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Determination of the degree of deacetylation of chitosan by capillary zone electrophoresis

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ABSTRACT

In this study we have developed a capillary zone electrophoresis (CZE) method for the determination of the degree of deacetylation (DDA) of chitosan. The electrophoretic mobilities of chitosans were measured by using the optimized CZE conditions. An internal standard, hexammine cobalt(III) chloride, was used to improve the precision of the electrophoretic mobility measurement. We have constructed a linear calibration curve between the CZE measured mobilities and the ^{1}H NMR determined DDAs ranging from 55.3% to 96.2%. Based on the established linear calibration equation and the CZE measured mobilities, we were able to obtain not only the average DDA values but also the DDA distribution profiles of the chitosan samples. Without prior sample treatment or purification, we have successfully employed the newly developed CZE method to characterize the reacetylation derivatives of chitosans and to detect the DDA variability in different lots of chitosan products.

1. Introduction

Chitosan, the cationic (1–4)-2-amino-2-deoxy-β-D-glucan, partly acetylated to the typical extent close to 0.25 is industrially produced from marine chitin, the second most abundant polysaccharide in nature (Muzzarelli, 2012). The degree of deacetylation (DDA) of chitosan represents the molar ratio of the $GlcNH_2$ repeating unit to the polymeric chain. When dissolved in acidic aqueous solution, chitosan behaves as a cationic polyelectrolyte due to the protonation of the amine groups on $GlcNH_2$ units. Chitosan has gained versatile applications in various industrial and biomedical fields, and the applicability is closely related to its DDA value. According to the literature, chitosan with higher DDA was more effective in removing chemical pollutants in wastewater (Chung, 2006), showed higher free radical scavenging activity on various radicals (Park, Je, & Kim, 2004), and was more efficient in initiating hemostasis (Yang et al., 2008). However, it was found that DDAs had different influences on the biodegradable and biocompatible properties of chitosans. Thus, the DDAs of chitosans should be tuned in order to achieve the optimal tissue engineering applications (Freier, Koh, Kazazian, & Shoichet, 2005; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Furthermore, opposite conclusions were drawn by different researchers regarding the effect of DDA on the azo dye adsorption capacity of chitosan (Saha, Karmaker, Ichikawa, & Fukumori, 2005; Wong, Szeto, Cheung, & McKay, 2008). Obviously, chitosan with accurately measured DDA and well-defined DDA distribution is essential to arrive at an undisputed conclusion of DDA effect on the efficacy and applicability of the chitosan samples.

Many analytical methods have been developed to measure the DDAs of chitosan samples (Kasaai, 2009), including titration methods (Zhang, Zhang, Ding, Zhang, & Liu, 2011), hydrolysis followed by HPLC analysis (Niola, Basora, Chornet, & Vidal, 1993), and various spectroscopic approaches such as NMR (Kasaai, 2010), UV–vis (Muzzarelli & Rocchetti, 1985; Wu & Zivanovic, 2008), and IR (Kasaai, 2008). For most of the current techniques used for DDA measurements, especially for the spectroscopic approaches, sample purification is usually required to avoid the spectral interference caused by the sample matrix (de Alvarenga, de Oliveira, & Bellato, 2010). Moreover, it is the average DDA value that is usually measured by these methods. Derived from the deacetylation reaction of chitin, the chitosan polymer chains, like the ordinary polydispersed synthetic polymers, inevitably possess some distributions in DDA and molecular weight. According to the literature, DDA distributions across the molecular weight distributions could be obtained by fractionating chitosan samples with semi-preparative size-exclusion chromatography (SEC), followed by DDA measurement for each SEC fraction with titration method (Berth & Dautzenberg, 2002) or ^{1}H NMR technique (Nguyen, Hisiger, Jolicoeur, Winnik, & Buschmann, 2009). Nevertheless, the resulting DDA in each fractionated sample was still an average DDA. The DDA distributions of the chitosan samples were still not available from these methods.

