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The use of multilayered capillaries for chiral separation by electrochromatography☆

Fumihiko Kitagawa*, Masato Kamiya, Koji Otsuka

Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

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ABSTRACT

Fused-silica capillaries were modified by the successively multiple ionic-polymer layer (SMIL) coating technique for a capillary electrochromatography (CEC) analysis of binaphthyl enantiomers. The SMIL coating capillaries consisting of three different polymers (A^+–B^-–C^+ coating) were prepared by the alternative deposition of positively charged chiral or achiral polymers and negatively charged DNA. Previous studies have indicated that DNA–cationic polypeptide or synthetic polymer complexes immobilized onto the inner surface of the capillary worked as the chiral stationary phases for 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BNP). In this study, to investigate the chiral recognition mechanism and optimize the CEC separation condition in the DNA–cationic polymer coating, effects of the chirality of the polymer unit, the strand of DNA, and the number of layer pairs on the separation were investigated. It should be noted that, since single stranded DNA (ssDNA) was more suitable to immobilize cationic polymers than double stranded DNA, the ssDNA–cationic polymer immobilized capillaries gave a stable electroosmotic flow and reproducible CEC analyses. As a result, a poly(ethyleneimine)–ssDNA–protamine (Prt) coating provided the best chiral separation of BNP. The high separation performance of the prepared capillary is discussed in terms of DNA/polycations interaction.

1. Introduction

Electrochromatography on capillary and microchip formats, which is the hybrid method of electrophoresis and liquid chromatography, plays an important role in the chiral separation since both the high efficiency of electrophoresis and the high selectivity of chromatography are coupled [1–12]. In capillary electrochromatography (CEC), many chiral selectors have been employed, such as cyclodextrins, crown ethers, peptides, proteins, polysaccharoses, and molecularly imprinted polymers [13–21]. These selectors are generally immobilized in packed, monolithic or open tubular (OT) capillaries in CEC. Among them, the OT capillaries can be prepared with a relatively simple procedure. To immobilize the chiral selectors onto the inner surface of the capillaries, covalent or dynamic coating techniques are mainly applied. By employing the covalent immobilization technique, robust and stable selector layers can be obtained. However, the covalent binding requires labor-intensive and time-consuming processes, which make the optimization of the separation conditions difficult. On the other hand, the dynamic coating via physical adsorption can easily provide the selector immobilized capillaries but needs frequent recoating due to its desorption from the inner surface. Thus, the introduction of a versatile method for stable coatings of the chiral selectors has been desired in OT-CEC.

Recently, a polyelectrolyte multilayer (PEM) [22–24] or successively multiple ionic-polymer layer (SMIL) [25–27] coating has been applied to the OT-CEC analysis of enantiomers [28–30]. These coatings onto the inner surface of a fused-silica capillary involve an electrostatic layer-by-layer deposition process with anionic and cationic polymers. Warner and co-workers reported that the PEM coatings prepared with alternating rinses of cationic poly(diallyldimethylammonium chloride) (PDADMAC) or poly(L-lysine) (PLL) and anionic poly(sodium *N*-undecanoyl-L-leucyl-valinate) or poly(sodium *N*-undecanoyl-L-leucyl-alaninate) were successfully employed to the OT-CEC chiral separations of binaphthyls, benzodiazepines, barbiturates, and β-blockers [28–30]. The PEM coating was very stable and allows more than 200–300 runs to be performed in the same capillary without recoating. In the PEM coating, therefore, only easy and rapid procedures are required to obtain robust immobilization of the chiral selectors.

In our previous study, the SMIL coating consisting of three different polymers (A^+–B^-–C^+ coating) was applied to the immobilization of polypeptides and proteins onto the inner surface of the capillaries to provide a high performance separation medium

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* Corresponding author. Tel.: +81 75 383 2449; fax: +81 75 383 2450.
E-mail address: fkitagawa@mbox.kudpc.kyoto-u.ac.jp (F. Kitagawa).

