per gram of silica was 7 mg when a 1 g/l solution was used. In order to check the binding of Ad-Dex-COOH to the poly-β-CD support, a poly(ethylene glycol) model polymer bearing one naphtyl terminal group (Nap-PEG) was injected onto the column before and after applying Ad-Dex-COOH. As expected, the retention of Nap-PEG decreased (about 20%) since the number of free β-CD cavities was lower after immobilization of the dextran derivative.

3.2. Characterization of the Ad-Dex-COOH silica support

The binding of Ad-Dex-COOH polymer to the grafted poly-β-CD was successful as the stationary phase exhibited cation-exchange properties. The ion-exchange capacity was measured from the adsorption front of tetramethylammonium chloride. At pH = 7.0, it was equal to 0.010 meq/g of support. Although COOH groups should be mainly ionized at this pH value [8], the measured ion-exchange capacity was lower than the theoretical value (0.025 meq/g) determined from the amount of polymer adsorbed on the support and its COOH content. It can be concluded that a great number of ionic groups were no more accessible to the ion-exchange process after immobilization of Ad-Dex-COOH by host–guest interactions. As expected, a lower ion-exchange capacity was observed at pH = 6.0 (0.005 meq/g) because of the decrease of the ionized groups. The adsorbed Ad-Dex-COOH layer was relatively stable since the columns could be used for more than 3 months for studying the elution behaviour of proteins.

3.3. Retention behaviour of proteins on the Ad-Dex-COOH silica support

As expected for an anionic immobilized layer, the positively charged proteins (cytochrome *c* and lysozyme) were retained, while the negatively charged protein (ovalbumin) was eluted at the column dead volume. The chromatographic behaviour of proteins was examined by studying the effect of salt concentration in the eluent.

When the retention process is only governed by electrostastic interactions, the retention of proteins is well described by the stoichiometic displacement model [9]. According to this model, which predicts the retention behaviour of proteins in ion-exchange chromatography, the logarithm of the capacity factor of a protein (k) is linearly related to the logarithm of the salt concentration (C_s) in the eluent

$$\log k = \log K_Z - Z \log C_s \quad (1)$$

where Z is the electrostatic interaction parameter and K_Z is a constant related to the ion-exchange capacity of the support and to the equilibrium constant of the chemical equilibrium reaction. With both electrostatic and hydophobic interactions between the protein and the support, an additive term was introduced [10] to account for the increase of protein retention at large salt concentration. The effect of the salt concentration on the protein retention factor is then described by the expression

$$\log k = \log K_Z - Z \log C_s + \gamma C_s \quad (2)$$

where γ is the hydrophobic interaction parameter.

The effect of NaCl concentration on protein retention is illustrated in Fig. 2 for two different pH values. An important decrease of protein retention was observed when the pH of the mobile phase was changed from pH = 7.0 to 6.0. Such a variation was to be expected as the ion-exchange capacity of the support was two-fold lower at pH = 6.0 than in neutral medium.

For cytochrome *c*, the plot of log k versus log C_s was well fitted by a straight line at pH = 7.0 and 6.0. The values obtained for the parameters log K_Z and Z are summarized in Table 1, together with their 95% confidence interval. At pH = 7.0, a non-linear fit of the three-parameter equation (Eq. (2)) was attempted, but the large confidence interval

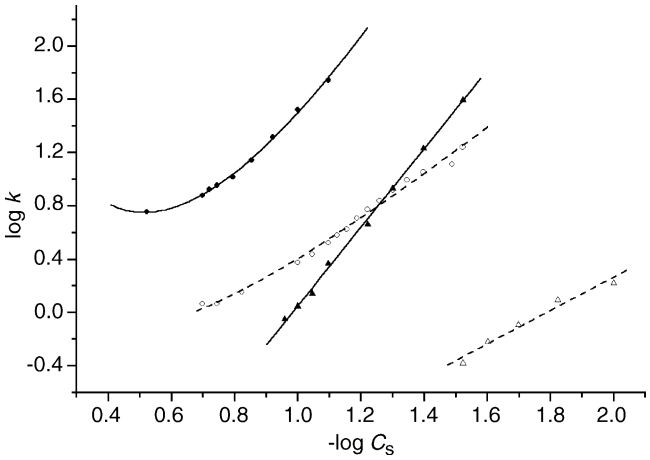

Fig. 2. Variation of the logarithmic retention factor of basic proteins with the logarithmic salt concentration at pH = 7.0 and 6.0. Stationary phase: Ad-Dex-COOH silica; eluent: NaCl in 0.02 M TRIS buffer; flow rate: 1 ml/min. (●) lysozyme and (▲) cytochrome *c* at pH = 7.0; (○) lysozyme and (△) cytochrome *c* at pH = 6.0.