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In this article we are proposing a newly developed method based on capillary zone electrophoresis (CZE) technique for the measurements of both average DDAs and DDA distributions of high molecular weight chitosan samples. CZE could achieve molecular weight (or the degree of polymerization, N) dependent separations for charged oligomers such as poly(styrene sulfonate) (PSS) (Cottet & Gareil, 2000; Cottet, Gareil, Theodoly, & Williams, 2000), ssDNA and ds DNA (Hoagland, Arvanitidou, & Welch, 1999; Stellwagen, Bossi, Gelfi, & Righetti, 2001). On the other hand, the electrophoretic migration behaviors of evenly charged high molecular weight polyelectrolytes were characterized by the N-independent electrophoretic mobility due to the same extent of N-dependent increases in both the effective charge and the frictional coefficient of the polyelectrolyte chains (Cottet et al., 2000; Hoagland et al., 1999; Stellwagen et al., 2001). Moreover, the mobilities of various partially charged polyelectrolytes consisting of randomly copolymerized ionic and nonionic monomers also exhibited similar N-independent property, and more importantly showed proportional increases with increasing charge densities of the polyelectrolytes, though to different extents in different charge density regimes or for different natures of the polyelectrolyte backbones (Cottet et al., 2000; Gao, Dubin, Sato, & Morishima, 1997; Hoagland, Smisek, & Chen, 1996). Therefore, with CZE operating under acidic condition, the resulting electrophoretic mobilities of the partially charged polycationic chitosans would presumably have similar charge density dependence as the aforementioned observations in the literature.

So far there have been only a few studies regarding the analysis of high molecular weight chitosans by CZE. Ban, Choi, Ryu, and Yoo (2001) employed a chitosan standard (DDA85%) and the optimized CZE conditions to determine the chitosan contents in rat plasma and foodstuff in 100 mM triethylamine (TEA)-phosphate buffer (pH 2.0). Fu, Huang, Zhai, Li, and Liu (2007) achieved baseline separation for a chitosan standard (DDA70%) and a carboxymethylation derivative of chitosan by using 50 mM sodium phosphate (pH 2.0) as the CZE run buffer. A commercial product was identified as mainly chitosan content because of its similar electrophoretic migration time as that of the chitosan standard. In these two studies, CZE results showed characterization of chitosan contents and identifications, but not the DDA determination for the real samples. Mnatsakanyan et al. (2013) reported a study on correlating DDAs (ranging from 72.6% to 96%) and electrophoretic mobilities of chitosan samples in 100 mM sodium phosphate buffer at pH 3.0. With their CZE conditions, a nonlinear correlation between mobility and DDA was concluded for the samples investigated in the study. Within about 20% DDA range studied in the article, the authors expressed the DDA variations of the polysaccharide chains in terms of the CZE electrophoretic mobilities. The method was not applied to the DDA analysis of real samples.

The construction of a calibration curve between mobility and DDA is essential to successfully utilize the CZE method to measure both the average DDAs and the DDA distributions of chitosan samples. In this study, we employed the ^{1}H NMR method to accurately determine the DDAs and optimized the CZE conditions for precise measurements of the electrophoretic mobilities of chitosan samples with various DDAs. Accordingly, a linear calibration curve was established and the developed CZE method was used to analyze various real chitosan samples.

2. Experimental

2.1. Chemicals and reagents

Chitosan samples were purchased from different suppliers. RC01065 was the product of BioSyntech (Laval, QC, Canada). Chitosan-low (LMW), chitosan-medium (MMW), and chitosan-high (HMW) were obtained from Aldrich (St. Louis, MO, USA). Chitosan-1000 (C-1000) and two lots of Chitosan-10 (C-10) were purchased from Wako (Osaka, Japan). C-10 samples with lot numbers ASJ1805 and DCR2568 were denoted by C-10A and C-10D, respectively. All other chemicals were of reagent grade. Hexammine cobalt(III) chloride ($Co(NH_3)_6Cl_3$, CZE internal standard denoted by IS), acetic anhydride, phosphoric acid, and sodium acetate were obtained from Sigma (St. Louis, MO, USA). Poly(ethylene oxide) (PEO, average molecular weight $\sim 1 \times 10^6$), sodium hydroxide, deuterium chloride, and deuterium oxide were purchased from Aldrich (St. Louis, MO, USA). Acetic acid, ammonium hydroxide, hydrochloric acid, and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Tris was the product of USB (Cleveland, OH, USA).