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Table 1
Polymers used in this study

Polymer	Abbreviation	M_W
Poly(ethyleneimine)	PEI	750,000
Poly(diallyldimethylammonium chloride)	PDADMAC	400,000–500,000
Protamine	Prt	4,250
Poly(L-arginine)	PLA	70,000–150,000
Poly(L-lysine)	PLL	300,000
Poly(D-lysine)	PDL	300,000
Sodium dextran sulfate	DxS	500,000
Double stranded DNA from salmon spermary	dsDNA	300,000–9,000,000
Single stranded DNA from salmon testes	ssDNA	180,000–250,000

for proteins and enantiomers in capillary zone electrophoresis and CEC [31]. We found that, when high-molecular-mass poly(ethyleneimine) (PEI) was employed as the first layer in the A^+–B^-–C^+ coating, even small-molecular-mass polypeptides (protamine, Prt) could be stably immobilized as the third layer. As a preliminary result, furthermore, the chiral separation of a binaphthyl compound in the Prt immobilized capillaries was strongly dependent on the second anionic polymer layer of the coating, i.e., the application of sodium dextran sulfate (DxS) and DNA as the second layer resulted in no and good chiral separation, respectively. These results indicated that DNA–polypeptide complexes play an important role in the chiral recognition but further investigations should be needed.

In this study, the SMIL A^+–B^-–C^+ coating consisting of DNA and several cationic polymers or polypeptides shown in Table 1 was applied to CEC to study the chiral recognition mechanisms.

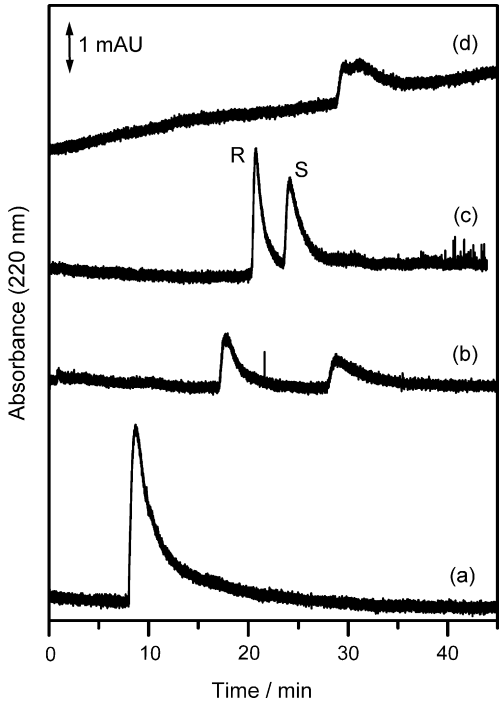

Fig. 1. Chiral separations of BNP in the (a) PEI–DxS–PLA, (b and c) PEI–dsDNA–PLA, and (d) PEI–dsDNA capillaries. BGS, 10 mM phosphate buffer (pH 4.0, (a), (b), (d) 0%, and (c) 20% methanol); sample concentration, 100 ppm; applied voltage, (a–c) −25 kV, (d) +25 kV; injection, 5 kPa, 1 s; applied voltage, −25 kV; detection, 220 nm; temperature, 15 °C. Data taken from Ref. [15].

Effects of the chirality of the polymer unit on the separation were investigated. To enhance the coating stability, single stranded DNA (ssDNA) was employed as the second layer and the comparison of the analytical performance with double stranded DNA was carried out. Furthermore, the number of layer pairs in the coating was varied to adjust the layer thickness. Finally, optical purity test of a binaphthyl compound was demonstrated under an optimized condition.

2. Experimental

2.1. Chemicals

PEI, PDADMAC, poly(L-arginine) (PLA), poly(L-lysine) (PLL), poly(D-lysine) (PDL), Prt, DxS, single stranded DNA (ssDNA, from salmon testes) and 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BNP) were obtained from Sigma–Aldrich (Tokyo, Japan), *N*,*N*′-dimethylformamide (DMF) from Nacalai Tesque (Kyoto, Japan), double stranded DNA (dsDNA, from salmon sperm) from Wako (Osaka, Japan). All reagents were of analytical or HPLC grade. The BGS used throughout the experiments was 10 mM phosphate buffer (pH 3.0–7.0). Sample solutions (50–100 ppm) were prepared by dilution of the standard solutions with the BGS. All solutions were prepared with deionized water purified by using a Direct-Q System (Nihon Millipore, Japan), and filtered through a 0.45-μm pore membrane filter prior to use.

