

Open-tubular Electrochromatographic Chiral Separation of Amino Acids Using an Organic Nanocrystals Immobilized Capillary

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The preparation of cinchonidine (CCND) nanocrystals and their immobilization onto the inner surface of a fused silica capillary was investigated for use in the enantioseparation by capillary electrochromatography. The CCND nanocrystals were prepared by an emulsion method that utilizes crystal growth in an oil-in-water emulsion without a stabilizer. As a result, white-colored aqueous dispersions of organic nanoparticles were obtained. SEM images showed that the prepared CCND nanocrystals were from 300 to 700 nm in size. When the obtained dispersion was introduced into a poly(diallyldimethylammonium chloride) (PDDAC) coated capillary, the nanocrystals were strongly adsorbed onto the inner surface due to an electrostatic interaction between cationic PDDAC and the negatively charged organic nanocrystals. In CCND nanocrystals coated capillary, CEC enantioseparations of racemic amino acids were successfully achieved. The reproducibilities of the detection times were acceptable with a relative standard deviation of less than 7%, indicating that stable nanocrystal coating could be obtained by our proposed method.

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Introduction

Cinchona alkaloids, which include quinine, quinidine, cinchonine, cinchonidine, and so on, are one of the most important chiral selectors in separation science. In high-performance liquid chromatography (HPLC), some of these chiral selectors and their derivatives immobilized silica packed columns are commercially available, and provide good enantioseparation performance.¹ On the other hand, the application of the cinchona alkaloids to the capillary electrophoresis (CE) separation of chiral compounds is still limited in spite of their superior chiral selectivity.² This is because these selectors are insoluble to water. In chiral CE analysis, conventional chiral selectors, such as cyclodextrins and crown ethers, are generally added to an aqueous background solution (BGS) as a pseudostationary phase (PSP). To use water-insoluble cinchona alkaloids as the PSP, a non-aqueous CE (NACE) mode is selected to dissolve the selectors in non-aqueous BGSs.³⁻⁶ However, slow and unstable electroosmotic flow (EOF) in the NACE mode sometimes offers a relatively poor analytical reproducibility. In addition, the cinchona alkaloids exhibit strong absorption in the UV region due to the quinoline ring, which interferes with UV detection of the target analytes. Hence, counter-current and partial-filling techniques have been employed to avoid a strong UV background in the cinchona alkaloids-based NACE mode.^{2,7}

To apply such water-insoluble and UV-absorptive chiral selectors to CE, capillary electrochromatography (CEC), which is a hybrid method of CE and HPLC, should be an alternative of the NACE mode. In CEC, the chiral selectors are immobilized in packed, monolithic or open-tubular (OT) capillaries.⁸⁻¹¹ Besides, both the high efficiency of CE and the high selectivity of HPLC are coupled in CEC; the immobilization of the chiral selectors onto the packed, monolithic materials or the inner surface of the capillary can avoid the UV background, according to the selector. Preinerstorfer *et al.* have reported the preparation of a monolithic silica capillary column modified with cinchona alkaloid derivatives to separate dipeptides in non-aqueous CEC.¹² In spite of high separation efficiencies, the monolithic capillary required labor-intensive and time-consuming synthetic processes for derivatization of the native cinchona alkaloid to immobilize onto the monolithic structure surfaces. So far, only this report has appeared to utilize the cinchona alkaloids as the chiral selector in CEC. Thus, the introduction of a versatile method to modify the water-insoluble and UV-absorptive chiral selectors has been desired in CEC.

Recently, the application of nanoparticles to OT-CEC as the chiral stationary phase has been reported.^{13,14} Dong *et al.* have developed a mesoporous silica nanoparticles immobilized capillary column with a coating of cellulose derivatives for the OT-CEC separation of nine pairs of enantiomers including acidic, neutral and basic racemates.¹³ Li *et al.* have immobilized bovine serum albumin-gold nanoparticle conjugates onto the separation microchannel to resolve ephedrine enantiomers in OT-microchip CEC.¹⁴ Due to the large surface area of the nanoparticles modified OT capillary and microchannel, a high

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enantioselectivity can be obtained. Hence, the use of nanoparticles as the stationary phase should be effective in chiral CEC.

