PEGylated Chromatography: Efficient Bioseparation on Silica Monoliths Grafted with Smart Biocompatible Polymers

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ABSTRACT Novel oligo(ethylene glycol)-based thermoresponsive stationary phases have been studied for the separation of bioanalytes. Well-defined copolymers of (2-methoxyethoxy)ethyl methacrylate and oligo(ethylene glycol) methacrylate were synthesized by atom-transfer radical polymerization in the presence of an N-succinimidyl-functionalized initiator. The reactive chain ends of these copolymers were then reacted with amino-functionalized silica monoliths. The formed composites were studied as chromatography materials. For instance, it was demonstrated that thermoresponsive oligo(ethylene glycol)-based stationary phases allow rapid, efficient separation of steroid and protein mixtures in pure water under isocratic high-performance liquid chromatographic elution.

KEYWORDS: thermoresponsive chromatography • bioseparation • thermoresponsive polymers • ATRP • grafting • HPLC

Thermoswitchable stationary phases have recently been described as an interesting option for controlling the separation of bioanalytes (1). These types of stationary phases are typically prepared by grafting thermoresponsive polymers onto preformed chromatographic supports (i.e., silica- or polymer-based beads or monoliths) (2). The main advantage of these smart chromatographic materials is that they can separate mixtures of biomolecules in a pure aqueous environment under isocratic conditions (3). Besides being a greener technology compared to conventional reversed-phase chromatography, which typically uses organic solvent gradients for the separation of bioanalytes (4), this chromatographic technique also has the advantage of avoiding denaturation of biomolecules because separation only takes place in water.

So far, poly(*N*-isopropylacrylamide) (PNIPAM) has been mainly exploited for preparing smart stationary phases (5). This thermoresponsive polymer exhibits a lower critical solution temperature (LCST) in water at approximately 32 °C and was therefore widely explored for preparing switchable materials for biological applications (6). One reason for the biomedical popularity of such polymeric materials is that it exhibits a LCST relatively close to body temperature; moreover, its insensitivity toward slight environmental changes such as the pH or concentration makes it desirable for hyperthermia-induced drug-delivery studies (7). However, PNIPAM is not fully bioinert. Indeed, this macromolecule contains multiple secondary amide functions (i.e., strong hydrogen-bond donors and acceptors), which may interact with natural polyamides such as proteins. This feature may be a severe limitation in some delicate applications, in particular for the bioseparation of proteins and enzymes (8).

Recently, oligo(ethylene glycol)-based thermoresponsive polymers have been proposed as an interesting alternative to PNIPAM (9). Indeed, these polymers display reversible phase transitions in water and, furthermore, are mainly composed of bioinert ethylene oxide units (i.e., poor hydrogen-bond donors and highly hydrated acceptors). Moreover, these interesting macromolecules can be easily synthesized using commercially available monomers. For instance, random copolymers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA) and oligo(ethylene glycol) methacrylate (OEGMA; $M_n \sim 475$ g/mol) exhibit a LCST in water, which can be precisely adjusted by varying the comonomer composition (10). Thus, thermoresponsive P(MEO_2MA-*co*-OEGMA) copolymers have been recently exploited for preparing a variety of smart biocompatible materials (11). In particular, it has been demonstrated lately that these polymers allow reversible control over bioadhesion (12). Thus, it was tempting to use these smart biocompatible coatings for developing innovative stationary phases. In the present communication, P(MEO_2MA-*co*-OEGMA)-modified silica monoliths have been tested for the chromatography of steroids and proteins.

