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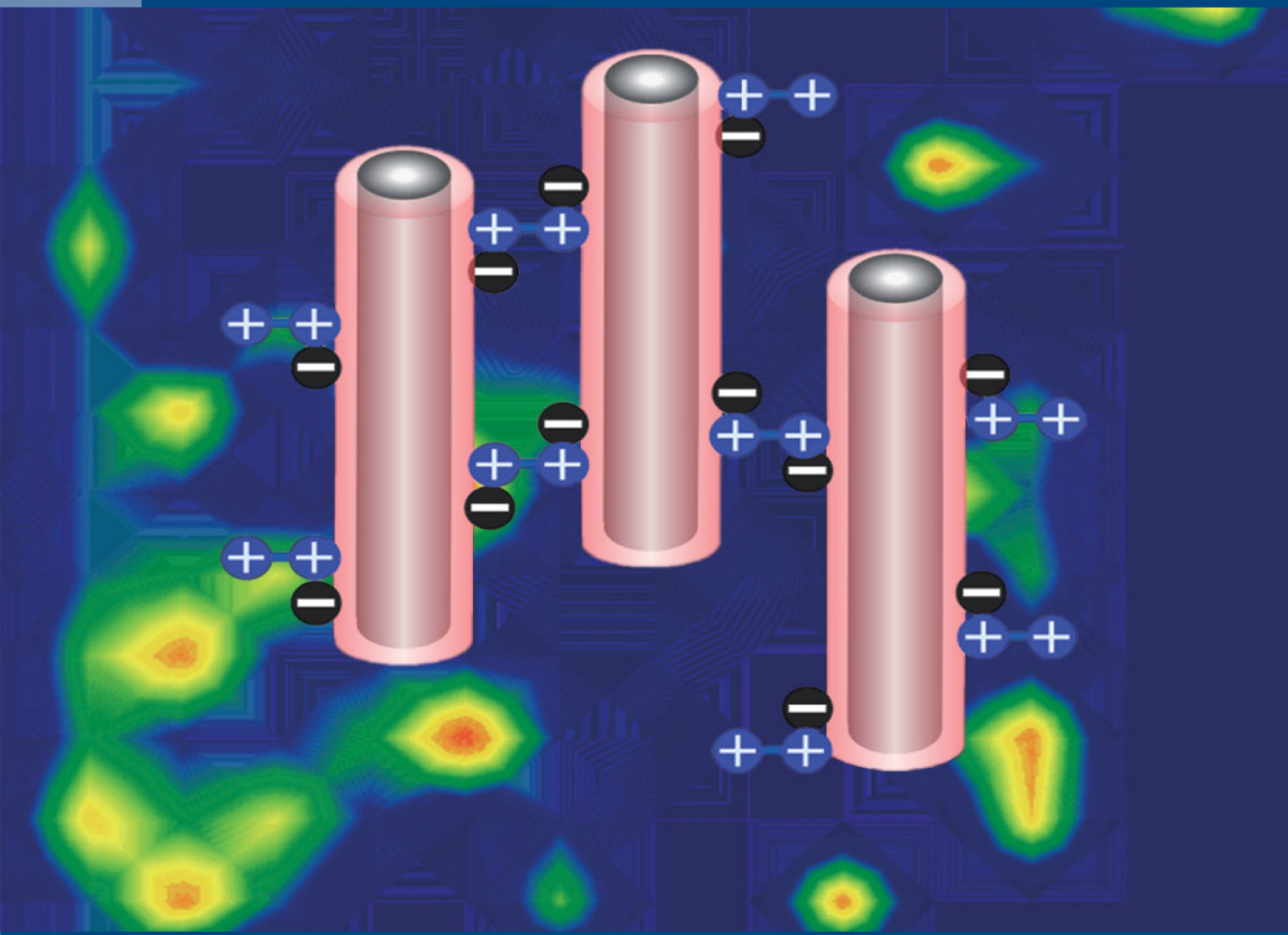

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Bingxiang Wang[1]
Weibo Chai[1]
Guosheng Ding[1,2]

[1]Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, P.R. China
[2]Analysis Center, Tianjin University, Tianjin, P.R. China

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Research Article

The application of functional silica nanoparticles to fulfill the rapid and improved enantioselective capillary electrophoresis separation of amino acid derivatives

