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In-capillary formation of polymer/surfactant complexes–assisted reversed-migration micellar electrokinetic chromatography for facile analysis of neutral steroids

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ABSTRACT

In this study we developed a novel approach, using in-capillary formation of polymer/surfactant complexes (IPSC)–assisted reversed-migration MEKC (RM-MEKC), for the analysis of neutral steroids. This process involved two sequential events: in-capillary polymer/surfactant complexes formation during sample preconcentration, followed by IPSC separation. The procedure began with a polymer-filled capillary. Initially, on-line preconcentration of the sample was performed at the sample plug. Meanwhile, free surfactants migrated to interact with polymers, forming polymer–surfactant complexes. Analytes were then kinetically partitioned between the mixed phases (micelles and polymer–SDS complexes). Sodium dodecyl sulfate (SDS) and poly(*N*-isopropylacrylamide) (PNIPAAm) were employed as pseudo-stationary phases (PSPs). This system allowed the successful separation of five steroids (testosterone, hydrocortisone 21-acetate, dexamethasone, prednisolone, hydrocortisone) in acetate buffer and the determination of urinary free hydrocortisone; it also exhibited excellent performance for sample on-line concentration. The limit of detection for hydrocortisone was 20.98 ng/mL (R^2=0.9995). The polymer size, concentrations, end-group charges, and SDS concentrations were evaluated. This IPSC/RM-MEKC system, which can be adopted in commercial CE instruments, is easy to operate, suitable for combination with several sample preconcentration options, sensitive, robust, and environmentally sustainable. We suspect that such systems might have potential applications in clinical analyses and in microanalytical devices.

1. Introduction

Neutral steroids are vital components of various physiological processes, such as cortisol, a glucocorticoid, responding to stress, gluconeogenesis and several metabolic regulations [1]. The determination of corticosteroids is, therefore, clinically important, especially in the diagnosis of endocrine and metabolic disorders [2]. Measurements of urinary free cortisol levels (typically 5–55 μg/24 h) [3] provide information allowing the diagnosis of Cushing's syndrome (hypercortisolism, hyperadrenocorticism) and Addison's disease (hypocortisolism, hypoadrenalism), which are life-threatening endocrine disorders [4]. Dedicated analytical approaches are required to detect low levels of these steroids, encouraging the development of novel means for simple, efficient, and sensitive analyses.

Regular methods for determination of free cortisol include protein binding assays [5], radioimmunoassays (RIAs) [6], and assays based on high-performance liquid chromatography (HPLC) and gas chromatography (GC) [7,8]. RIAs are used widely for measurements of serum cortisol, but are unsuitable for urinary specimens because unknown interfering urinary substances can lead to two- to three-fold overestimation of the levels relative to those determined using HPLC [9]. The specificity of chromatographic (e.g., liquid chromatography (LC) or GC) approaches toward urinary neutral steroid analysis can be improved through sample preparation involving either liquid–liquid or solid phase extraction [10–12]. Alternative analytical techniques for steroid determination have also been reported, including capillary electrophoresis (CE) coupling with micellar electrokinetic chromatography (MEKC) [13] and related approaches [14–17], electrospray ionization mass spectrometry (ESI-MS) [18], and polymeric-mixed micelles [19,20].

The selectivity of MEKC—namely the balance in the polar and hydrogen bonding interactions between the analyzed components and the pseudo-stationary phases (PSPs) and the bulk solvent—determines the quality of the separation event. Judicious selection of the components of the PSPs and the additives (modifiers) incorporated in the aqueous phase is critical for achieving optimal resolution.

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The chemical selectivity of the PSPs can be modified through the addition of organic modifiers (e.g., co-solvents: usually 20–30%, cyclodextrins, or urea) [21]; additionally, the application of mixed micelles (in so-called mixed phases MEKC), using anionic and nonionic surfactants, can significantly improve the resolution of the separation of corticosteroids [20,22]. Moreover, the adoption of copolymer [23] and polymeric surfactants [24] have also been explored, although the preparation of polymeric micelles requires extra energy.

In addition to the selectivity, the detection sensitivity also plays a vital role in the performance of CE. On-line sample preconcentration techniques can improve the detection sensitivity of CE, which generally features a limited sample injection volume. Increasing the sample size is one way to compensate for low detection sensitivity. Reversed-migration micellar electrokinetic chromatography (RM-MEKC) is an approach that involves on-line preconcentration and efficient removal of the sample matrix that is inherent in large-volume sample stacking [25]. The basic principle of RM-MEKC is that the migration of anionic micelles (anode at the detector end) is faster than that of the electroosmotic flow (EOF); the velocity of the EOF is usually decreased by using low-pH buffers. The micelles present in the background electrolyte (BGE), but not in sample zone (S), perform several separation modes, including sweeping operation [26]. Sweeping using low conductivity sample matrices works well for analytes with low to moderate retention factors such as steroids [27–30].

In this study, we developed a novel strategy for separation improvement—the in-capillary formation of polymer/surfactant complexes (IPSC)—that is comparable with the addition of modifiers in the running buffer; it allows fine-tuning of the microenvironment to manipulate the migration and retention of analytes. This novel, facile, mixed-phase system (IPSC) was combined with RM-MEKC for the on-line pre-concentration and separation of neutral steroids with distinct polarity. The IPSC procedure in MEKC involves two steps, starting with a polymer-filled capillary. Initially, on-line preconcentration of the sample is performed at S; free surfactants migrate to interact with the polymers to form IPSC. Next, sample partitioning occurs once the polymer–surfactant complexes confront the analyte-bearing surfactant micelles. An IPSC-gradient is constructed along the capillary. The polymer segments of the IPSC may partially penetrate the micelles [31]. Through interactions with the IPSC, the analytes will have many opportunities to undergo partitioning to attain optimal resolution. The sequential IPSC formation and sample partitioning provides a novel approach for separation.