A series of P(MEO_2MA-*co*-OEGMA) copolymers with variable chain lengths and compositions were synthesized by atom-transfer radical polymerization in the presence of the initiator *N*-succinimidyl 2-bromoisobutyrate (13). The formed copolymers were characterized by size exclusion chromatography (SEC), ^{1}H NMR, and turbidimetry (Table 1). All of

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Received for review July 8, 2009 and accepted August 18, 2009

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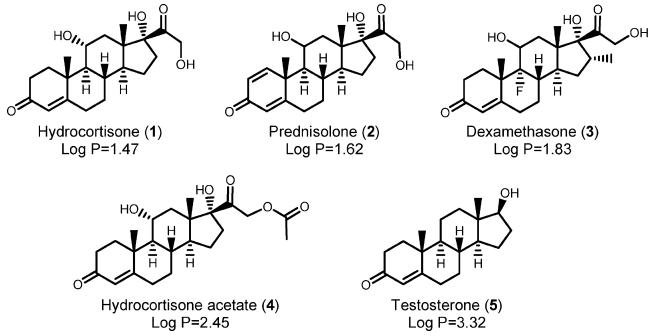
DOI: 10.1021/am900461a

Table 1. Characterization of the Copolymers P(MEO_2MA-*co*-OEGMA) and of the Corresponding Modified Silica Monoliths

	$[OEGMA]_0/[MEO_2MA]_0$[a]	$DP_{n,th}$[b]	M_n[c]	M_w/M_n[c]	cloud point[d] (°C)	grafting density[e] ($\mu g/m^2$)	grafting density (chains/nm^2)
a	10:90	100	18 100	1.28	41	233	0.007 14
b	10:90	75	15 690	1.41	38	402	0.017 70
c	10:90	50	12 310	1.30	39	371	0.024 80
d	10:90	25	6 220	1.36	40	305	0.039 20
e	5:95	100	18 120	1.66	33	250	0.009 26
f	15:85	100	17 040	1.41	43	235	0.007 15

[a] Experimental conditions for polymer synthesis: in an ethanol solution, 60 °C, $[initiator]_0:[CuCl]_0:[Bipy]_0 = 1:1:2$. [b] Theoretical degree of polymerization $DP_{n,th} = ([OEGMA]_0 + [MEO_2MA]_0)/[initiator]_0$. [c] Measured by SEC. [d] Measured by turbidimetry. [e] In a typical reaction, approximately 0.05 g of polymer was dissolved in 1 mL of *N*,*N*-dimethylformamide (1 mL/cycle). Parallel to the in situ grafting of the copolymer, grafting was also performed on a free-standing piece of aminated monolith. Grafting densities were calculated from elemental analysis data using the following formula: (% $C_p \times 10^6$), where % C_i = increase in the carbon percent of the aminated silica from elemental analysis, % $C_{i(theory)}$ = calculated weight percent of carbon in the initiator, % C_p = increase in the carbon percent after polymerization, % $C_{p(theory)}$ = calculated weight percent of carbon in the monomer, and A = specific surface area (m^2/g). Grafting density in chains/$m^2 = m_p N_A/M_n$, where m_p = amount of grafted polymer on the silica monolith, N_A = Avogadro's number, and M_n = number-average molecular weight of the grafted polymer (g/mol) (14).

Scheme 1. Chemical Structures of the Steroids Involved in the Separation Process and Their Partition Coefficients (log *P*)



these techniques confirmed the formation of well-defined copolymers with controlled chain lengths, chain ends, and cloud points in water. The reactive *N*-succinimidyl ester chain ends of the polymers were then reacted in situ with amino-functionalized silica monoliths via standard amide coupling chemistry. Before and after modification, the monoliths were analyzed by elemental analysis and Fourier transform infrared (FT-IR) spectroscopy.

According to elemental analysis, the concentration of amino groups on the silica monolith was 453 $\mu g/m^2$. After coupling of P(MEO_2MA-*co*-OEGMA) thermoresponsive polymers, grafting densities in the range of 233−402 $\mu g/m^2$ were measured (Table 1). The fact that the polymers were successfully grafted onto the monolith surfaces was also confirmed by FT-IR (Figure S1 in the Supporting Information). For instance, the spectrum of the modified monolith showed new adsorption bands corresponding to the amide functions at 1700 cm^{-1} ($\nu_{C=O}$) and 1540 cm^{-1} (δ_{N-H}).