In this study, diamino moiety functionalized silica nanoparticles with the size of 118 ± 12 nm were successfully synthesized and directly introduced into a chiral capillary electrophoresis system to improve the enantioseparation of 9-fluorenyl methoxycarbonyl derivatized amino acids using norvancomycin as chiral selector. Under acidic background electrolyte conditions, functional silica nanoparticles can be readily adsorbed onto the inner surface of bare silica capillary column through electrostatic interaction to form a dynamic coating, resulting in a reversed anodic electro-osmotic flow (i.e. from cathode to anode). As expected, chiral amino acid derivatives (usually negatively charged) can be rapidly separated under co-electro-osmotic flow conditions in the current separation system. Furthermore, the column performance and detection sensitivity for the enantioseparation were also obviously improved because the adsorption of chiral selector of norvancomycin to the capillary wall was greatly suppressed. Some important factors influencing the separation, such as the coating thickness, background electrolyte concentration, functional silica nanoparticles concentration, and the organic modifier were also investigated and the optimized separation conditions were obtained.

Keywords: Amino acid derivatives / Capillary electrophoresis / Enantioseparation / Norvancomycin / Silica nanoparticles
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1 Introduction

Amino acids play essential roles in nature. Therefore, the analysis of amino acids is one of the most challenging topics in multiple areas of science such as clinical and pharmaceutical studies, neuroclinical applications, agricultural and food sciences [1, 2]. The naturally found protein amino acids are generally composed of L enantiomers, while D enantiomers can be found in plants, bacterial cells, or in antibiotics. The amino acid enantiomers usually have identical chemical and physical properties (except for optical rotation) but possess different biological activities. Therefore, the separation of amino acid enantiomers (or their derivatives) is of great importance nowadays.

At present, numerous resolution methods, such as crystallization, extraction, membrane separation, enzymatic, and chromatographic methods have been developed for the enantioseparation of amino acid enantiomers [2]. Among many widely used chromatographic techniques, CE is very powerful due to its several well-known advantages, such as high separation efficiency, short analysis time, flexibility in separation conditions, and extremely small volume requirements for sample and separation media [3, 4]. Various chiral selectors have been extensively employed to separate amino acid enantiomers by CE [5, 6], and some review papers on the CE separation of enantiomers have been published [7–9].

Macrocyclic antibiotics have gained popularity as chiral selectors that have been widely used for the analysis of enantiomers [10–15]. Nevertheless, when macrocyclic antibiotics were used in traditional CE, the efficiencies were relatively poor because of the adsorption of macrocyclic antibiotics to the capillary wall. Furthermore, the strong

Correspondence: Dr. Guosheng Ding, School of Pharmaceutical Science and Technology, Tianjin University, 92 Weijin Road, Tianjin 300072, P. R. China
E-mail: dgs@tju.edu.cn
Fax: +86-22-27405694

Abbreviations: dASNP, diamino moiety functionalized silica nanoparticle; **DDW**, double-distilled water; **FMOC-Cl**, 9-fluorenylmethoxycarbonyl chloride; **NPs**, nanoparticles; **NPCE**, NP capillary electrophoresis; **NVC**, norvancomycin hydrochloride; **PDDMAC**, poly(diallyl dimethylammonium chloride); **SiNPs**, silica nanoparticles; **TEOS**, tetraethoxysilane

UV adsorption of macrocyclic antibiotics usually causes decreased detection sensitivity [16]. When racemates with negative charges are to be separated, the migration times are relatively long because analytes migrate against the EOF.

To overcome these disadvantages, some strategies have been proposed as possible solutions, such as modifying the capillaries with permanent or dynamic polycationic coating [17, 18], using pH above the zero mobility of macrocyclic antibiotics [19] and utilizing the partial filling technique [20, 21]. However, the preparation of traditional permanent coating layer is usually a time-consuming process. Although polymer coating is easy to prepare, its stability is always a concern, especially when CE separation is connected with ESI-MS detection. Using a higher pH is easy to operate; however, high pH has been proved to be harmful to the stability of macrocyclic antibiotics. The partial filling technique is very useful to improve the UV detection sensitivity; however, the optimization process is somewhat complex and time consuming to avoid the coelution of interaction phase (or chiral selectors) with analytes.