The PSPs used herein were (poly(*N*-isopropylacrylamide), PNIPAAm) and sodium dodecyl sulfate (SDS), due to their interaction at the isopropyl groups and the alkyl group, [32]. The amide bonds and isopropyl side groups of PNIPAAm could function as hydrophilic and hydrophobic matrices, respectively, to assist the partitioning of the neutral steroids against surfactants. SDS functioned in two phases: initially it acted as a single PSP to perform sweeping and then it was involved in the mixed-phase separation. We tested the ability of this system to separate five neutral steroids: hydrocortisone (H), prednisolone (P), dexamethasone (D), testosterone (T), and hydrocortisone 21-acetate (H_{21}) dissolved in acetate buffer (Fig. S1). Factors that influenced the performance of this approach were investigated: (i) the properties of the polymer, such as its end group modification (charge/polarity) and molecular weight; (ii) the nature of the polymer–surfactant interaction; and (iii) on-line sample preconcentration. This design demonstrated excellent ability to perform sample preconcentration and separation. We applied this system successfully with a commercial CE instrument for the routine analysis of urinary cortisol. We suspect that this technique will also have potential applications in microfluidic systems.

2. Materials and methods

2.1. Apparatus and reagents

PNIPAAm-assisted MEKC experiments were performed using an Agilent HPCE system (Waldbronn, Germany) with an inline UV detector (wavelength set at 254 nm). Data were collected using 3D-CE ChemStation software. Polyimide-coated untreated fused-silica capillary tubing (total length, 58.5 cm; effective length to the detector, 40 cm; internal diameter, 50 μm; external diameter, 357 μm) was purchased from Alltech Associates (Illinois, USA). All experiments were conditioned at 25 °C except where specifically indicated. Samples were injected under 50 mbar pressure for 5 s; the electrophoretic system was operated under negative polarity and a constant voltage (−25 kV). The separation was performed in the MEKC system by using an inlet buffer of 8 mM SDS and 20 mM acetate (pH 5.0) and a separation buffer of 0.3% (w/v) PNIPAAm and 20 mM acetate (pH 5.0).

A new capillary was pretreated with 1 N KOH for 60 min, followed by rinsing with water for another 60 min. Between runs, the capillary was conditioned with 0.5 N KOH for 5 min, with water for 3 min, and with separation buffer for 4 min. At the end of the day, the capillary was flushed with 0.5 N KOH for 3 min and with water for 5 min. In all cases, a pressure of 50 mbar was applied.

N-Isopropylacrylamide (NIPAAm), 2-phenylethanethiol (2-PET), 3-mercaptopropionic acid (MPA), 2-aminoethanethiol (AET), and tetrahydrofuran (THF) were purchased from Acros (Gleen, Belgium). 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from SHOWA Chemicals (Tokyo, Japan). Mercaptoethanol (ME), P, D, H, H_{21}, and citric acid monohydrate were purchased from Sigma (St. Louis, MO, USA). T and SDS were purchased from Fluka (Buchs, Switzerland). Sodium citrate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Methanol (MeOH) was purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Riedel–de Haën. All reagents were of analytical grade and all working solutions were prepared in ultra-pure water, obtained from a Milli-Q Millipore system (Billerica, MA, USA).

2.2. Preparation of running buffer and standard solution

Stock solutions of the five steroids D, H_{21}, T, P, and H were prepared at 0.5 mM by dissolving in MeOH. Standard solutions were obtained through appropriate dilution with sample buffer (60 mM acetate buffer (pH 5.0) containing 10% MeOH). Acetate buffer, containing sodium acetate and acetic acid (1 M) in Milli-Q water, was adjusted to pH 5.0. The separation buffer containing PNIPAAm (3 mg/mL; 0.3%, w/v) was prepared by dissolving the required amount of polymer in 20 mM acetate buffer (10 mL). The inlet buffer containing SDS (230.7 mg/mL) was prepared by dissolving the required amount of the surfactant in 20 mM acetate buffer (10 mL). All solutions were passed through a 0.22 μm filter (Millipore, Bedford, MA, USA) prior to use. Standards were stored at 4 °C until required for use.

2.3. Synthesis of PNIPAAm and the measurement of polymer–surfactant complexes: specific viscosity and particle sizes

PNIPAAm was prepared through radical polymerization of NIPAAm in THF. A mixture of NIPAAm (5.0 g, 44.2 mmol), MPA (424 mg, 4.0 mmol), and AIBN (6564 mg, 4.0 mmol) in THF (50 mL) was degassed through several freeze/thaw cycles. The reaction bottle was immersed in liquid N_2 and sealed under reduced pressure for 5 min. The bottle was then removed from the liquid N_2 to melt the reaction mixture. Meanwhile, the accumulated gases were released. After two or three of freeze/thaw cycles, no further bubbling was observed during the melting

process. The contents of the reaction bottle were maintained under negative pressure and then the bottle was heated at 70 °C for 2 h. After evaporation of the solvent, the reaction mixture was poured into ice-cold (0 °C) Et_2O to precipitate the polymer. The polymer was further purified through two or three THF/Et_2O cycles to remove the monomer, radical initiator, and chain transfer agent. Finally, a white crystalline product was obtained after drying at 40 °C. The molecular weight (M_w) of the obtained PNIPAAm was determined through gel permeation chromatography (GPC) in THF at 25 °C, using a Waters 515 HPLC pump (Milford, MA, USA) equipped with a Waters Styragel HR3 and Styragel HR4 column. The molecular weight calibration curve was established using polystyrene standards having molecular weights of 1.2, 3.25, 10.2, 28, 68, 195, 490, and 1080 kDa. The values of M_w of the four PNIPAAm samples were determined to be 113, 968, 1957, 2441, 3385, and 7170 g/mol (Da).