All the chitosan samples used as standards for constructing the calibration curve between electrophoretic mobility and DDA were purified according to the following procedures similar to the literature methods (Berth & Dautzenberg, 2002; Schatz, Pichot, Delair, Viton, & Domard, 2003). The chitosan powder was first dissolved in 5% acetic acid and filtered by suction with a mixed cellulose ester filter (5-μm pore size, Advantech MSF, Japan). In this study, all the chitosan standards were totally dissolved and according to the electropherograms shown in Fig. S1, no apparent sample loss was found during the sample purification process. Chitosan in the filtrate was then precipitated by adding 30% NH_4OH and 90% methanol. After centrifuging the solution and removing the supernatant, the chitosan precipitate was washed several times with 90% methanol until the solution pH became neutral. The chitosan precipitate was then ice-dried and ready for use in CZE and ^{1}H NMR measurements.

2.2. Deacetylation and reacetylation of chitosan samples

The deacetylation of chitosan was similar to the literature method used for the alkaline deacetylation of chitin (Mima, Miya, Iwamoto, & Yoshikawa, 1983). 70% NaOH aqueous solution was preheated to 130 °C in an oil bath. The mixture of 1:20 ratio of chitosan sample (g) to NaOH volume (mL) was stirred and refluxed for 10 min. The reaction was stopped by quenching to an ice bath and the sample powder was washed with ddH_2O several times to reach neutral pH. The DDA of chitosan thus obtained was about 95%. The product obtained from the deacetylation reaction of MMW was denoted by MMW-deA.

The reacetylated chitosan samples were prepared by following the method of Berth and Dautzenberg (2002). The homogeneous reacetylation reaction between chitosan and acetic anhydride was performed in an acetic acid solution containing 67% methanol with constant stirring at room temperature for 24 h. The extent of reacetylation of the amine groups was determined by the reaction completeness and the molar ratio of the added acetic anhydride to the amine groups on the chitosan chain. The product derived from the reacetylation reaction of MMW by using the reactant molar ratio (acetic anhydride/amine) of 0.2 was denoted by MMW-reA0.2. Similarly, MMW-deA-reA0.05 denoted the derivative of MMW sample which was first deacetylated to DDA95% and then reacetylated by using 0.05 molar ratio of acetic anhydride/amine.

2.3. Apparatus

2.3.1. Capillary electrophoresis for mobility measurement

CZE experiments were performed on a Beckman P/ACE MDQ CE system (Fullerton, CA, USA) equipped with a diode array detector to record signal at 200 nm and a coolant circulating cartridge to keep capillary temperature at 25 °C. A bare fused silica capillary column (Polymicro Technologies, Phoenix, AZ, USA) of 30-cm total length (effective length: 20 cm) × 100-μm I.D. was assembled

in the cartridge. Prior to the first use, the capillary column was conditioned by the sequential rinses of methanol (10 min), 1 N HCl (10 min), ddH_2O (2 min), 1 N H_3PO_4 (10 min), ddH_2O (2 min), and the CZE run buffer (10 min). A Tris-phosphate buffer was used as the CZE run buffer, which was prepared by titrating 100 mM phosphoric acid solution with 100 mM Tris to pH 2.0 monitored by a pH meter. Chitosan sample and the freshly prepared internal standard $Co(NH_3)_6Cl_3$ were mixed in 5% acetic acid and diluted to the final concentrations of 0.1 mg/mL and 10 μM, respectively. The sample mixture was then introduced into the capillary by hydrodynamic injection at 0.3 psi for 3 s. CZE measurement was carried out by applying a constant voltage of +4.5 kV. Between CZE runs, capillary was first rinsed at 20 psi with 5% acetic acid for 5 min, followed by successively flushing with 1 N HCl for 5 min and 0.03% PEO for another 5 min for dynamic coating of PEO onto the capillary inner wall. Prior to sample injection, 100 mM Tris-phosphate buffer was filled into the capillary at 20 psi for 1.5 min. After the last run of the CZE experiments, capillary column was rinsed with 5% acetic acid and ddH_2O at 20 psi for 10 min, respectively, and then blown dry with nitrogen gas.