In this study, organic nanocrystals from a native cinchona alkaloid, cinchonidine (CCND), were prepared by reprecipitation¹⁵ and emulsion methods¹⁶ developed by Nakanishi's group. In these methods, hydrophobic compounds can be nanocrystallized and stably dispersed in water. Such a stable dispersion may be due to a relatively high zeta potential on the surfaces of the organic nanocrystals,^{17–19} which is advantageous for immobilizing onto the base material surfaces by an electrostatic layer-by-layer (LbL) deposition technique.^{20,21} Oikawa *et al.* reported that the LbL deposition of cationic poly(diallyldimethylammonium chloride) (PDDAC) and negatively-charged poly(diacetylene) nanocrystal dispersion has been successfully applied to the fabrication of multilayered thin films.^{18,19,22} By utilizing these unique characteristics of the organic nanocrystals, the water-insoluble native CCND is expected to be electrostatically immobilized onto the capillary surface easily and stably without any organic synthetic processes. To our knowledge, furthermore, no study has been reported on the application of organic nanocrystals to separation science. In the present paper, the prepared CCND nanocrystals were modified onto the inner surface of the fused silica capillary for the enantioseparation of amino acids in OT-CEC.

Experimental

Chemicals

(–)-CCND, PDDAC ($M_w = 400000 - 500000$) and *N*-(3,5-dinitrobenzoyl)-DL-leucine (DNB-DL-Leu) were obtained from Sigma-Aldrich (Tokyo, Japan), toluene, acetonitrile and DL-phenylalanine (DL-Phe) from Nacalai Tesque (Kyoto, Japan), thiourea from Wako (Osaka, Japan). Thiourea was used as an EOF marker. BGSs used throughout the experiments were 10 mM phosphate buffer (pH 5.0 – 8.0). All solutions were prepared with deionized water purified by using a Direct-Q System (Nihon Millipore, Japan), and filtered through a 0.45- μ m pore membrane filter prior to use.

Instrumentation

All CE experiments were performed on a P/ACE MDQ system (Beckman Instruments, Fullerton, CA) equipped with a diode-array UV detector. Fused-silica capillaries with 40 cm (effective length; 30 cm) $\times$ 50 μ m i.d. obtained from Polymicro Technologies (Phoenix, AZ) were used. Samples were hydrodynamically injected at 0.5 psi for 5 s (injection volume, 9.0 nL). The applied voltage and the temperature were set at +20 kV and 25°C, respectively. UV detection was performed at 200 or 230 nm.

In the preparation of the organic nanocrystals by an emulsion method, ultrasonic wave irradiation was carried out using a UD-200 ultrasonic disrupter (TOMY DIGITAL BIOLOGY, Tokyo, Japan). The sizes and morphologies of the nanocrystals were evaluated by field-emission scanning electron microscopy (FE-SEM; JSF 7400F JEOL, Japan). A UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) was used to observe the transmittance of the nanocrystal dispersions.

Procedure

CCND organic nanocrystals were prepared by the reprecipitation¹⁵ and emulsion methods.¹⁶ In the reprecipitation method, 400 μ L of a 5 mM CCND acetonitrile solution was added dropwise using a microsyringe into 10 mL of vigorously

stirred water at 60°C. The temperature was maintained for a few minutes, and gradually lowered to room temperature under stirring to obtain a white-colored aqueous dispersion of CCND nanocrystals. In the emulsion method, 6 mL of a 20 mM CCND toluene solution was added dropwise by a microsyringe to 100 mL of water at 60°C, followed by ultrasonic agitation and mechanical stirring. After ultrasonic agitation for 20 min, the temperature was gradually lowered to room temperature under mechanical stirring. A toluene layer formed by stopping the stirring and breaking the emulsion was removed, and finally a clear aqueous dispersion of the CCND nanocrystals was obtained.

The prepared CCND nanocrystals were immobilized onto the inner surface of the capillary by the LbL deposition technique.^{18–22} A bare fused-silica capillary was activated with 1 M NaOH. A solution of 7.5% (w/w) PDDAC in Tris-HCl buffer (pH 8.0) was pumped through the capillary for 30 min, followed by rinsing with water for 15 min. Then, the prepared CCND nanocrystals dispersion was introduced into the capillary. Finally, the prepared capillary was conditioned with a BGS for 30 min prior to the CE measurement.