The specific viscosity of 0.3% (w/v) PNIPAAm solutions in water containing SDS at various concentrations were measured using glass capillary viscometers (Cannon-Fenske Routine Viscometers; size: 50) at 25 °C. The particle sizes of 0.3% (w/v) PNIPAAm solutions in acetate buffer (20 mM, pH 5.0) containing SDS at various concentrations (0, 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 0.75, 1, 1.25, 2.5, 5, 10, 15, 18, 20 mM) were measured by Dynamic light scattering (Malvern ZS 90, USA). All solutions were passed through a 0.22 μm filter before use.

2.4. Real sample extraction

Human urine samples were collected for 24 h and stored without preservatives at −20 °C until use. The steroids were extracted with 6 mL (500 mg) Bakerbond C18 cartridges (J.T. Baker, Phillipsburg, NJ, USA), which had been activated by treating sequentially with MeOH (3 mL) and deionized water (3 mL). After application of urine samples (2 mL), the cartridges were washed sequentially with deionized water (3 mL) and hexane (2×6 mL^2). After the cartridge had been vacuumed for 4 min to remove the organic solvent, the steroids were eluted with CH_2Cl_2/EtOAc (4:1, 6 mL) as the eluting solvent. The eluates were collected and dried under N_2. Samples were dissolved in sample buffer to make 10-fold concentration. All solutions were passed through a 0.22 μm filter prior to use. 6α-methylprednisolone (50 μM) was added as the internal standard.

3. Results and discussion

3.1. IPSC-assisted RM-MEKC system

Fig. 1 outlines the design of our IPSC-assisted RM-MEKC system for the separation of neutral steroids. The capillary was first filled with negatively charged semi-telechelic PNIPAAm (M_w 1957 Da; 0.3%) in 20 mM acetate buffer (pH 5.0) (Fig. 1A). SDS was used as the analyte carrier. The separation process involved two sequential steps. In the first step, the system performed on-line sample preconcentration through sweeping and established the formation of IPSC (polymer–SDS complex). Analytes (five steroid standards: 1, T; 2, H_{21}; 3, D; 4, P; 5, H; 50 μM in 60 mM acetate buffer (pH 5.0) containing 10% MeOH) were introduced at 50 mbar (Fig. 1B). After applying a voltage at −25 kV, SDS (8 mM in 20 mM acetate buffer, pH 5.0) was introduced to sweep the analytes. Meanwhile, free SDS migrated forward to interact with the polymers to form IPSCs (Fig. 1C). The cathode and anode were assigned at the inlet and outlet ends, respectively. The EOF was slowed down by lowering the pH of the BGE (to pH 5.0) to facilitate the migration of SDS micelles toward the detection end. In addition, sample loading was increased with the raise of BGE concentration in S (Fig. S2).

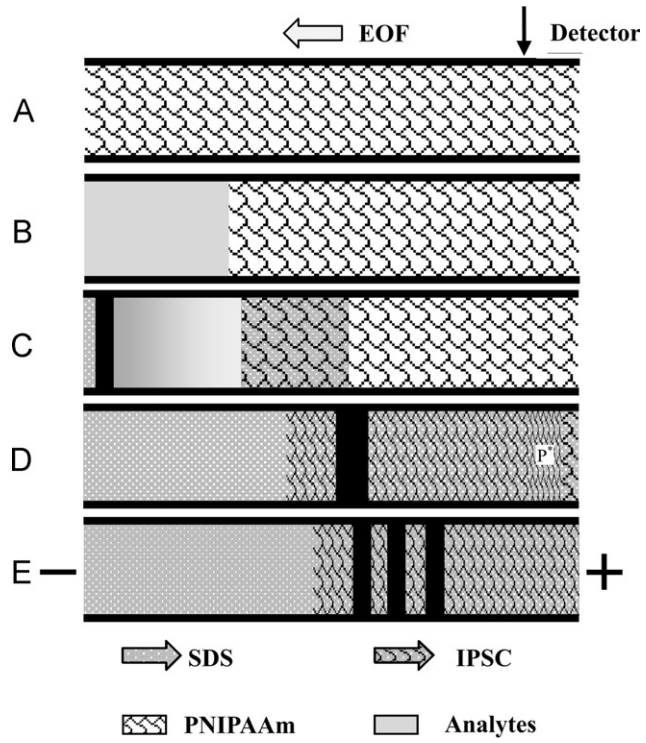

Fig. 1. Schematic representation of the operation of the autoassembling polymer/surfactant complexes assisted RM-MEKC system assisted: (A) capillary filled with 0.3% (w/v) PNIPAAm (M_w 1957 Da) in 20 mM acetate buffer (pH 5.0), (B) analytes (50 μM) dissolved in sample buffer (60 mM acetate buffer, pH 5.0, 10% MeOH) were introduced at 50 mbar (5 s), (C). the starting of IPSC/RM-MEKC system. Sample on-line preconcentration was firstly executed, followed by the formation of IPSC after the application of voltage at −25 kV, and (D and E) analytes interacted with IPSCs to be separated. Steroid standards (50 μM in sample buffer): 1, testosterone (T); 2, hydrocortisone 21-acetate (H_{21}); 3, dexamethasone (D); 4, prednisolone (P); 5, hydrocortisone (H). P*: the system peak. Experimental conditions: inlet buffer (20 mM acetate buffer, pH 5.0, 8 mM SDS), outlet buffer (20 mM acetate buffer, pH 5.0, 0.3% w/v PNIPAAm). Sample zone (60 mM acetate buffer, pH 5.0). Capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection 50 mBar, 5 s.