2.3.2. ^1H NMR for DDA measurement

NMR samples were prepared by dissolving 6 mg of the purified chitosan in 980 μL of D_2O and 20 μL of DCl. After dissolution, approximately 600 μL of the chitosan solution was transferred to an NMR tube for the DDA measurement. ^1H NMR spectra were acquired on a Bruker 600 MHz NMR spectrometer. The ^1H NMR measurements were carried out based on the method of Lavertu, Darras, and Buschmann (2012) and Lavertu et al. (2003). A representative ^1H NMR spectrum for chitosan sample HMW is presented in Fig. S2.

3. Results and discussion

3.1. Optimization of CZE conditions

CZE analysis of high molecular weight cationic polyelectrolytes usually encountered the problem of analyte adsorption onto the inner wall of the fused silica capillary, which would cause unstable electroosmotic flow (EOF), tailing and broadening sample peaks, and short lifetime of capillary column. To achieve high precision measurement of the electrophoretic mobility of chitosan, the optimal CZE conditions were selected such that the chitosan adsorption could be best reduced. In the following sections we would elucidate the optimization of several CZE conditions such as capillary dimension and inner wall coating, CZE run buffer, chitosan concentration, and electric field strength.

3.1.1. Capillary diameter, length, and PEO coating

In principle, capillary column with smaller diameter and hence larger surface area to volume could define narrower sample zone, dissipate heat more efficiently, and consequently achieve higher separation efficiency in CZE. However, capillary with larger specific surface area and longer length, along with injection of higher sample concentration, would facilitate the adsorptive interaction between the analyte and the capillary inner wall. Therefore, it was concluded that for the CZE separation of macromolecules having the tendency of adsorbing to the capillary wall, larger capillary provided no less separation resolution but greater detection sensitivity than the smaller capillary (Ermakov, Zhukov, Capelli, & Righetti, 1995; Schure & Lenhoff, 1993). To lower the possibility of sample adsorption, we employed relatively shorter and larger capillary of 30-cm total length (effective length: 20 cm) × 100-μm I.D. for the analysis of the polycationic chitosan. Narrower capillary (50-μm I.D.) with longer effective lengths of 30 cm and 40 cm were also tested, but resulted in broader peaks with poor signals, and consequently caused bad repeatability. Similar results were also found by Ban et al. (2001). The optimized capillary dimension allowed us to shorten the electrophoretic migration time and to reduce the injected concentration of chitosan sample for more precise mobility measurement.

Capillary inner wall coating was usually carried out to prevent analyte adsorption. PEO has been widely used as a coating material to bind the silanol group of the capillary column through covalent bond or by the formation of hydrogen bond (Iki & Yeung, 1996). In this study, an acid wash precondition procedure combined with the physical coating of PEO was adopted to refresh the capillary column between runs. The PEO coating could effectively eliminate the EOF and avoid the adsorption of the positively charged chitosan chains on the capillary wall. With the capillary column treatment method, we were able to perform repeatable CZE measurements and to greatly enhance the lifetime of capillary column. Without PEO coating, gradually longer elution time was observed for the test chitosan sample (C-1000) after 10 consecutive CZE runs, while with PEO coating, repeatable elution time could be obtained for at least 50 runs.

3.1.2. CZE run buffer

CZE run buffer at pH 2.0 was chosen to fully protonate the amine group of the chitosan sample and to suppress the dissociation of the silanol group of the capillary inner wall so as to prevent the electrostatic attraction between the capillary wall and the chitosan sample. It was found that CZE run buffer with higher concentration could effectively reduce the analyte adsorption by providing higher concentration of counterions (Ermakov et al., 1995). Appropriately adjusted buffer compositions could result in more symmetrical peak shape of the analyte (Oudhoff, Buijtenhuijs, Wijnen, Schoenmakers, & Kok, 2004). To prepare the test buffers, 100 mM phosphoric acid was titrated to pH 2.0 with 100 mM sodium hydroxide, sodium acetate, sodium tetraborate, and Tris, respectively. Among the test buffers, Tris-phosphate buffer generated relatively lower current and more symmetrical peak shape. In addition to the 100 mM Tris-phosphate buffer, we prepared two more buffers at 50 and 200 mM for the CZE analysis of C-1000 sample with DDA79.8% measured by ^1H NMR. Electropherogram of smoother baseline and more symmetrical peak shape was obtained when using the 100 mM Tris-phosphate buffer. Therefore, we selected 100 mM Tris-phosphate aqueous solution with pH 2.0 as the CZE run buffer throughout this study.