2.2. Immobilization of ionic polymers onto capillary surface

Fused-silica capillaries with 80.0 cm (effective length; 67.8 cm) × 50 μm i.d. obtained from Polymicro Technologies (Phoenix, AZ, USA) were used. The SMIL coating of ionic polymers onto the inner surface of the capillary was carried out as reported previously [31]. Briefly, the capillary was activated with 1 M NaOH. A solution of PEI was pumped through the capillary for 15 min, followed by rinsing with water for 15 min and being left for 5 min. Then, the capillary was rinsed with an anionic polymer solution to form the second layer. Finally, a cationic polymer solution was introduced into the capillary to form the third layer. The concentrations of PEI, Prt, PLA, PLL, PDL, PDADMAC, DxS, dsDNA, and ssDNA solutions were set at 5.0, 2.5, 2.5, 2.5, 2.5, 5.0, 5.0, 1.0, and 0.9% (w/w), respectively. The prepared capillary was conditioned with the BGS prior to the CEC measurement. The capillary on which polymer A^+, B^-, and C^+ was attached as the first, second, and third layer, respectively, was named A–B–C capillary.

2.3. Apparatus

CEC experiments were performed on a CAPI-3300 (Otsuka Electronics, Hirakata, Japan). On-column UV detection was carried out at 220 nm. The temperature was kept constant at 15 °C. Since the CEC separations were carried out in the capillaries attached the cationic polymer as the outermost layer, reversed polarity mode was used at −25 kV. Sample injections were made with a pressure of 5 kPa for 1 s.

3. Results and discussion

3.1. Effect of third cationic polymer layer on CEC separation of BNP enantiomers

In our previous study [31], we found that the chiral separation of BNP in the PEI–polyanion–Prt capillary was strongly dependent on the second anionic polymer layer (Fig. 1). When sodium dextran

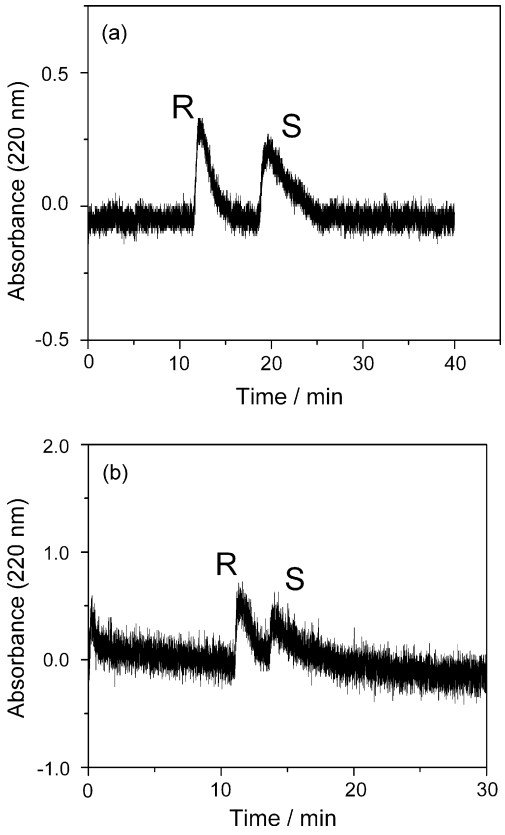

Fig. 2. Chiral separations of BNP in (a) PEI–dsDNA–PLL and (b) PEI–dsDNA–PDL capillaries. BGS, 10 mM phosphate buffer (pH 4.0). Other conditions as in Fig. 1.

sulfate (DxS) was used as the second layer, no chiral separation was attained (Fig. 1a). On the contrary, the application of DNA gave a good chiral separation in OT-CEC (Fig. 1c). These results indicated that DNA–polypeptide complexes formed at the inner surface of the capillary play an important role in the chiral separation of BNP.

To investigate the effect of the chirality of the cationic polymers immobilized onto the third layer on the selectivity, the PEI–dsDNA–PLL and PEI–dsDNA–PDL capillaries were applied to the CEC separation of racemic BNP. As a result, BNP was successfully separated in the both capillaries (Fig. 2). Although the resolution in the PEI–dsDNA–PDL capillary was lower than that in the PEI–dsDNA–PLL capillary, the migration order, i.e., *R* and *S* enantiomers migrated first and second, respectively, was identical in these capillaries. In the PEI–dsDNA–PLA and PEI–dsDNA–Prt capillaries, the migration order was same as the PEI–dsDNA–PDL and –PLL capillaries. Thus, the selectivity in the chiral separation of BNP was independent of the chirality of the amino acid units of the third layer polymer.