In the second step, the system aimed to execute efficient sample separation through kinetic analyte partitioning between the micelles and IPSCs. The IPSCs-gradient was provided along the capillary. The interactions among the analytes, surfactants, and IPSCs to perform separation could be monitored through an overlapped spectroscopic absorption profile measured at 254 and 210 nm for the analytes and PNIPAAm, respectively (Fig. 2). There was no separation occurred without the presence of polymer. The polymer–surfactant interaction was revealed by the increase in viscosity and the changes in particle sizes that occurred upon the addition of SDS (Figs. S3 and S4). The PNIPAAm strands in the capillary migrated to the detector upon interacting with the SDS units (Fig. 2). The peak labeled P* that appeared prior to the peaks of the samples was the system peak. (Fig. 1D). Polymer units that accumulated sufficiently large amounts of negatively charged surfactants were pushed back to the detection end. Desirable resolution was achieved as a result of the modified selectivity contributed by the SDS micelles and IPSCs. The preconcentrated analytes carried by the SDS micelles were partitioned between the micelles and IPSCs (Fig. 1E).

3.2. Effect of polymer properties on system performance

3.2.1. Effect of polymer end groups

To explore the effect of the polymer's net charge on the performance of this system, we examined polymers with various

distinctly charged end groups, prepared using appropriately charged chain transfer reagents bearing positive ($-NH_3^+$), neutral ($-C_6H_5$, –OH), and negative (–COOH) charges (Fig. 3). We obtained low or no resolution with polymer possessing positively and neutrally charged end groups. At pH 5.0, the positively charged polymer (PNIPAAm–NH_3^+) would have interacted electrostatically with the negatively charged capillary wall, resulting in a significant baseline disturbance and unsatisfactory separation. Poor resolution and disturbed baselines were also evident when using the phenyl-terminated neutrally charged polymer (PNIPAAm–C_6H_5), due to hydrophobic complexation of this modified polymer with the capillary wall. In contrast, the neutral polymer presenting terminal hydroxyl groups (PNIPAAm–OH) provided a certain degree of separation, but without sufficient resolution. The greatest resolution among our tested systems was that observed when applying the polymer presenting carboxyl end groups (PNIPAAm–COOH). Therefore, PNIPAAm–COOH was used for further study.

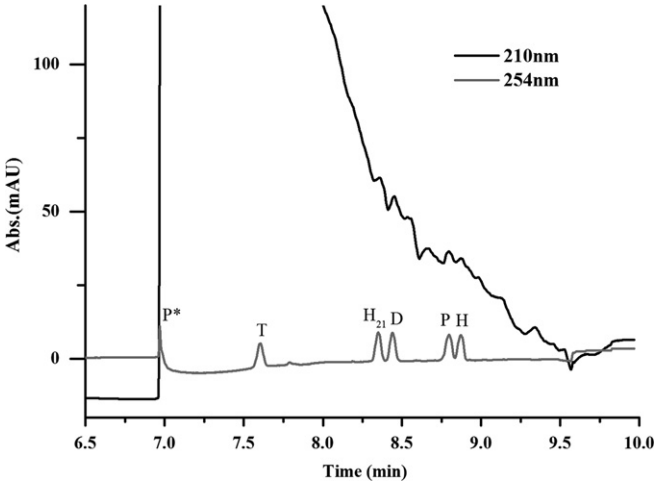

Fig. 2. Overlaid electrogram of PNIPAAm and neutral steroids. The wavelengthes for PNIPAAm and analytes were of 210 and 254 nm, respectively. Inlet buffer: 20 mM acetate buffer, pH 5.0, 8 mM SDS; separation buffer: 20 mM acetate buffer, pH 5.0, 0.3% PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection: 50 mbar, 5 s.

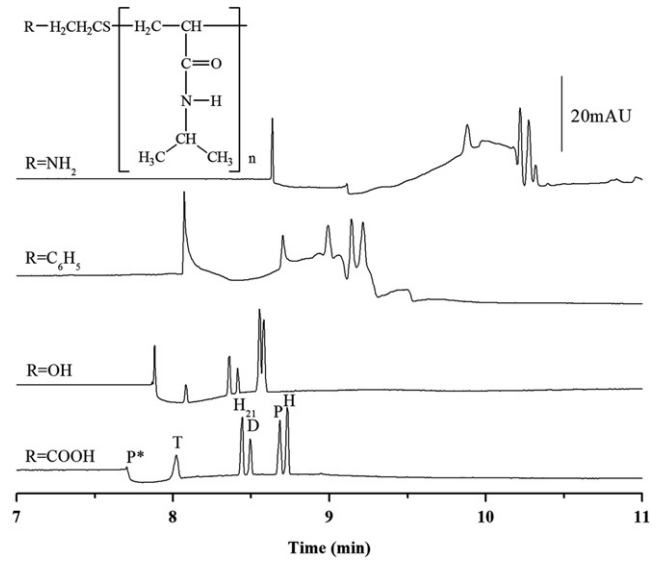

Fig. 3. Effect of PNIPAAm end group charges on steroid separation. The chain transfer reagents provided PNIPAAM derivatives with (i) positive ($-NH_3^+$); (ii) neutral ($-C_6H_5$ and –OH); and (iii) negative (–COOH) terminal charges. Inlet buffer: 20 mM acetate buffer, pH 5.0, 8 mM SDS; separation buffer: 20 mM acetate buffer, pH 5.0, 0.3% PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection: 50 mbar, 5 s.