Nanoparticle capillary electrophoresis (NPCE) is the combination of nanoparticles (NPs) and CE technology in which NPs are used as stationary or pseudostationary phases to alter EOF, increase column capacity, and improve separation selectivity and column efficiency [22, 23]. In comparison with conventional CE and CEC techniques, such as packed CEC, NPCE is easy to optimize, does not require time-consuming particle packing or retaining frits, and has the advantage of an unused interaction phase for every new analysis. Furthermore, through orthogonal interface design, charged analytes are pulled out of the electrospray plume and accelerated toward the inlet to the mass spectrometer by electrical forces while the high-molecular-weight NPs do not diverge from the electrospray plume, so ESI-MS can be directly used for detection after the NPCE separation despite the continuous flow of NPs into the interface [24, 25].

In the past few years, several kinds of NPs have been successfully introduced into chiral CE [26–29]. For example, four pairs of dinitrophenyl-labeled amino acid enantiomers and three pairs of drug enantiomers were analyzed by Yang et al. [26] using cyclodextrin-modified gold NPs as the chiral selector. Chip-based enantioselective separation of tryptophan enantiomers was achieved in <50 s using β-cyclodextrin-conjugated graphene oxide magnetic nanocomposites as stationary phase by Liang et al. [27]. Recently, Yue et al. [28] synthesized and utilized surface molecularly imprinted silica nanoparticles (SiNPs) to fulfill the highly efficient enantioseparation of racemic tryptophan. However, time-consuming and tedious synthesis procedures are usually involved in these methods, which will definitely restrict their wide applications.

In recent years, functional SiNPs have received considerable attention, and had been successfully used as additives for the enhanced separation of different kind of compounds in CE. In comparison with other kinds of NPs such as organic polymer based and metallic NPs, functional SiNPs have many advantages, such as good UV transmittance, high stability, large surface area, and easy postmodification with different functional groups [22, 23].

In this work, diamino moiety functionalized silica NPs (dASNPs) were introduced as additives in a chiral CE system for the enantioseparation of amino acid derivatives. Under suitable BGE conditions, dASNPs were adsorbed onto the inner surface of the capillary to form a dynamic coating, resulting in higher column performance, shortened analyte migration time, and increased detection sensitivity. As far as we know, there is no paper to date reporting the use of functional SiNPs to enhance the enantioselective separation in CE.

2 Materials and methods

2.1 Reagents and apparatus

All chemicals were of analytical grade unless noted otherwise. Double-distilled water (DDW) purified by a Milli-Q system (Millipore, USA) was utilized throughout the experiment. Tetraethoxysilane (TEOS; 98%) and *N*-(β-aminoethyl)-γ-aminiopropyl triethoxysilane (99%) were purchased from Guotai Huarong New Chemical Materials (Zhangjiagang, China). Ammonia (28 wt%) and tris(hydroxyl methyl) aminomethane (Tris) were from Tianjin Yuanli Chemicals (Tianjin, China). The amino acids and 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) were obtained from J&K Chemical Technology (Beijing, China). Norvancomycin hydrochloride (NVC) was purchased from North China Pharmaceutical (Shijiazhuang, China). Boric acid, phosphoric acid, sodium hydroxide, potassium hydroxide, potassium dihydrogen phosphate, and hydrochloric acid were from Beijing Chemical Reagent Company (Beijing, China). Ethanol, methanol, acetonitrile, pentane, thiourea were purchased from Tianda Kewei (Tianjin, China). Poly(diallyl dimethylammonium chloride) (PDDMAC) was obtained from Aladdin Reagent (Shanghai, China).

The TEM images of dASNPs were obtained with a JEM100CXII transmission electron microscope (JEOL, Japan). The samples for TEM were prepared by placing a drop of colloidal solution on carbon-coated copper grid and dried at room temperature. The SEM images were obtained with a NOVA NanoSEM 430 scanning electron microscope (FEI, USA). The size distribution was obtained by using ImageJ software (V1.47).

All CE experiments were carried out on a self-assembled system equipped with an F203–1AC high-voltage power supply (Tianjin Hengbo High Voltage Power Supply Plant, Tianjin, China) and a UV 3000 detector (Chuangxin Tongheng Science and Technology, Beijing, China). Data were collected and analyzed using a CXTH-3000 chromatography data system (Chuangxin Tongheng Science and Technology, Beijing, China). Capillaries of 75 μm id and 375 μm od (Yongnian Optic Fiber. Hebei, China) were used throughout the experiment.