An achiral cationic polymer, PDADMAC, was employed to form the dsDNA–polycation complex stationary phase for the CEC separation of racemic BNP (Fig. 3). In the PEI–DxS–PDADMAC capillary, BNP enantiomers were not separated as with the PEI–DxS–PLA. On the other hand, the PEI–dsDNA–PDADMAC capillary provided the chiral separation of BNP at pH 4.0–7.0. The migration order of the enantiomers in the PEI–dsDNA–PDADMAC capillary was also identical with those with the PEI–dsDNA–chiral polycation capillaries. These results suggested that the chiral selectivity for BNP would be mainly determined by not the chirality of the cationic polymer immobilized in the outermost layer but the helicity of the dsDNA–cationic polymer complexes. It has been reported that dsDNA–linear cationic polymer complex exhibits a highly ordered structure and a strong optical activity based on the helicity of the dsDNA chain [32–34]. Since BNP is completely deprotonated at pH > 4.0, stronger interactions with the dsDNA–cationic polymer complexes are expected than those with the negatively charged DNA. The enhanced electrostatic interactions may result in a chiral recognition between the BNP atropisomers and α-helix structure based on dsDNA.

3.2. Effect of second DNA layer on CEC chiral separation

To investigate the effect of the second layer on the chiral separation, we compared dsDNA with ssDNA. Since it has been reported that ssDNA exhibits stronger interaction with polycations than dsDNA due to higher chain flexibility of ssDNA [35,36], a stable immobilization of cationic polypeptides is expected by employing ssDNA as the second layer. For evaluating the immobilization of PLA, the electroosmotic flow (EOF) rate measurements were carried out. The determined electroosmotic mobility (μ_{eo}) is plotted against the number of runs (Fig. 4). In the PEI–DxS–PLA and PEI–dsDNA–PLA capillaries, gradual decreases in μ_{eo} were observed. On the other hand, stable EOF was obtained in the PEI–ssDNA–PLA capillary. To compare the coating stability, a degradation ratio ($\%\Delta\mu_{eo}$) was calculated from the following equation [24]:

$$\%\Delta\mu_{eo} = \frac{\mu_{eo1} - \mu_{eo25}}{\mu_{eo1}} \times 100 \quad (1)$$

where μ_{eo1} and μ_{eo25} are the electroosmotic mobility at the first and 25th run, respectively. The $\%\Delta\mu_{eo}$ in the PEI–dsDNA–PLA capillary was 12.6%, while the use of ssDNA as the second layer provided improved stability with the $\%\Delta\mu_{eo}$ of 4.4%. This result demonstrated that polypeptides could be immobilized in a stable way by using ssDNA as the binder between the first PEI layer and the outer layer.

When the PEI–ssDNA–Prt capillary was employed in the CEC analysis of BNP, a better separation was observed relative to the PEI–dsDNA–Prt as shown in Fig. 5. This result was contrary to our expectation since the ssDNA–polycation complex could not form

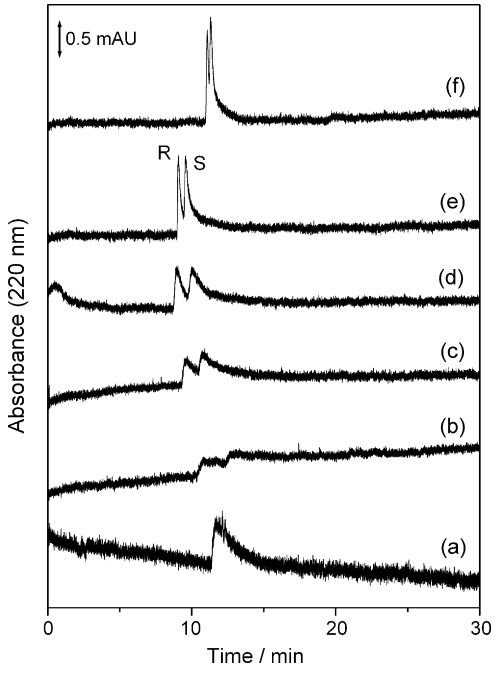

Fig. 3. Chiral separations of BNP in the (a) PDADMAC–DxS–PDADMAC (pH 4.0), (b–f) PDADMAC–dsDNA–PDADMAC capillaries at pH (b) 3.0, (c) 4.0, (d) 5.0, (e) 6.0, and (f) 7.0. Other conditions as in Fig. 1.