3.2.2. Effect of temperature

PNIPAAm is a thermoresponsive polymer having a lower critical solution temperature (LCST) near 32 °C. At temperatures above its LCST, the polymer strands aggregate to form a cluster having a hydrophobic surface, resulting in precipitation from the solution. Its LCST does, however, vary upon the addition of surfactants [32]. Thus, we wondered whether reveal temperature would affect the resolution of our separation system. Table 1 reveals that the number of theoretical plates and the resolution of the analytes both increased upon elevating the temperature. The migration of SDS accelerated upon increasing the temperature, possibly attributable in part to the decreased viscosity, as evidenced by the increased retention time of P* upon increasing the temperature from 20 to 30 °C. At 35 °C (Fig. S5A); however, a significantly large interference peak appeared next to P*, presumably originating from polymer aggregation. Based on these results, we employed ambient conditions in our subsequent experiments to minimize interference.

3.2.3. Effects of polymer size, polymer concentration, and SDS concentration on polymer–surfactant interactions

Our developed system exploited polymer–surfactant interactions, forming IPSCs, to implement sample separation. We expected, therefore, that the polymer size, polymer concentration, and SDS concentration would all be critical parameters affecting the system's performance. According to the classical polymer–surfactant binding equation Eq. (1) describing the competition between free micelle formation and micelle–polymer interactions, the total surfactant concentration (X_t) can be expressed as

$$X_t = X_1 + X_f + X_b = X_1 + g_f(K_f X_1)^{g_f} + nX_P g_b\left[\frac{(K_b X_1)^{g_b}}{1+(K_b X_1)^{g_b}}\right] \quad (1)$$

where K_f and K_b are the intrinsic equilibrium constants for micelle formation and for the binding of surfactants onto polymers, respectively, and are associated with the aggregation equilibrium. The second term, g_f, represents the average aggregation number of the free micelles. The third term, g_b, represents the average size of micelles. The relative values of K_b, K_f, g_b, and g_f govern the complexation behavior of the polymer and surfactant.

We partitioned the total surfactant concentration into three terms representing the dispersed surfactant (X_1), the surfactant in free micelles (X_f), and the surfactant in polymer-bound micelles (X_b) [33]. In the third term, X_p represents the total polymer concentration in solution. We postulated that each polymer molecule featured n binding sites for micelles of average size g_b. The magnitude of n would be roughly proportional to the size or molecular weight of the polymer, but it would also be influenced by the conformational changes of the polymer resulting from inter-micelle interactions [34]. The values of n, X_p, and nX_p would all affect the binding behavior of the surfactant to the polymer. Accordingly, different degrees of polymer–surfactant binding might have influenced the resolution of the separation.

To examine the effect of the same mass concentration of the polymer, but with different molecular weights, we tested polymers having molecular weights of 113, 968, 1957, 2441, 3385, and 7170 g/mol at a concentration of 0.3% (w/v). Fig. S5B reveals that no separation occurred when using the NIPAAm monomer (113 g/mol) as the separation matrix. Extended retention times, but insufficient resolution, were observed when the polymer's molecular weight was greater than 2441 g/mol. For large polymers,

Table 1
Theoretical plate number and resolution of five steroid standards in different conditions.

		Theoretical plate number					Resolution	
		Testosterone	Hydrocortisone 21-acetate	Dexamethasone	Prednisolone	Hydrocortisone	*R*1	*R*2
BGE (mM)	15	560,000	45,000	190,000	320,000	360,000	–	–
	20	140,000	1,100,000	2,000,000	1,100,000	1,100,000	3.0	3.0
	25	1,800,000	1,400,000	2,000,000	1,300,000	1,500,000	2.8	2.7
	30	2,000,000	2,100,000	2,400,000	1,800,000	2,000,000	2.5	2.5
	40	880,000	1,800,000	3,700,000	2,400,000	2,500,000	2.2	2.1
SDS (mM)	6	540,000	410,000	460,000	470,000	370,000	4.5	3.5
	8	1,400,000	1,100,000	2,000,000	1,100,000	1,100,000	3.0	3.0
	10	1,100,000	1,100,000	1,400,000	1,500,000	1,500,000	1.5	2.4
	12	1,700,000	1,800,000	2,100,000	2,200,000	2,100,000	1.5	2.3
	15	410,000	1,300,000	1,700,000	2,700,000	22,000	0.9	1.8
PNIP (%)	0.15	950,000	830,000	1,400,000	780,000	820,000	2.6	1.8
	0.30	1,200,000	1,200,000	2,400,000	1,200,000	1,400,000	2.9	3.1
	0.45	900,000	890,000	1,400,000	1,000,000	1,300,000	2.8	7.4
	0.60	730,000	890,000	850,000	1,100,000	1,100,000	2.9	4.6
Temp (°C)	20	920,000	620,000	1,000,000	710,000	720,000	–	
	25	1,100,000	780,000	1,600,000	1,000,000	970,000	–	
	30	1,300,000	1,100,000	1,700,000	1,200,000	1,200,000	–	
	35	1,400,000	2,500,000	2,100,000	1,000,000	1,300,000	–	

*R*1:resolution between Hydrocortisone 21-acetate and Dexamethasone.
*R*2:resolution between Prednisolone and Hydrocortisone.

the movement of the micelles was impeded, resulting in prolonged retention times and insufficient resolution, as observed in the cases using polymers having values of M_n of 7171 and 3385 g/mol. The resolution was not significantly improved until the polymer size was decreased to 1957 g/mol.