Results and Discussion

Preparation of the CCND nanocrystals

To prepare the CCND nanocrystals immobilized capillary, we compared two preparation methods for the organic nanocrystals. First, the precipitation method,¹⁵ which is known to be a simple and easy preparation technique, was applied to the preparation of the CCND nanocrystals. When an acetonitrile solution of CCND was added to hot water, white precipitations formed immediately. After cooling to room temperature under mechanical stirring, a clear and white-colored aqueous dispersion of the CCND nanoparticles was obtained. However, the formation of large aggregations was gradually observed as the supernatant became transparent, indicating that the CCND nanoparticles aggregated under the static condition, *i.e.*, without mechanical stirring. Since such an unstable dispersion of the nanocrystals should cause clogging in the capillary at the surface immobilization step, we investigated the application of the emulsion method to the preparation of CCND nanocrystals.

In the emulsion method, an organic solution containing the target compounds at high temperature is emulsified without a stabilizer, and then cooled to room temperature.¹⁶ During the cooling, nucleation and crystal growth occur inside the emulsion to produce an aqueous dispersion of nanocrystals. It has been reported that the size distribution of nanocrystals in the emulsion method becomes narrower than that in the reprecipitation method.²³ Thus, it is anticipated that a more sophisticated emulsion method will give a stable dispersion of the organic nanocrystals. In our study, toluene-in-water emulsion was used to prepare the CCND nanocrystals. As a result, a clear and white-colored aqueous dispersion was obtained. By measuring the SEM images, it was clarified that the prepared CCND nanocrystals were from 300 to 700 nm in size, as shown in Fig. 1a. Although the aqueous dispersion containing the CCND nanocrystals with a broader size-distribution was obtained by the emulsion method, which differed from the previous report,^{16,23} a very stable nanocrystals dispersion was successfully prepared. To evaluate the stability of the dispersion, the optical transmittances at 532 nm of the CCND nanocrystals dispersions prepared by the reprecipitation and emulsion methods were plotted against the time (Fig. 1b). In the dispersion obtained by the reprecipitation method, the transmittance was immediately

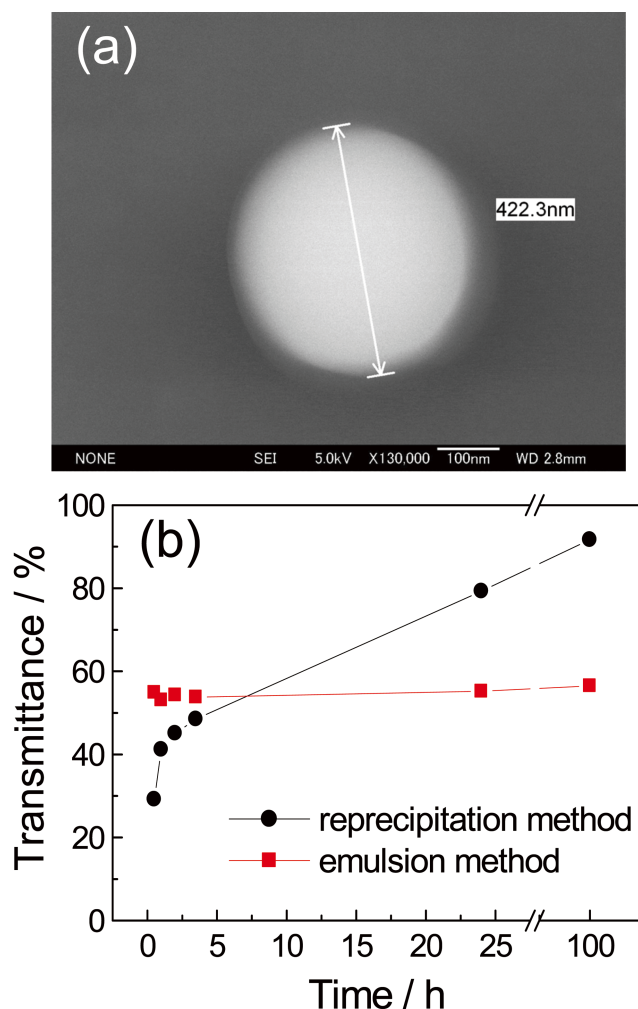


Fig. 1 (a) Typical FE-SEM image of the CCND nanocrystals obtained with the emulsion method and (b) time courses of the optical transmittance at 532 nm of the CCND nanocrystal dispersions prepared by the reprecipitation and emulsion methods.