Table 2
Separation and retention parameters in the CEC analyses of BNP with the prepared capillaries

	M_w of polypeptide	μ_{eo} ($cm^2/V\,s$)	N_R	N_S	k_R	k_S	α	R_S
PEI–dsDNA–PDL	300,000	-3.8×10^{-4}	1200	1900	1.8	2.4	1.3	1.8
PEI–ssDNA–PDL		-3.9×10^{-4}	620	360	1.2	1.7	1.4	2.1
PEI–dsDNA–PLA	110,000	-2.9×10^{-4}	1400	950	1.9	3.0	1.7	2.4
PEI–ssDNA–PLA		-3.1×10^{-4}	790	460	1.8	3.3	1.8	2.6
PEI–dsDNA–Prt	4,250	-2.3×10^{-4}	110	100	2.2	3.1	1.3	0.6
PEI–ssDNA–Prt		-3.3×10^{-4}	1500	1400	2.4	5.0	2.1	5.2

the α-helix structure. Recently, it has been reported that ssDNA adsorbed on the PEI immobilized surface hybridizes with their complementary strands in a solution during the PEM formation process, which allows the formation of dsDNA with rearranging the surface structure [37]. Taking this phenomenon into account, it was considered that the PEI–ssDNA–Prt capillaries contained both ssDNA and dsDNA regions on the surface. When Prt interacted with the partially distributed dsDNA region, helical dsDNA–Prt complexes would form on the capillary surface. As a result, the PEI–ssDNA–Prt capillary showed the chiral separation ability for BNP enantiomers. It should be noted that the best chiral separation of BNP was obtained in the PEI–ssDNA–Prt capillary among all the combination of ionic polymers listed in Table 1. However, the enhanced separation by employing ssDNA was attained only in the PEI–DNA–Prt capillaries. As shown in Table 2, only a slight increase in resolution (R_S) of BNP by applying ssDNA as the second layer was observed in comparison with dsDNA in the PDL- and PLA-coated capillaries. To discuss this enhancement effect, the retention factor (k) and selectivity factor (α) were estimated. The k used in liquid chromatography is not simply available in CEC due to the coupled electrophoretic migration. Taking the electrophoretic mobility (μ_{ep}) of the analytes into consideration, k can be calculated from the following equation [38]:

$$k = \frac{(1 + \mu_{ep}/\mu_{eo})t_r - t_0}{t_0} \quad (2)$$

where t_r and t_0 are the detection time of the analyte and the EOF marker, respectively. Assuming that the μ_{ep} values determined in the bare capillaries are the same with those in the coated capillaries and the electrophoretic mobilization of the analytes in the stationary phase can be neglected, the values of k and α can be estimated as summarized in Table 2. As can be seen in the table, large changes in the α and R_S values between ssDNA and dsDNA were not observed in the PEI–DNA–PDL and PEI–DNA–PLA capillaries, while in the PEI–DNA–Prt capillary they were considerably increased by employing ssDNA. This smaller difference in the separation and retention characteristics between the PEI–ssDNA–PDL or –PLA and PEI–dsDNA–PDL or –PLA capillaries might be curious. We anticipated the larger difference in the separation performance among these capillaries since the surface structures of the ssDNA–cationic polymer immobilized capillaries, which are consisting of both the ssDNA and dsDNA regions as mentioned above, would be considerably different from those of the dsDNA–polycation capillaries. As one possible reason, this may be due to the differences in the molecular weight of DNA and cationic polymers.

It is well known that polycations interact with both ssDNA and dsDNA, and condense them into compact and ordered nanoparticles in a free solution [39–41]. It has been also reported that a smaller polycation condensed DNA into huge aggregates up to 2 μm in size, while a larger polycation condensed DNA into compact particles with diameters of 80–100 nm [42]. As discussed in our previous study, if DNA chains are not completely immobilized in the first PEI layer region and extruded towards the outer water phase to give polymer brushes, the electrostatic interaction between DNA

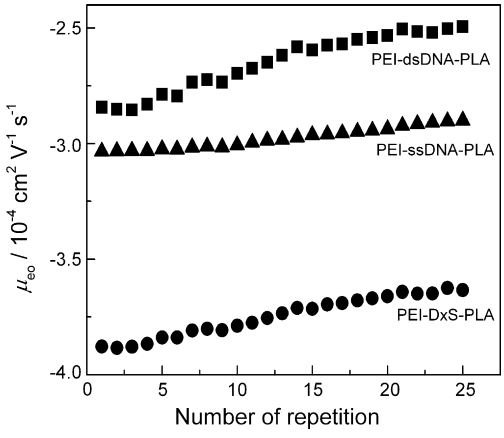

Fig. 4. Stability of the prepared capillaries. The μ_{eo} values were determined from the migration time of DMF in 10 mM phosphate buffer (pH 4.0).