Furthermore, we fixed the polymer size to examine the effect of polymer concentration. The higher the polymer concentration, the early the appearance of P*, indicating that elevated levels of IPSCs were formed and migrated toward outlet end. As displayed in Fig. S5C, we tested a series of PNIPAAm (1957 g/mol) concentrations (0, 0.15, 0.30, 0.45, 0.60%) for separation of the steroids. No separation occurred in the absence of the polymer. Increasing the polymer concentration enhanced the strength of the polymer–surfactant interactions, resulting in a wider elution window and adjusted resolution (Table 1). Based on these results, we conclude that larger-sized polymers limited the migration of the SDS micelles, leading to extended sample retention or lower resolution. Moreover, SDS micelles accumulated in these larger polymers, leading to decreased resolution (Fig. S5B, Table 1).

The SDS concentration (tested from 6 to 15 mM) was another factor that modulated the polymer–surfactant interactions. Elevated levels of this surfactant enriched the negative charges, accelerated micelle migration, and compressed the elution window forward (Fig. 4). The column efficiency improved upon increasing the SDS concentration up to 12 mM, as evidenced by the increased numbers of theoretical plates for the five steroids. Improved sweeping was evidenced by the increase in the peak height; the peak height of H increased 2.5-fold while comparing the peak heights for SDS concentration of 6 mM and 12 mM. The resolutions of *R*1 and *R*2 (H_{21}/D and P/H, respectively) decreased, however, upon increasing the SDS concentration (Table 1). We suspect that the rapid movement of the SDS micelles limited analyte partition between the IPSCs and micelles and, thereby, compressed the elution profiles and decreased the resolution of the separation. Decreased analyte partitioning presumably resulted from increased binding of the surfactants to the polymers; covering the polymer stands with a large number of surfactant moieties (e.g., for SDS at 15 mM; Table 1) would decrease the degree of sample partitioning and focusing. Although the peaks were well separated at a low surfactant concentration (6 mM), the separation process took quite a while to complete

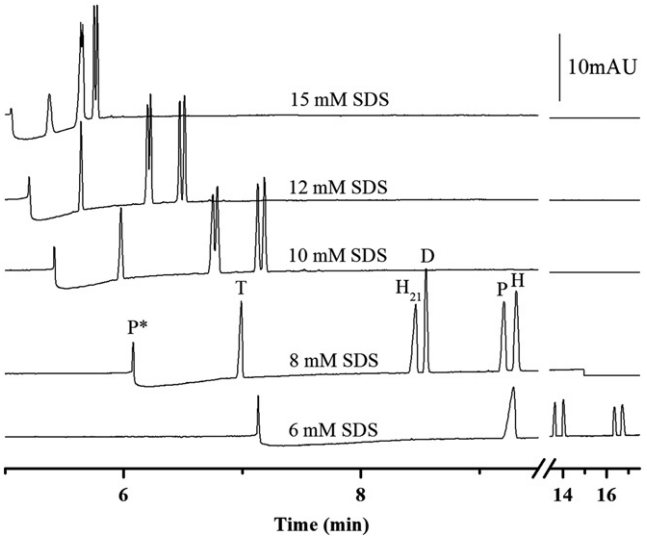

Fig. 4. Effect of SDS concentrations (6, 8, 10, or 12 mM) on steroid separation. Inlet buffer: 20 mM acetate buffer, pH 5.0, assigned SDS concentrations; separation buffer: 20 mM acetate buffer, pH 5.0, 0.3% w/v PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection: 50 mbar, 5 s.

(17 min). Thus, the balance between the concentrations of SDS and PNIPAAm critically affected the separation of these steroids.

PNIPAAm possesses amphiphilic properties because it features amido (hydrophilic) and isopropyl (hydrophobic) groups. Analytes could be partitioned between micelles and polymers and separated based on their retention factor, *k*. The hydrophilic groups of the analytes could form hydrogen bonds with PNIPAAm; their hydrophobic groups could interact with both PNIPAAm and the micelles. For the five neutral steroids (T, H_{21}, D, P, H) tested in the study, T possessed the highest value of *k* for SDS micelles; accordingly, it eluted first. On the other hand, the more-hydrophilic steroids interacted more with the polymers and were eluted later. Thus, increasing the polymer concentration improved the resolution of the more-oxidized steroids, such as that of P and H (from 3.1 to 4.6 at polymer contents of 0.3 and 0.6% w/v, respectively; Table 1; Fig. S5C).