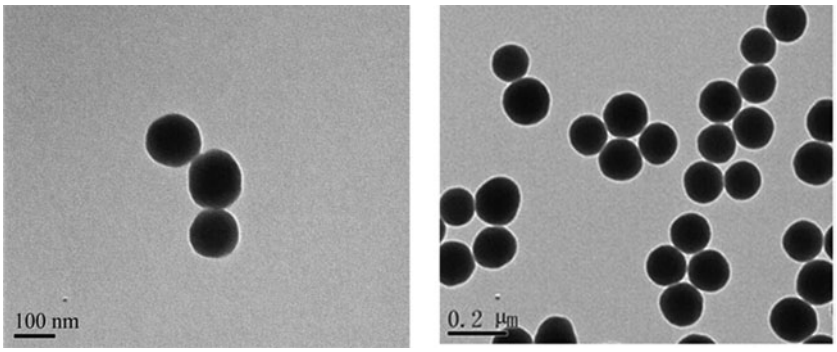

Figure 1. TEM images of dASNPs with different magnification.

2.2 Synthesis and characterization of dASNPs

dASNPs were prepared according to our previous work [23]. The preparation procedure is as follows: a total amount of 2 mL of TEOS was added to a conical flask in the presence of 25 mL of ethanol and 1.2 mL of ammonia under stirring. After 24 h at 30°C in thermostatic water bath, 100 μL of TEOS was added to the mixture, and the reaction lasted for another 30 min. Then 100 μL of *N*-(β-aminoethyl)-γ-aminiopropyl triethoxysilane was added dropwise. The obtained mixture was allowed to react for another 24 h, the particles thus formed were centrifuged and washed with ethanol and DDW repeatedly, and then vacuum-dried at 80°C for 6 h.

2.3 Derivatization procedures

The analytes used in this study were prepared from amino acids through a well-known procedure [30]. First, amino acid (200 μL, 2 mM) was added to FMOC-Cl (200 μL, 10 mM) in 0.2 M borate buffer (pH 9.0) and reacted for 2 min, and then extracted with pentane (0.5 mL) to remove excess of regent. The obtained water layer was diluted with ten times of water and the sample thus prepared was ready for use.

2.4 Experimental procedures

BGE was prepared by dissolving a certain amount of sodium dihydrogen phosphate in DDW, adjusted to the appropriate pH value with 0.1 mol/L KOH or 1.0 mol/L H_3PO_4, and then certain amounts of NVC were added. All solutions were filtered through a 0.45 μm nylon membrane and degassed by ultrasonication before use.

A high-concentration NP method was used to modify the capillary before chiral separation, i.e. the previously pretreated capillary was flushed with high-concentration dASNP suspension in phosphate BGE (5.0 mg/mL, pH 3.0) for 5 min using a manual syringe pump, then the pressure was released and the capillary was allowed to stand for 10 min. The procedure can be repeated for several cycles to obtain a stable coating with certain thickness.

For comparison, PDDMAC-coated capillary was prepared according to the literature [31]. The detailed procedure is as follows: Coating solutions were freshly prepared by dissolving PDDMAC at 0.2% w/v in 20 mM Tris aqueous solution adjusted to pH 8.3 with 0.01 M HCl, the capillary was then rinsed for 10 min with the polyelectrolyte-coated solution followed by a 2 min rinse with the BGE and stabilization under 10 kV voltage in the BGE.

A new capillary with the total length of 45 cm and effective length of 35 cm was made by scraping off 3∼5 mm of the polymer outside the capillary at an appropriate place. Before use, the capillary was flushed with methanol, DDW, 1 mol/L NaOH, DDW, 1 mol/L HCl, DDW for 20 min each. Separation voltage was set at 10 or −10 kV. The analytes were injected by hydrostatic pressure injection at altitude intercept of 15 cm for 5 s; between runs, the capillary was rinsed with BGE using medical syringe for several minutes. Detection wavelength was set at 254 nm unless stated otherwise.

Selectivity (a) and resolution (Rs) were calculated using equations $a = t_2/t_1$ and $\mathrm{Rs} = 1.18\,(t_2 - t_1)/(w_2 + w_1)$, where t_1 and t_2 are the migration times of the two enantiomers in the order of time, and w_2 and w_1 are peak widths at half height of enantiomer peaks.