The chromatographic performances of these P(MEO_2MA-*co*-OEGMA)-grafted stationary phases were then evaluated in aqueous media under isocratic conditions. First, a mixture of five steroids was investigated (Scheme 1). Upon examination of their log *P* values, these analytes may be ranked by increasing hydrophobicity: hydrocortisone (**1**) ∼ prednisolone (**2**) < dexamethasone (**3**) < hydrocortisone acetate (**4**) < testosterone (**5**). Figure 1a shows the elution profiles for the separation of these mixtures of analytes below and above the LCST of the column packed with composite e (Table 1, entry e). It can be observed that below the LCST of this column analytes **1** and **2** can be separated, while more hydrophobic analytes **3** and **4** are eluted in a single peak. Analyte **5**, which has a much higher log *P* value, is eluted in a separate peak from the others. Significantly, changing the temperature of the column above its LCST led to the separation of all five steroids. This, together with an increase in the retention time for the more hydrophobic analytes, seems to indicate that the driving forces for separation above LCST are hydrophobic−hydrophobic interactions between the analytes and the stationary phase. In that regard, the present materials behave similarly to PNIPAM-modified columns (2). Nevertheless, at low temperature, the P(MEO_2MA-*co*-OEGMA)-grafted stationary phases allow a better separation of hydrophilic analytes with close log *P* values than their PNIPAM counterparts (2).

In addition, the influence of some macromolecular parameters (e.g., molecular weight or comonomer composition) on the separation of the steroid mixture was investigated. For example, we observed that the composites prepared with a polymer of high molecular weight (Table 1, entry a) require only a small grafting density (0.007 14 chains/nm^2) on the silica support in order to achieve reasonable separation of the five steroids at 55 °C. On the other hand, with a lower molecular weight polymer (Table 1, entry d), the grafting density had to be increased (0.0392 chains/nm^2) to observe a similar performance (Figure 1b) (15). Thus, to be able to achieve optimal control of the hydrophobicity of the column, leading to an efficient separation, higher molecular weight polymers are preferred because a hydrophobic column would require a lower grafting density. Indeed, overgrafting may lead to the blocking of mesopores.

As mentioned, one important advantage of the P(MEO_2-MA-*co*-OEGMA) copolymer is the possibility of tuning their thermosensitivity by adjusting their composition. In Figure 1c, the chromatograms for the separation of the five steroids on two columns packed with composites prepared at differ-

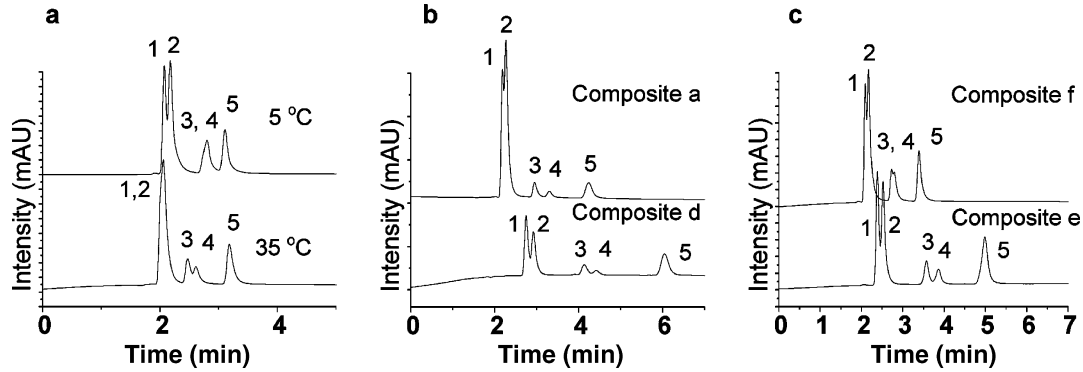


FIGURE 1. Elution profiles and changes in the retention times on five aqueous mixtures of steroids upon variation of (a) the temperature on composite e, (b) the molecular weight at 55 °C, and (c) the comonomer ratio at 35 °C.