3.3. Effect of BGE concentration on system performance

In RM-MEKC, the BGE concentration markedly affects the migration of polymers and SDS. We examined the effect of the BGE on our system by using a series of concentrations from 15 to 40 mM (Fig. 5). Similar to the effect of the SDS concentration, the elution profile was compressed upon increasing the BGE concentration; in contrast, all of the peaks exhibited valley-to-valley separations that were considerably different from those resulting from the effects of the SDS concentration. In addition, the numbers of theoretical plates of the analytes increased upon increasing the concentration of the BGE (up to 30 mM), especially for T, which exhibited the best performance among all of the tested steroids at a low BGE concentration (15 mM). The resolutions of R1 and R2 decreased (from 3.0 to 2.2 and from 3.0 to 2.1, respectively) upon increasing the BGE concentration (Table 1). These observations suggested that an increased BGE concentration accelerated the SDS micelles as a result of their encountering lower counter forces. The optimal resolution was achieved at a BGE concentration of 20 mM (Table 1), according to the following equation defining the resolution Eq. (2):

$$Rs = \frac{N^{1/2}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k_2}\right)\left(\frac{1-(t_{eo}/t_{mc})}{1+(t_{eo}/t_{mc})}K_1\right) \quad (2)$$

where N is the number of theoretical plates; α is the selectivity factor (k_1/k_2); k_1 and k_2 are the retention factors; and t_{eo} and t_{mc} are the migration times of a non-retained solute and of a micelle, respectively.

3.4. Effect of sample preconcentration

Fig. 6 reveals the effect of sample preconcentration: the peak heights of hydrocortisone (H) and cortisone (C) increased upon increasing the injection size (for injection times of 100, 200, and 300 s, the injection lengths were 0.56, 1.12, and 1.68 cm, respectively). This system provided excellent sample preconcentration and resolution behavior after large sample size injections. Moreover, the improved sensitivity of this system was evident from the peak height enhancement factors ((peak height treatment/peak height typical) × dilution factor) of 200 for H and 150 for cortisone relative to those obtained using conventional MEKC.

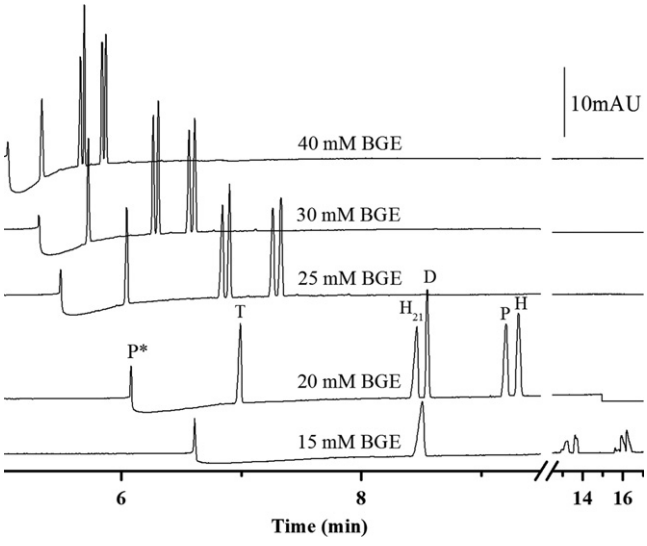

Fig. 5. Effect of the BGE concentrations (15, 20, 25, 30, or 40 mM) on steroid separation. Inlet buffer: 20 mM acetate buffer, pH 5.0, 8 mM SDS; separation buffer: assigned acetate buffer concentrations, pH 5.0, 0.3% w/v PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection: 50 mbar, 5 s.

3.5. Real sample analysis

We recorded standard curves for H and C after injecting samples (5 s; injection length: 0.28 mm) dissolved in 10% MeOH in 60 mM acetate buffer (pH 5.0) into the system, obtaining baseline-resolved peaks (S/N=3; Table 2). The limit of detection (LOD) and limit of quantification (LOQ) for H were 20.98 ng/mL and 69.92 ng/mL (R^2=0.9995; n=9), respectively. For cortisone, the LOD and LOQ were 18.43 ng/mL and 61.43 ng/mL (R^2=0.9997; n=9), respectively. The relative standard deviations of intra-day and inter-day assays were all less than 5% (Table 3).

Human urine samples were collected for 24 h and stored at −20 °C (Fig. 7), we extracted H and cortisone using a C8 solid phase extraction cartridge and dissolved them in 10% MeOH in 60 mM acetate buffer. The peaks of these two steroids were confirmed by spiking sample standards (1.3 and 2.6 μg/mL; 5 μL), and their concentrations were determined by standard addition method through comparing with an internal standard, 6α-methylprednisolone (1.3 μg/mL, 5 μL). After fitting the standard curve generated from the concentrations of the standards and the peak height and multiplying by the sample volume (24 h), we determined the urinary concentrations of H and cortisone to be 20.6 and 26.04 μg/24 h, respectively—possibly indicating low 11-β-hydroxysteroid dehydrogenase activity (11-β-HSD2 catalyzes the conversion of cortisol to cortisone) in the volunteer, because the ratio of H to cortisone (0.79) was above normal (0.5) [35].