3 Results and discussion

3.1 Characterization of dASNPs

TEM images of dASNPs are shown in Fig. 1. It is clear from the images that dASNPs are sphere-like and uniform in shape with the size of 118 ± 12 nm. The C, H, and N contents of dASNPs, determined by elemental analysis, were 4.68, 1.86, and 1.23%, respectively, indicating that diamino moiety has been successfully incorporated into dASNPs during the sol–gel process. According to the N content, it could be deduced that the diamino moiety incorporated into dASNPs was about 0.44 mmol/g.

3.2 Separation of FMOC amino acids on bare capillary

The enantioseparations of representative FMOC amino acids on bare capillary column are shown in Supporting Information Fig. S1. It is clearly shown that the adsorption of NVC

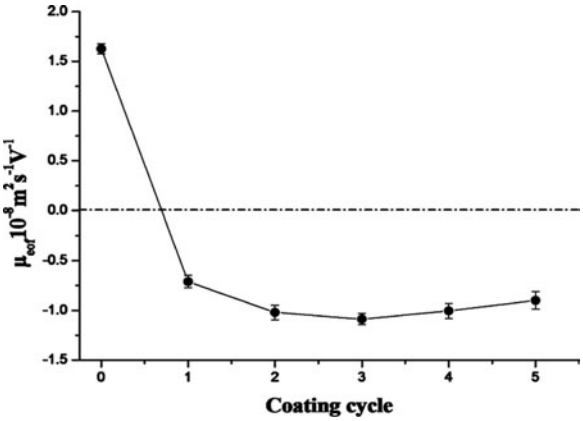

Figure 2. The effect of coating cycles on EOF. Experimental conditions: 30 mM phosphate BGE (pH 6.0) with 0.3 mg/mL dASNPs and 1.25 mM NVC, detection wavelength, 254 nm; separation voltage, −10 kV. Other conditions are stated in Section 2.4.

onto the bare capillary wall resulted in poor separation efficiency and strong UV detection interference. Furthermore, longer analysis times (>20 min) were necessary due to the migration of analytes in the opposite direction to the EOF.

3.3 Effect of the thickness of coating layer on EOF

In CE, the control of EOF is usually an important factor for achieving optimal separation because analytes move through the capillary by EOF drive, as well as self-electrophoretic mobility if they are charged [32, 33]. Figure 2 shows the plots of EOF versus the coating cycles. After being treated with dASNPs, the cathodic EOF reversed to anodic under same BGE conditions.

To prepare coating capillary column with good column repeatability, control of the coating thickness is usually necessary [34]. In current experiment, the coating thickness can be controlled by the cycles of repetitive coating procedure. When increasing the cycles of repetitive coating, the thickness would increase accordingly. In Fig. 2, the influence of the thickness of dASNPs coating on EOF was investigated. When the capillary was repetitively treated for 1∼3 cycles, the anodic EOF increased accordingly. However, the EOF would not increase when the cycles of repetitive coatings was further increased above 3. Figure 3 shows the SEM images of the inner wall of the capillary column before (A and B) and after being treated with dASNPs (C and D).

3.4 Effect of concentration of dASNPs on enantioseparation

The dASNPs coating thus prepared can be considered as a dynamic coating, just as other kinds of NPs coating [35]. To maintain the stability of the coating layer, it is necessary to add suitable amount of dASNPs to BGE. The effects of the concentration of dASNPs in phosphate BGE on enantioseparation are shown in Supporting Information Fig. S2.

It is clearly shown in Supporting Information Fig. S2 that Rs increased with the increase in the concentration of dASNPs from 0.1 to 0.3 mg/mL. Further increasing the content of dASNPs would induce decreased resolution and degraded detection sensitivity, mainly because of the aggregation and sedimentation of dASNPs in separation system. The same trend was also found when we investigated the effect of the