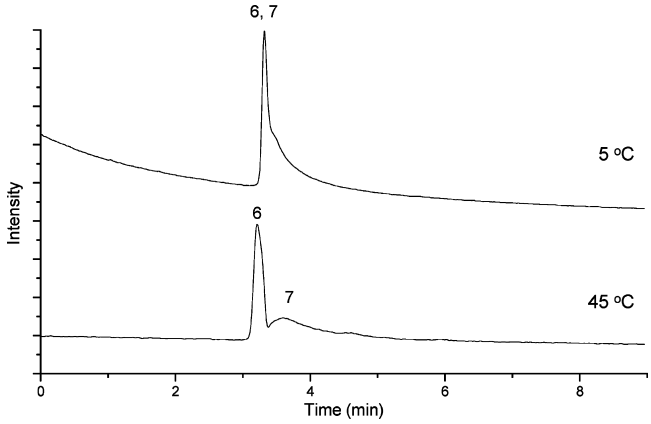


FIGURE 2. Elution profiles and changes in the retention times with temperature variation on an aqueous mixture of two proteins, lysozyme and myoglobin, using composite e.

ent comonomer compositions and thus having different LCSTs are shown. For the polymer with a lower LCST (Table 1, entry e), separation of the more hydrophobic analytes **3** and **4** can be already achieved at lower temperatures (33 °C), while the composite with a higher LCST (Table 1, entry f) can only perform this separation above 43 °C with a worse resolution. Thus, we could show that the separation temperature is closely correlated to the LCST of the polymer and that this could simply be adjusted by changing the comonomer composition of the P(MEO_2MA-*co*-OEGMA) thermoresponsive polymer.

Last, P(MEO_2MA-*co*-OEGMA)-grafted stationary phases were tested for protein chromatography. Previous attempts to separate proteins using thermoresponsive stationary phases employed PNIPAM in combination with ion-exchange polymers such as acrylic acid, using a combination of hydrophobic and ionic interactions (14). Thus, in our preliminary experiments, we chose the most hydrophobic column (Table 1, entry e) to attempt the separation of the two proteins with relatively close hydrophobicities, lysozyme (**6**) and myoglobin (**7**). At a temperature below the LCST of column e, the two proteins were eluted in a single peak. With an increase in the temperature, they achieved near-baseline separation (Figure 2) in a relatively short elution time based only on simple polymers, in contrast to that of the PNIPAM–acrylic acid analogue. Also, their relatively short elution time is in contrast to that of the pure PNIPAM analogue, which showed extensive retention times. A reason for this observation may be the fact that the nonlinear poly(ethylene glycol) (PEG)-like-based polymer backbone is chemically inert, in contrast to the PNIPAM analogue, which contains the amide bond, leading to some nonspecific interaction and extensive retention on the column. The absence of such interactions thus led to the fast elution of proteins on P(MEO_2MA-*co*-OEGMA) columns. However, the broader peaks indicating tailing may also suggest the involvement of different types of interactions between proteins instead of just hydrophobic–hydrophobic forces, as demonstrated in the case of steroid separation.

In conclusion, we have reported for the first time the preparation and chromatographic evaluation of a PEG-related thermoresponsive stationary phase, leading to the successful separation of a mixture of five steroids based on a simple temperature switch under environmentally friendly aqueous conditions. P(MEO_2MA-*co*-OEGMA) displays interesting separation properties that are different from those of PNIPAM, combining the separation of both hydrophilic and hydrophobic bioanalytes. In addition, this polymer is biocompatible and the scope of its applications can be further extended to biomedical technology. By further optimization of our system, proteomics based on isocratic water conditions may one day overcome the current limitations.

Acknowledgment. We thank Dr. Karin Cabrera (Merck Darmstadt) for providing rehydroxylated monolith columns. J.-F.L and Z.Z. thank the Fraunhofer Society and the German Research Foundation (Grant DFG Sfb 448) for financial support.