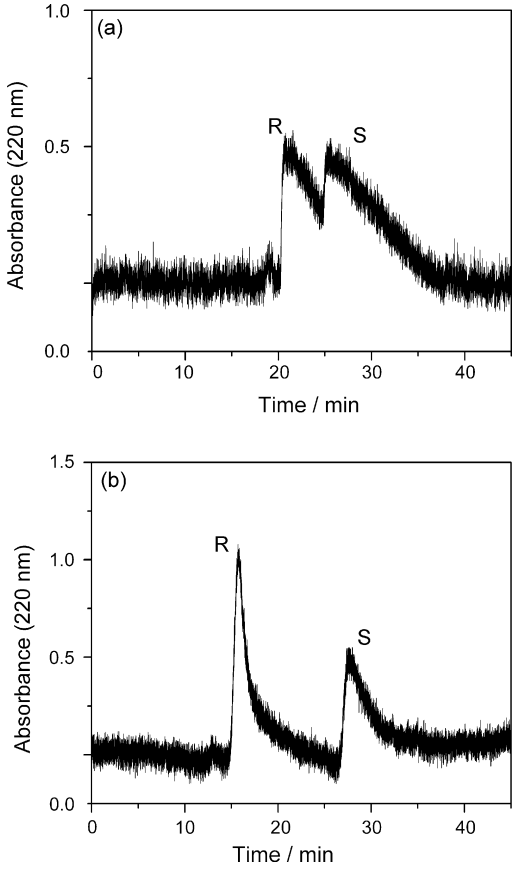

Fig. 5. Chiral separations of BNP in (a) PEI–dsDNA–Prt and (b) PEI–ssDNA–Prt capillaries. Conditions as in Fig. 2.

Table 3
Effect of the number of layer pairs on the separation and retention parameters in the CEC analyses of BNP with an ssDNA/Prt multilayer

Number of layer pairs[a]	%RSD of t_R	N_R	N_S	k_R	k_S	α	R_S
1	0.51	2900	1800	1.4	2.5	2.2	4.3
2	4.4	760	600	2.3	3.8	1.7	2.4
3	2.9	240	230	3.3	3.9	1.2	1.3

[a] PEI was coated as the first layer.

brushes and PDL or PLA results in the formation of condensed DNA–polypeptide particles on the inner surface of the capillary [33,34]. Such high condensation ability of these cationic polypeptide would provide a resembled surface structure with packed DNA–polypeptide particles in the both ssDNA and dsDNA immobilized capillaries despite ssDNA is quite smaller than dsDNA used in this study. Thus, this may be one possible reason why the μ_{eo}, k, α, and R_S values obtained with the PEI–ssDNA–PDL and PLA capillaries were almost identical with those with the PEI–dsDNA–PDL and PLA, respectively. On the other hand, the DNA condensation ability of Prt is lower than PLA and PDL because Prt is a small. Hence, the DNA–Prt complexes would not form a tightly packed structure but remain a brush-like structure whose chains are oriented to the outer water phase. As a result, the surface structure strongly depended on the molecular weight of DNA, so that the apparent differences in the separation and retention parameters appeared between ssDNA– and dsDNA–Prt immobilized capillaries. Since a brush-like and helical DNA–Prt complex structure on the capillary surface would be favorable for the chiral recognition of BNP, the PEI–ssDNA–Prt capillary showed the best and reproducible separation performances for the racemic BNP on the basis of the stronger ssDNA/Prt interaction and lower condensation ability of Prt. The separation performance would be affected by the ionic strength of the polymer solution for preparing the SMIL capillary due to changes of the condensation ability of cationic polymers [43], and this possibility is being examined.