increased during the first 1 h, and a gradual increase for up to 100 h was observed. This increased transparency of the supernatant indicated that the amount of the nanocrystals decreased accompanying the formation of large aggregations. On the other hand, the transmittance of the dispersion prepared by the emulsion method remained almost constant during 100 h, demonstrating the superior stability of the dispersion. After 5 days, actually, the dispersion remained white-colored and large aggregations were scarcely found. Such a stable dispersion should be suitable for the surface modification inside the capillary.

Immobilization of the CCND nanocrystals onto the capillary surface

For immobilizing the CCND nanocrystals onto the inner surface of the fused-silica capillary to form the chiral stationary phase, the LbL deposition technique was utilized. The stable dispersion obtained from the stabilizer-free emulsion method indicated that the CCND nanocrystals would be sufficiently charged. Hence, the electrostatic interaction between ionic polymers and charged nanocrystals can give a stable immobilization onto the capillary surface.²¹ In this study, cationic PDDAC was first selected as the binding layer between

Table 1 Electroosmotic mobilities (μ_{eo})^a in the prepared capillaries^b

Coating	μ_{eo} (cm ² /V·s)	%RSD (n = 6)
Bare fused silica capillary	$+2.9 \times 10^{-4}$	0.67
After treatment with PDDAC	-2.0×10^{-4}	1.0
After treatment with CCND	$+2.4 \times 10^{-4}$	0.95

a. Minus sign represents a reversal EOF.

b. EOF marker, 50 ppm thiourea; BGS, 10 mM phosphate buffer (pH 5.0). Other conditions are as in the Experimental section.

the CCND nanocrystals and the fused-silica surface.

The modification of the capillary was evaluated by the EOF rate measurements by using phosphate buffer (pH 5.0) as the BGS. Table 1 gives the electroosmotic mobility (μ_{eo}) in the bare, the PDDAC modified, and the CCND nanocrystals immobilized capillaries. The μ_{eo} values in the bare and the PDDAC modified capillaries were estimated to be $+2.9 \times 10^{-4}$ and -2.0×10^{-4} cm²/V·s, respectively. The negative μ_{eo} value clearly showed that the EOF was reversed by the coating with cationic PDDAC. After the CCND nanocrystals immobilization, on the other hand, the cathodic EOF was observed and μ_{eo} was calculated to be $+2.4 \times 10^{-4}$ cm²/V·s at pH 5.0. This re-inversion of the EOF direction indicated that the CCND nanocrystals were negatively-charged and immobilized onto the PDDAC layer. It should be emphasized that the negatively-charged CCND nanocrystals in water was curious, since the CCND molecule has a protonatable tertiary amine moiety in the quinuclidine ring ($pK_a \sim 10$).²⁴ This might be due to a preferential adsorption of anions onto the nanocrystals. In water, hydroxide ions tend to be strongly attracted to hydrophobic solid surfaces, generating a negative zeta potential.²⁵ If such preferential adsorption onto the nanocrystals exceeds protonation of the quinuclidine ring of CCND, they would be negatively-charged. Actually, the μ_{eo} of the CCND nanocrystals immobilized capillary was increased from $+2.4 \times 10^{-4}$ to $+3.2 \times 10^{-4}$ cm²/V·s with increasing pH of the BGS from 5.0 to 8.0. The increased EOF rate at higher pH may indicate preferential adsorption of hydroxide anions onto the CCND nanocrystals. In the present stage, the reason for the negative zeta potential of the CCND nanocrystals is still unclear, and thus further schematic studies on the electrostatic structure of the nanocrystal surface should be made.

In the CCND nanocrystals immobilized capillary, the relative standard deviation (RSD) of μ_{eo} was good, less than 1%, as shown in Table 1. The good repeatability of the EOF rate showed stable immobilization of the nanocrystal layer. Therefore, these results demonstrated that the modification of the CCND nanocrystals was successfully achieved by the electrostatic interaction *via* the cationic PDDAC binding layer, and that the modified capillary can be employed to OT-CEC.