Supporting Information Available: Experimental procedures, measurements and analysis, and an additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

(1) (a) Kanazawa, H.; Yamamoto, K.; Kashiwase, Y.; Matsushima, Y.; Takai, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. *J. Pharm. Biomed. Anal.* **1997**, *15*, 1545–1550. (b) Kikuchi, A.; Okano, T. *Prog. Polym. Sci.* **2002**, *27*, 1165–1193.
(2) (a) Roohi, F.; Antonietti, M.; Titirici, M. M. *J. Chromatogr., A* **2008**, *1203*, 160–167. (b) Roohi, F.; Titirici, M. M. *New J. Chem.* **2008**, *32*, 1409–1414.
(3) Kanazawa, H. *Anal. Bioanal. Chem.* **2004**, *378*, 46–48.
(4) (a) Hennessy, T. P.; Boysen, R. I.; Huber, M. I.; Unger, K. K.; Hearn, M. T. W. *J. Chromatogr., A* **2003**, *1009*, 15. (b) Skudas,

R.; Grimes, B. A.; Machtejevas, E. E.; Kudirkaite, V. K.; Kornysova, O.; Hennessy, T. P.; Lubda, D.; Unger, K. K. *J. Chromatogr., A* **2007**, *1144*, 72.
(5) Mendes, P. M. *Chem. Soc. Rev.* **2008**, *37*, 2512–2529.
(6) De las Heras Alarcón, C.; Pennadam, S.; Alexander, C. *Chem. Soc. Rev.* **2005**, 276–285.
(7) Lutz, J. F. *Polym. Int.* **2006**, *55* (9), 979–993.
(8) Wu, J. Y.; Liu, S. Q.; Heng, P. W. S.; Yang, Y. Y. *J. Controlled Release* **2005**, *102*, 361–372.
(9) (a) Lutz, J. F. *J. Polym. Sci., Part A: Polym. Chem.* **2008**, *46*, 3459–3470. (b) Aoshima, S.; Kanaoka, S. *Adv. Polym. Sci.* **2008**, *210*, 169–208.
(10) (a) Lutz, J. F.; Akdemir, O.; Hoth, A. *J. Am. Chem. Soc.* **2006**, *128*, 13046–13047. (b) Lutz, J. F.; Hoth, A. *Macromolecules* **2006**, *39*, 893–896.
(11) (a) Jonas, A. M.; Glinel, K.; Oren, R.; Nysten, B.; Huck, W. T. S. *Macromolecules* **2007**, *40*, 4403–4405. (b) Chen, G.; Wright, P. M.; Geng, J.; Mantovani, G.; Haddleton, D. M. *Chem. Commun.* **2008**, 1097–1099. (c) Lee, H. I.; Lee, J. A.; Poon, Z.; Hammond, P. T. *Chem. Commun.* **2008**, 3726–3728. (d) Magnusson, J. P.; Khan, A.; Pasparakis, G.; Saeed, A. O.; Wang, W.; Alexander, C. *J. Am. Chem. Soc.* **2008**, *130*, 10852–10853. (e) Chanana, M.; Jahn, S.; Georgieva, R.; Lutz, J. F.; Bäumler, H.; Wang, D. *Chem. Mater.* **2009**, *21*, 1906–1914. (f) Chen, G.; Amajjahe, S.; Stenzel, M. H. *Chem. Commun.* **2009**, 1198–1200. (g) Pietsch, C.; Hoogenboom, R.; Schubert, U. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 5653–5656.
(12) (a) Wischerhoff, E.; Uhlig, K.; Lankenau, A.; Börner, H. G.; Laschewsky, A.; Duschl, C.; Lutz, J. F. *Angew. Chem., Int. Ed.* **2008**, *47*, 5666–5668. (b) Wischerhoff, E.; Glatzel, S.; Uhlig, K.; Lankenau, A.; Lutz, J. F.; Laschewsky, A. *Langmuir* **2009**, *25*, 5949–5956.
(13) Nicolas, J.; San Miguel, V.; Mantovani, G.; Haddleton, D. M. *Chem. Commun.* **2006**, 4697–4699.
(14) Kanazawa, H.; Nishikawa, M.; Mizutani, A.; Sakamoto, C.; Morita-Murase, Y.; Nagata, Y.; Kikuchi, A.; Okano, T. *J. Anal. Chem.* **2008**, *1191*, 157–161.
(15) The differences in the retention times are due to different grafting densities because the comonomer ratios MEO_2MA/OEGMA are comparable in these samples.

AM900461A