As one of the useful chacteristics of the SMIL coating technique, multilayers can be prepared onto the inner surface of the capillary to adjust the layer thickness. To estimate the effect of the number of layer pairs on the chiral separation of BNP, we prepared the PEI–(ssDNA–Prt)$_n$ (n = 1, 2, 3) capillaries. In all the prepared capillaries, the CEC separations of racemic BNP were attained and the separation and retention parameters are summarized in Table 3. Upon increasing the number of layer pair, the retention factor was increased, whereas plate number (N), α, and R_S values were decreased. This may be due to disordering of the surface structure with increasing the number of layer pairs. It should be emphasizing that the degree of the peak tailing was increased with increasing the number of layer pairs, which indicated the irreversible retention of BNP by the ssDNA–Prt stationary phase. In preparing the PEI–(ssDNA–Prt)$_n$ capillaries, furthermore, the yield was reduced with increasing the number of layer pairs since the larger aggregation of the DNA–Prt complexes formed by the replicate injections of the modification solutions sometimes clogged up the capillaries. This may be disadvantage of the developed method, so that further optimization of the modification condition, e.g., polymer concentration, temperature, and flow rate, should be needed to improve the preparation yield. When the successfully prepared PEI–ssDNA–Prt capillary was employed to the CEC analysis, the run-to-run repeatability of the detection time of the *R* enantiomer was good with the relative standard deviation (RSD) of 0.51%. Thus, the three layer coating of the PEI–ssDNA–Prt brought the best chiral separation performance in the SMIL capillaries investigated in this study.

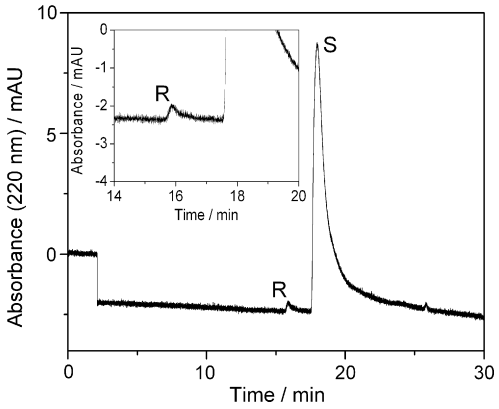

Fig. 6. CEC analysis of 5.0 μg/mL (*R*)-BNP and 495 μg/mL (*S*)-BNP obtained with the PEI–ssDNA–Prt capillary. Conditions as in Fig. 5. Inset shows an expanded electropherogram around the *R* enantiomer peak.

3.3. Application to optical purity test

By using the PEI–ssDNA–Prt capillary, the evaluation of lower amounts of one enantiomer beside higher amounts of the other was carried out, which is important to determine the optical purity of a compound. As shown in Fig. 6, the chiral separation was attained and the minor *R* enantiomer with 1.0% level can be detected along with 99% major *S* enantiomer. Based on the ratio of the peak area, the enantiomeric excess of (*R*)-BNP over (*S*)-BNP was calculated to be 97.9%. The obtained enantiomeric excess agreed very well with a known concentration of (*R*)-BNP and (*S*)-BNP of the injected sample.

4. Conclusions

The SMIL-coated capillaries consisting of DNA–cationic polymer or polypeptide were successfully applied to the CEC chiral separation of BNP. The migration order of (*R*)- and (*S*)-BNP was independent of the optical activity of the third polymer layer polymer in the PEI–dsDNA–PLL and –PDL capillaries. In the PEI–dsDNA–PDADMAC capillary, the chiral separation of BNP could be attained in spite of using an achiral polymer as the third layer. These results indicated that the helix structure of the DNA–cationic polymer complexes played an important role in the chiral separation. By employing ssDNA as the second layer in place of dsDNA, the stable coating was obtained to provide reproducible CEC analyses. The use of ssDNA as the binder in the SMIL coating will be effective to immobilize cationic polypeptides or proteins onto the capillary surface for not only the CEC stationary phase but also for bioanalyses such as affinity assays. Among all the combination of ionic polymers tested in this study, the PEI–ssDNA–Prt capillary gave the best chiral separation of BNP. This high separation performance of the ssDNA–Prt complex immobilized capillary is discussed on the basis of the ssDNA/polycations interaction. The number of layer pairs in the SMIL coating was also an important factor in the chiral separation. In the PEI–ssDNA–Prt capillary, furthermore, the CEC separation of 1% *R*-enantiomer in excess of 99% *S*-enantiomer could be achieved. Studies are in progress to apply the DNA–polypeptide-coated SMIL capillaries to a wide range of enantioseparations.

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