(100% of relative error for the γ value) indicates that a straight line is sufficient for describing this retention process. The important decrease of *Z* between pH = 7.0 and 6.0 could be explained by the decrease of the ion-exchange capacity of the support.

For lysozyme retention at pH = 7.0, the non-linear dependence of log *k* versus log C_s indicates a mixed retention mode involving both electrostatic and hydrophobic interactions. The retention parameters were determined with a non-linear regression analysis from the best fit of Eq. (2) on the experimental data (Table 1). The 95% confidence intervals on the parameter evaluations are also given. The large value of the γ coefficient indicates strong hydrophobic interactions with the support. At pH = 6.0, the hydrophobic effects are weak so that the plot of log *k* versus log C_s is well fitted by a straight line. However, the parameters (log K_Z and *Z*) obtained by linear regression analysis (Table 1) differ from those evaluated when fitting the three-parameter equation. This significant difference indicates that the hydrophobic interaction cannot be really neglected when analyzing these retention data. Both the electrostatic and the hydrophobic constants are quite low, more than half lower than those evaluated at pH = 7.0.

Table 1
Retention parameters of proteins on the Ad-Dex-COOH silica support

Protein	pH	log K_z	*Z*	γ (l/mol)
Cytochrome *c*	7.0	−2.9 ± 0.1	3.0 ± 0.1	0
		−3.4 ± 0.6	3.3 ± 0.3	2.2 ± 2.3
	6.0	−2.2 ± 0.2	1.3 ± 0.1	0
Lysozyme	7.0	−2.9 ± 0.2	3.9 ± 0.2	5.5 ± 0.4
	6.0	−1.1 ± 0.1	1.5 ± 0.1	0
		−1.7 ± 0.2	1.9 ± 0.1	1.9 ± 0.7

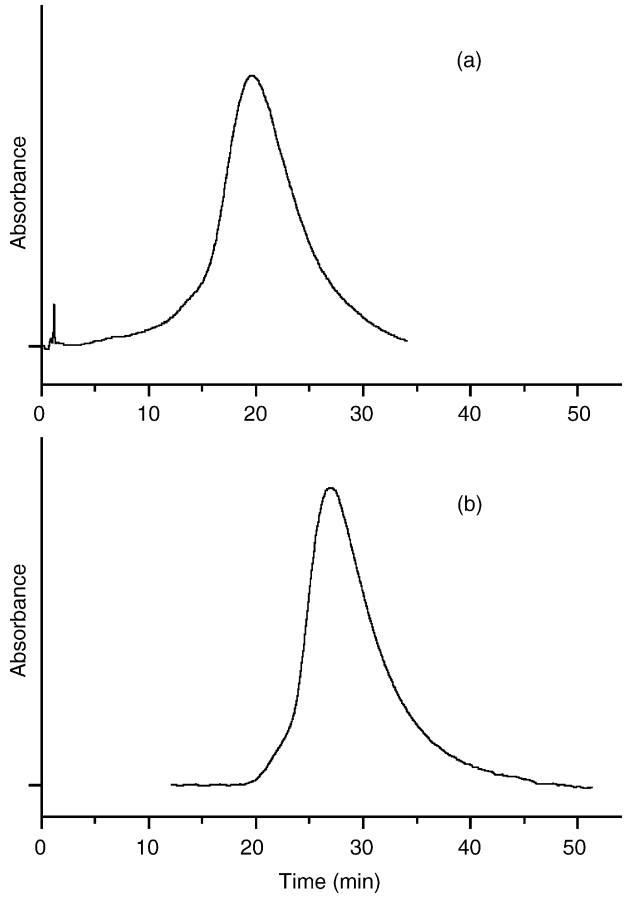

Fig. 3. Comparison of lysozyme isocratic elution on Ad-Dex-COOH silica and on a cation-exchange stationary phase. Buffer: 0.02 M TRIS buffer at pH = 7.0; displacing salt: NaCl. Flow rate: 1 ml/min. (a) Column (100 mm × 4.6 mm): Ad-Dex-COOH adsorbed on poly-β-CD-grafted silica (30 nm porosity). Eluent: 0.12 M NaCl in buffer. (b) Column (150 mm × 4.6 mm): dextran sulfate and hexadimethrine bromide adsorbed on silica (30 nm porosity) [12]. Eluent: 0.3 M NaCl in buffer.

4. Discussion

The Ad-Dex-COOH polymer was successfully immobilized on the poly-β-CD support as a negatively charged stationary phase was obtained. The charge density of the support is quite small (−0.005 C/m^2 at pH = 6.0), much below that of the commercial weak cation exchangers (−0.22 C/m^2 for the Zorbax WAX-300 at pH = 6.0 [10,11]). As a consequence, the salt concentration needed to elute the proteins was much lower than that generally used to separate proteins (Fig. 2). At pH = 7.0, the ion-exchange capacity of this support (−0.01 C/m^2) was significantly lower than that observed with a cation exchange stationary phase (−0.11 C/m^2) produced by coating a silica matrix of 30 nm porosity with two different polyelectrolytes of opposite charges [12], a polyanion (dextran sulfate) adsorbed on a first immobilized cationic layer (hexadimethrine bromide).