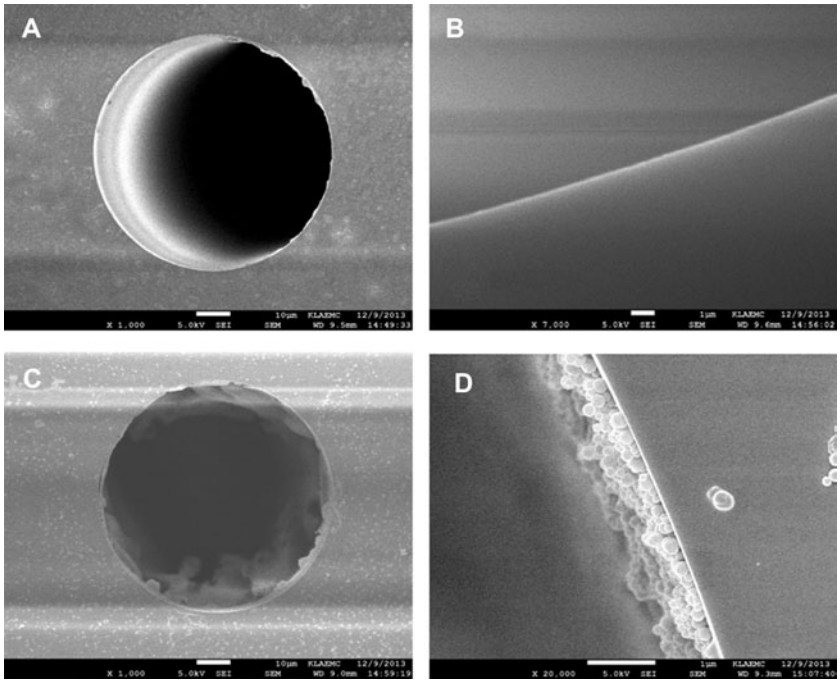

Figure 3. SEM images of inner wall of the capillary before (A and B) and after high-concentration NPs (HCNPs) treatment with different magnifications.

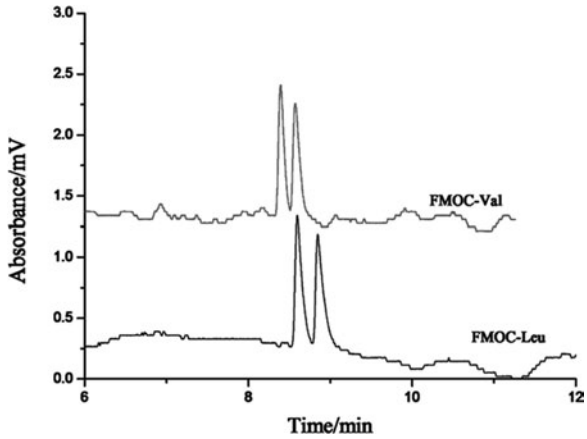

Figure 4. The enantioseparation of FMOC amino acids on dASNPs-coated capillary column. Experimental conditions: 10 mM phosphate BGE (pH 5.5) with 0.3 mg/mL dASNPs and 1.25 mM NVC; other conditions are stated in Section 2.4.

concentration of dASNPs upon *a*. Based on these experimental data, 0.3 mg/mL of dASNPs was selected for subsequent experiments.

3.5 Effect of pH on enantioseparation

The pH of the BGE is a key factor in chiral separation [36] for it may affect the EOF, charges of analytes, NVC, and dASNPs in the current separation system. The effect of pH on enantioseparation was investigated in the pH range of 4.5∼6.5, which was typically utilized for the enantioseparation of acidic racemates using NVC as chiral selector [15].

The results shown in Supporting Information Fig. S3 reveal that the migration time prolonged with the increase of the pH value. This is quite different from the tendency in BGE without dASNPs added. This trend may result from the EOF dependence on the protonation of the dASNPs absorbed on the inner wall of the capillary. Besides, at low pH (pH 4.5), Rs of analytes decreased greatly; as pH increased from 5.0 to 6.5, enantiomer resolution did not change markedly. This phenomenon may ascribe to the fact that the ionization of analytes is restrained at lower pH, leading to a weaker interaction between enantiomers and chiral selectors. However, higher pH results in longer analysis times and decreased column performance. As a compromise, pH 5.5 was selected as the suitable pH for subsequent experiments.

3.6 Effect of concentration of BGE on enantioseparation

The concentration of phosphate BGE can influence the EOF, separation selectivity and peak shape. The effect of BGE concentration in the presence of 0.3 mg/mL dASNPs was investigated, and the results are shown in Supporting Information Fig. S4. It is experimentally shown that with the increase of the BGE concentration, the migration times for analyte were gradually prolonged; this is mainly due to the decrease of EOF. The experiments also show that the ionic strength of the BGE had little impact on the resolution of the analytes. However, with the increase of BGE concentration, the baseline stability was affected and the separation repeatability was worsened, mostly because of the occurrence of more Joule heating during separation process. Thus, 10 mM phosphate BGE was chosen to secure rapid and stable separation.