Application to the OT-CEC chiral separation of amino acids

In a preliminary study of the OT-CEC chiral separation using the CCND nanocrystals immobilized capillary, DNB-DL-Leu and DL-Phe were selected as test analytes, since they are UV-absorptive and commercially available amino acids. DNB-DL-Leu was also well investigated in previous studies on the cinchona alkaloids-based NACE and CEC separation. Figure 2 shows electrochromatograms of these amino acids obtained with the bare and the nanocrystals immobilized capillary. In the bare capillary, these amino acid enantiomers were detected as a single peak, while by coating the CCND

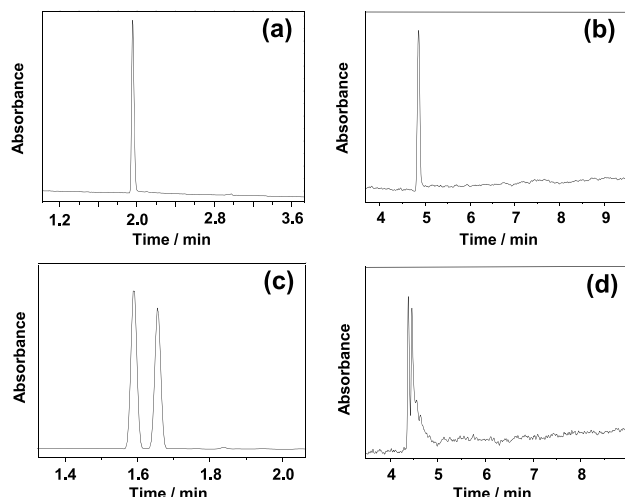


Fig. 2 OT-CEC analyses of amino acids obtained with (a, b) the bare fused-silica and (c, d) the CCND nanocrystal immobilized capillary. BGS, 10 mM phosphate buffer (pH 5.0); analytes, (a, c) 50 ppm DNB-DL-Leu, (b, d) 15 ppm DL-Phe.

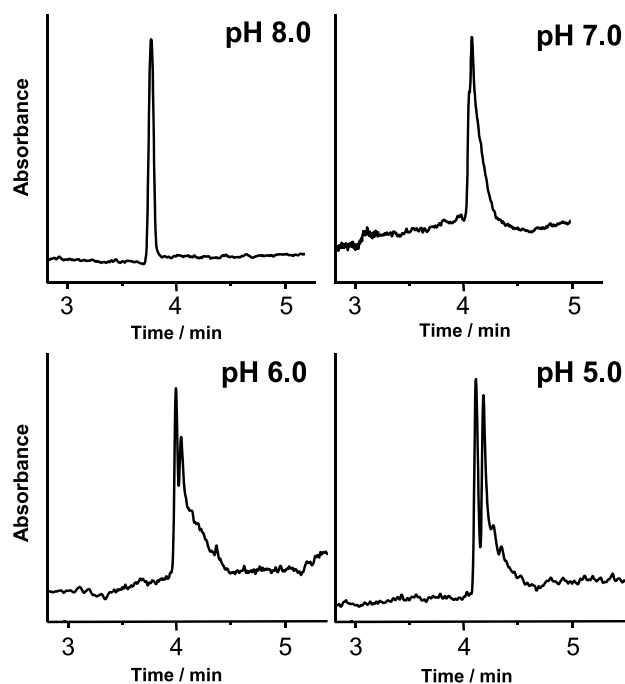


Fig. 3 Effect of the pH on the chiral separation of DL-Phe in CCND nanocrystals immobilized capillary. Other conditions are the same as in Fig. 2.

nanocrystals, the enantioseparations were achieved at pH 5.0. This clearly showed that CCND still retained the chiral selectivity in spite of nanocrystalization. It should be emphasized that such chiral separations were not observed in a molecular CCND physically-coated capillary, which was prepared by introducing a CCND methanolic solution into the bare capillary for 20 min, due to an insufficient amount of the chiral selector. The results indicated that chiral separation was attained on the basis of the increased phase ratio by immobilizing the nanocrystals with higher surface areas.