4. Conclusion

We have explored the merits of using polymer–surfactant interactions to develop a novel IPSC/RM-MEKC system for the analysis of neutral steroids. This mixed-phase system employed SDS and PNIPAAm as PSPs, with analyses performed through a two-step process: sample preconcentration/IPSC formation followed by IPSC-gradient kinetic separation. The detection limit of H (20.98 ng/mL; R^2=0.9995) was much lower than that of its normal physical concentration (5–55 μg/24 h) [32]. This system, which can be adopted in commercial CE instruments, is easy to operate, sensitive,

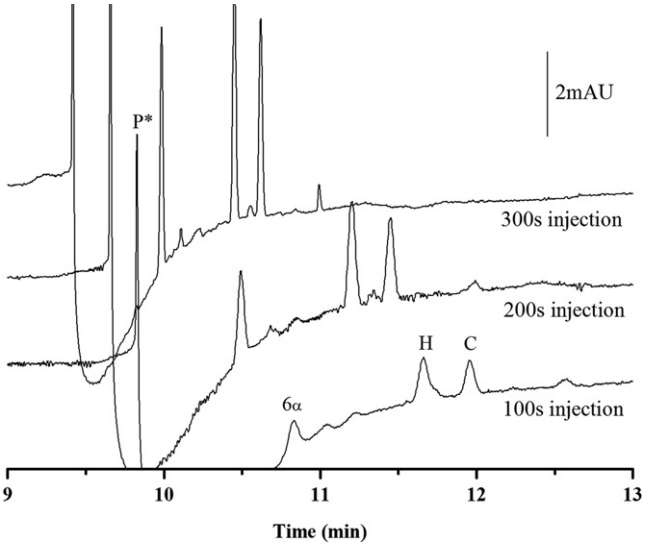

Fig. 6. Effect of sample on-line preconcentration by sweeping. MEKC electropherograms were obtained after analytes (0.50 μM) dissolved in sample buffer (60 mM acetate buffer, pH 5.0, 10% MeOH) were injected for different lengths of time (100, 200, or 300 s). Inlet buffer: 20 mM acetate buffer, pH 5.0, 8 mM SDS; separation buffer: 20 mM acetate buffer, pH 5.0, 0.3% w/v PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection, 50 mbar.

Table 2
Linear correlation, correlation coefficients (R^2), linear range, limits of detection (LOD) and limit of quantization (LOQ).

Analyte	Linear correlation (y=ax+b)	Correlation coefficient (R^2)	Linear range (μg/mL)	LOD (ng/mL)[a]	LOQ (ng/mL)[a]
Cortisone	$y=0.0069x-0.0579$	0.9995	0.04–0.64	18.43	61.43
Hydrocortisone	$y=0.0061x+0.0149$	0.9999	0.04–0.64	20.98	69.92

y: H/6α peak area ratio (mAU × s); x: amount concentration (ng/mL).
[a] $S/N=3$, $S/N=10$.

Table 3
RSDs (%) of intra-day and inter-day assays for hydrocortisone and cortisone.

Concentration (ng/mL)	Hydrocortisone % RSD				Cortisone % RSD			
	Intra-day		Inter-day		Intra-day		Inter-day	
1280	–	2.3	4.8	4.9	–	3.0	4.9	4.2
640	1.4	1.2	2.4	3.4	4.9	1.2	4.9	4.2
320	3.2	2.6	4.1	3.4	2.6	3.4	5.0	3.4
160	4.6	3.7	4.9	4.9	2.5	2.8	3.5	4.0
80	3.4	1.8	1.4	3.0	3.4	2.0	1.8	3.3

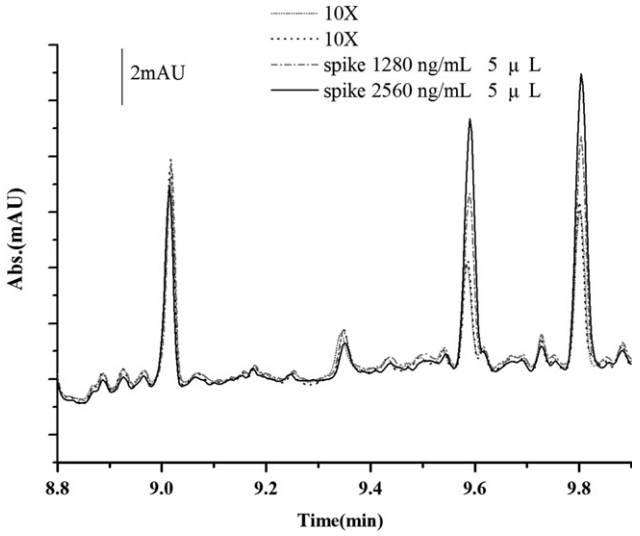

Fig.7. Determination of urinary free H. Urinary free H was extracted in a C18 cartridge and concentrated (10-fold); 6α-methylprednisolone (1.3 μg/mL, 5 μL) was added as the internal standard; steroid concentrations were determined using a standard addition method. Inlet buffer: 20 mM acetate buffer, pH 5.0, 8 mM SDS; separation buffer: 20 mM acetate buffer, pH 5.0, 0.3% PNIPAAm (1957 Da); sample zone: 60 mM acetate buffer, pH 5.0; capillary length: 40 cm (effective); separation voltage: −25 kV; sample injection: 50 mbar, 300 s.

and robust and can be combined with several sample preconcentration techniques. No pre-coating treatments or complicated, laborious, and time-consuming immobilization procedures are required. The aqueous-phase operation of this system requires only a minimal amount of organic solvent, making it environmentally friendly. We suggest potential applications of this technique in clinical analyses and in microanalysis devices.

Novelty statement

This study reports the first demonstration of the use of in-capillary formation of polymer/surfactant complex (IPSC)–assisted reversed-migration MEKC (RM-MEKC), for the analysis of neutral steroids. This IPSC/RM-MEKC system, determining LOD (20.98 ng/mL) and preconcentration factor (200 ×) for hydrocortisone, is easy to operate in commercial CE instruments, robust, environmentally sustainable, and may perform different sample preconcentration options.

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Appendix A. Supporting information

Supplementary information associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.036.

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