3.7 Effect of organic additive on enantioseparation

The presence of organic additive will change the viscosity and permittivity of the BGE, as well as the EOF of the separation system. Moreover, the enantiorecognition process involved in CE can be strongly influenced by the presence of organic modifier [37]. Therefore, the effect of two classical organic additives, namely methanol and acetonitrile, were tested to investigate the influence of organic modifier on enantioseparation.

The experimental results are shown in Fig. S5. As can be observed, while acetonitrile dramatically reduced the enantioresolution, methanol only had little impact on the enantioresolution. With the increase of the fraction of acetonitrile in BGE, the resolution decreased rapidly and was completely lost at 30% of the organic modifier. To reduce the analysis time, no organic modifier was added in the BGE.

Table 1. Related values of the investigated racemates under different chiral separation systems

	Bare capillary				dASNPs-coated capillary				PDDMAC-coated capillary			
FMOC amino acids	t_1/t_2 (min)	Rs	*a*	N_{eff}[a)] (plates/m)	t_1/t_2 (min)	Rs	*a*	N_{eff} (plates/m)	t_1/t_2 (min)	Rs	*a*	N_{eff} (plates/m)
Leu	20.78/21.98	2.24	1.06	71 945	8.62/8.90	1.69	1.03	144 689	6.59/6.78	1.03	1.12	88 490
Phe	20.71/23.39	4.30	1.12	69 429	7.22/7.65	2.20	1.06	102 203	5.85/6.10	1.04	1.36	37 256
Ala	20.56/24.67	7.75	1.25	74 843	6.63/7.08	4.22	1.07	224 365	5.52/5.82	3.05	1.05	175 741
Ser	22.37/23.19	1.48	1.04	78 691	8.46/8.67	1.41	1.02	177 100	5.24/5.40	0.52	1.01	153 708
Val	21.25/22.23	2.10	1.04	99 476	8.47/8.73	1.49	1.03	133 229	5.74/5.82	0.47	1.01	91 432

a) $N_{eff} = 5.54(t_1/w_1)^2$.

3.8 Separation of FMOC amino acids under optimized conditions

In the presence of dASNPs coating, all the FMOC amino acids tested were baseline resolved within 10 min, and at the same time, detection sensitivity and column efficiency were all increased, although the resolution were partially decreased (as shown in Fig. 4 and Table 1). Repeatability of the enantiomeric separation was studied by five sequential injections of FMOC-Leu as model a compound (Fig. S6). The RSDs for retention time (the first enantiomer, L isomer), *a* and Rs were 0.58, 0.03, and 3.5%, respectively. These data suggested the separation system was stable, and the method established here can be used for actual applications.

Under acidic BGE conditions, the inner wall of the coated capillary would be positively charged as a result of the protonation of the amine functional groups on dASNPs [38]. Accordingly, the EOF of the separation system would be reversed (i.e. from cathode to anode). In the current separation system, the EOF was determined to be -1.22×10^{-8} m^2 s^{-1} V^{-1}, i.e. from cathode to anode. Due to electrostatic repulsion, the adsorption of NVC on capillary wall was suppressed; the detection sensitivity was increased two times more than before. Since the negatively charged analytes migrated in the same direction with the EOF (co-EOF), shorter analysis time was needed.

3.9 The comparison with PDDMAC coating layer

Cationic charged polyelectrolyte PDDMAC had been frequently used to prepare dynamic coating for the separation of different compounds [39–41]. To show the advantages of current coating capillary column, enantioseparation of FMOC amino acids on PDDMAC-coated capillary was also tested and the results are shown in Fig. Supporting Information S7 and Table 1. Although migration times were shorter on the PDDMAC-coated capillary, most analytes were not baseline resolved probably due to the stronger interaction between PDDMAC and acidic analytes, which may be unfavorable for enantiorecognition.

4 Concluding remarks

This paper demonstrated that functional SiNPs can be favorably utilized as a coating layer material for chiral separation in CE. In the presence of dASNPs coating, significant improvements in column performance and detection sensitivity for the chiral separation of FMOC amino acids were obtained in comparison with NVC-alone cases with other conditions unchanged.

The method established here can also be extended to the enantioseparation of other compounds when using different functional SiNPs and chiral selectors. Research in relation to this is underway in our lab.

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The authors have declared no conflict of interest.

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