At pH 5.0, a baseline separation of DNB-DL-Leu was achieved in the prepared capillary (Fig. 2a). The good chiral separation

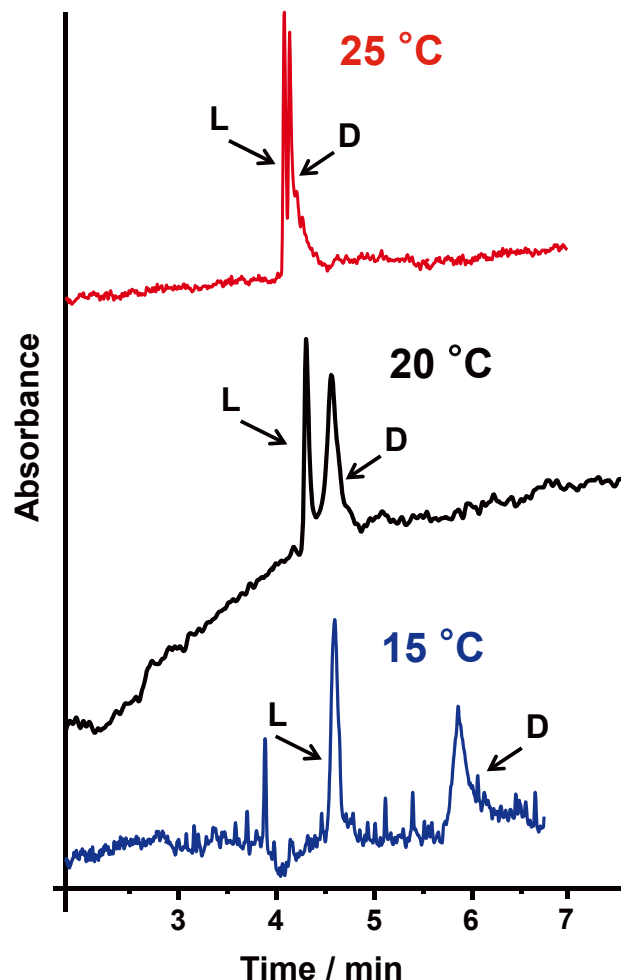


Fig. 4 Effect of the temperature on the chiral separation of DL-Phe in the CCND nanocrystals immobilized capillary. Other conditions are the same as in Fig. 2.

of DNB-DL-Leu at pH 5.0 agreed well with previous reports on the chiral HPLC and CEC separations of DNB-derivatized amino acids in cinchona alkaloids-based chiral columns at pH 5 – 7.^{12,26} On the other hand, the resolution of DL-Phe was apparently poor, as shown in Fig. 2b. To improve the resolution, the effect of the pH on the chiral separation of DL-Phe was investigated. As a result, at pH 6.0 a partial separation was observed, while at pH 7.0 and 8.0 only a single peak appeared, as shown in Fig. 3. At pH 4.0 reproducible measurements were difficult, probably due to desorption of the nanocrystals from the capillary surface, even in measurements of the EOF rate. These results showed that the best chiral separation of DL-Phe was obtained at around pH 5.0. It has been reported that the ion pairing between positively-charged tertiary amine moiety in the quinuclidine ring of CCND and negatively-charged carboxylate group in the amino acids and the π - π interaction between the quinoline ring of CCND and the phenyl ring of DL-Phe plays a crucial role in the chiral selectivity of the cinchona alkaloids-based chiral column.^{26,27} In the present condition, the best ion pairing and the π - π interaction would be attained at pH 5.0.

To obtain a baseline separation of DL-Phe, the effect of the temperature was studied. As shown in Fig. 4, the chiral resolution was increased upon decreasing the temperature, resulting in baseline separation at 15°C. Such a temperature

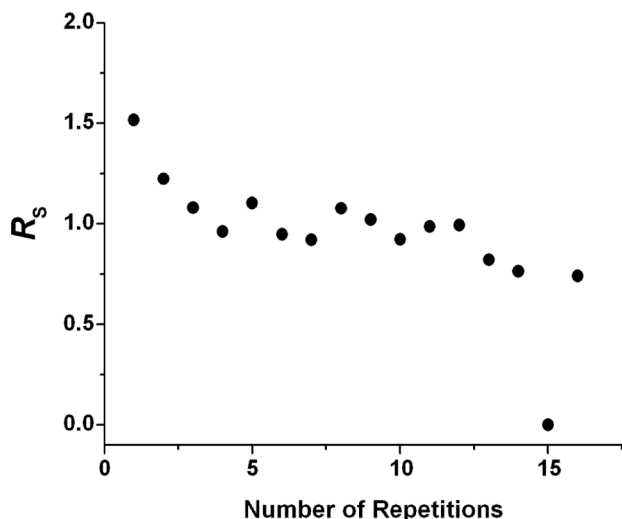


Fig. 5 Effect of the number of repetitions on the chiral resolution of DL-Phe in the CCND nanocrystals immobilized capillary. Conditions are the same as in Fig. 2.