Fig. 3 compares the lysozyme peak eluted under isocratic conditions on both stationary phases. With 0.12 M

of NaCl in the buffer, the capacity factor ($k = 20.6$) is close to that observed when lysozyme was injected on the cation-exchange column, with an eluent buffer containing 0.3 M of NaCl in the eluent. For both lysozyme peaks, a large band broadening was noticed, explained by the slow kinetic mass transfer exchanges in the pores of 30 nm size [12]. Similar values of the theoretical plate heights were found with $H = 0.24$ cm in Fig. 3(a) for the Ad-Dex-COOH silica column (100 mm × 4.6 mm) and $H = 0.11$ cm in Fig. 3(b) with the dextran sulfate column (150 mm × 4.6 mm) tested. However, an increase of bandwidth was observed when higher salt concentrations were used to elute lysozyme from the Ad-Dex-COOH silica column. This important loss of efficiency did not permit to use the Ad-Dex-COOH column for separating proteins under increasing salt gradient conditions.

When comparing the retention behaviour of cytochrome *c* and lysozyme, a larger *Z* value was found for lysozyme. This result agrees with the "net charge concept", as the p*I* of lysozyme is larger than that of cytochrome *c*. The effect of salt concentration on the retention behaviour of lysozyme shows the important role of hydrophobic interactions. The increase of bandwidth at larger salt concentration reveals a support heterogeneity, with the presence of hydrophobic binding sites. For cytochrome *c* instead, the linear dependence of log *k* versus log C_s indicates negligible hydrophobic effects, explained by the hydrophilic nature of this protein.

With the Ad-Dex-COOH silica support, hydrophobic interactions with the linker layer (1,4-diisocyanatobutane) permitting the grafting of cyclodextrin to the diol support are probably mainly responsible for the retention behaviour of lysozyme. In order to evaluate the extent of hydrophobic interactions in the absence of ionic groups, lysozyme and cytochrome *c* were analyzed on a neutral reference column prepared by immobilizing Ad-Dex (7% Ad–0% COOH) onto a poly-β-CD-column. With an eluent containing 0.1 M NaCl, the retention factor of lysozyme decreased from 2.0 when injected onto the initial poly-β-CD column to 0.2 when injected onto the Ad-Dex-coated column. When studying this Ad-Dex-coated support, cytochrome *c* was almost eluted at the column void volume even in presence of high salt concentrations (2 M ammonium sulfate), demonstrating the absence of hydrophobic interactions between cytochrome *c* and the stationary phase. On the contrary, the retention of lysozyme increased with salt concentration ($k = 0.22$ and 6.2 at 0.1 M and 1 M NaCl, respectively). The hydrophobic effect observed for lysozyme could result from interactions with the adamantane groups. However, its retention factor with 0.1 M NaCl in the eluent is one magnitude order lower than that observed on the initial poly-β-CD column. This result indicates a relatively good shielding of the poly-β-CD support with the Ad-Dex coating, but the presence of unmasked hydrophobic groups is revealed by the retention behaviour of lysozyme at large salt concentration.

5. Conclusion

An original procedure of polymer immobilization by formation of inclusion complexes was tested by adsorbing an anionic polymer (Ad-Dex-COOH) onto the poly-β-CD-grafted silica support. The immobilization of the polymer was successful as the stationary phases exhibited cation-exchange properties and the characteristics of the stationary phase were evaluated by studying the chromatographic behaviour of model proteins. The retention of positively charged proteins was mainly governed by electrostratic interactions with the negative charges of the immobilized polyanion, but hydrophobic effects were revealed from the chromatographic behaviour of lysozyme. The hydrophobicity of the stationary phase could probably arise from the linkers used for grafting poly-β-CD to silica. The main problem with these supports for separating proteins by ion-exchange chromatography was the band broadening of elution peaks caused by hydrophobic interactions and slow mass transfer kinetics. Further work is now needed to optimize the properties of the resulting stationary phases. The band broadening of protein peaks could be reduced by increasing the support porosity, decreasing the molecular weight of the immobilized polymer and reducing the hydrophobicity of the initial poly-β-CD-grafted silica support.

The results presented here clearly show that it is possible to immobilize polymers with functional moieties on β-cyclodextrin supports via the inclusion of an adamantane substituent group into β-cyclodextrin cavities. The supports are easy to prepare under mild conditions (aqueous media, room temperature), and are quite stable when using aqueous mobile phases. The approach could be extended to immobilize polymers with various substituent groups for the preparation of a large variety of supports for affinity purifications or HPLC separations.

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