dependence reflected a strongly enthalpy-driven process in the ion-pair formation between cinchona alkaloids and amino acids, which agreed well with previous reports on the enantioseparation of amino acids in the cinchona alkaloids-based selectors.^{28,29} Considering the results obtained in this study, the chiral-recognition mechanism on the CCND nanocrystals surface resembled with that in the conventional cinchona alkaloids-based chiral columns.

Although the decrease in the temperature was effective to improve the chiral resolution, on the other hand, poor analytical repeatabilities of the detection time and the resolution were obtained. Furthermore, serious baseline noises and fluctuations were observed at 15°C. This unstable baseline may be due to an insufficient equilibration time. It is well-known that it takes a very long time for the equilibration of CEC columns under the measuring condition, *i.e.*, the column is filled with the running BGS at the selected temperature, to obtain reproducible separations and stable baselines. In the present condition, the equilibration time was set at 30 min, which may be insufficient. For CE users, however, a very long equilibration time, *e.g.*, overnight, at constant and low temperature prior to run is not practical. Furthermore, the long equilibration may cause a gradual dissolution and desorption of the nanocrystals. In the nanocrystals immobilized capillary, hence, an alternative approach to improve the resolution was desired. As a preliminary result, the increase in the effective separation length from 30 to 40 cm gave a baseline separation of DL-Phe at pH 5.0 and 25°C, but considerable tailing of the second migrating peak was observed. Thus, more precise optimization of experimental conditions, such as BGS, pH, temperature, capillary length, immobilized amount and size control of the nanocrystals, and so on, should be needed for the practical use of the nanocrystals immobilized capillary.

The endurance of the CCND nanocrystals coating was investigated by conducting consecutive runs using the same capillary. The repeatabilities of the detection times of the amino acids were acceptable with RSDs of less than 7% ($n = 10$). To demonstrate the separation repeatability, the resolution (R_s) of DL-Phe was plotted against the number of repetitions (Fig. 5). At the first and second runs, a higher R_s was obtained, while from the third to twelfth runs, almost constant resolutions were

observed. Low R_s at the fifteenth run may have been due to a sample injection failure, since the obtained single peak was quite broad. These results indicated that a stable nanocrystal-coating could be obtained by our proposed method. At the present stage, however, the application data in the prepared capillary is still limited. Additionally, the column-to-column reproducibility of the chiral separation and the batch-to-batch reproducibility of the preparation of the CCND dispersion are not acceptable. Therefore, further optimizations of the preparation of the nanocrystals, the immobilization onto the capillary surface and the separation conditions are expected to provide stable, reproducible, and high-performance analyses of various enantiomers in the nanocrystals-based separation capillary column.

Conclusion

Cinchona alkaloid nanocrystals coated capillaries were successfully applied to the OT-CEC chiral separation of DNB-DL-Leu and DL-Phe. When the nanocrystals were prepared by the emulsion method, a stable dispersion was obtained, which was suitable for surface modification inside the capillary. By employing PDDAC as the binding layer, the CCND nanocrystals were well immobilized onto the inner surface of the capillary *via* the electrostatic interaction between the cationic polymer layer and negatively-charged CCND nanocrystals. In the separation of racemic DNB-DL-Leu and DL-Phe on the prepared capillary, the chiral-recognition mechanism on the CCND nanocrystals surface resembled that in the conventional cinchona alkaloids-based chiral columns. Based on capillary modification by the electrostatic interaction between cationic polymer binding layer and the nanocrystallized CCND, the water-insoluble cinchona alkaloid could be applied to the aqueous OT-CEC chiral analysis without any complicated organic synthesis. Studies are in progress to apply the cinchona alkaloid nanocrystals coated capillaries to a wide range of enantiomers.

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