3.1.3. Sample concentration

The amine group of chitosan reveals its basic nature and thus can possibly increase the pH of the sample solution, especially for the sample with high DDA and high concentration. Moreover, chitosan behaves as a cationic polyelectrolyte with high conductivity after protonation. Therefore, injection of high concentration chitosan into capillary column may perturb the applied electric field strength and causes peak deformation. According to laser light scattering study (Pa & Yu, 2001), chitosan polymer chains tended to interact with each other through hydrogen bonding, hydrophobic interaction, and ionic binding, and thus led to the formation of large size clusters, which would be more evident at high sample concentrations. The strong intermolecular interactions might affect the electrophoretic migration behaviors of the chitosan polymer chains. Thus, we prepared nine C-10A sample solutions with concentrations ranging from 0.05 to 9.0 mg/mL to investigate the concentration effects on the electrophoretic migration times and peak shapes of the chitosan samples. A constant voltage of +4.5 kV was applied across the capillary column of 30-cm × 100-μm with a 200 nm UV detection window at 20-cm from the injection end. The detailed CZE conditions are described in the experimental Section

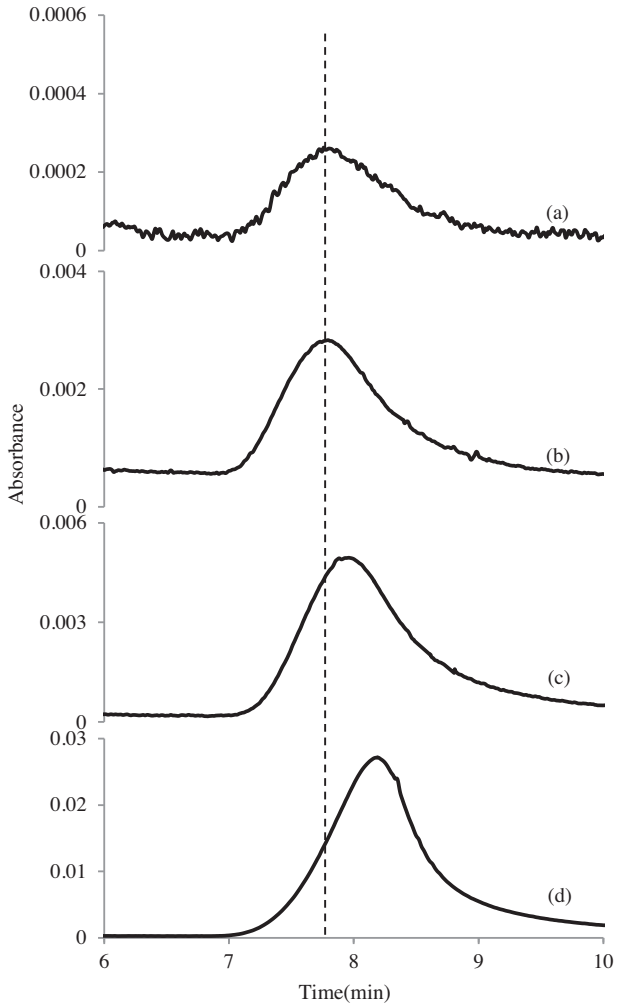

Fig. 1. Effect of chitosan concentration on electrophoretic migration time and peak shape. Electropherograms of C-10A with injected concentrations of (a) 0.05 mg/mL, (b) 0.5 mg/mL, (c) 1.0 mg/mL, and (d) 5.0 mg/mL in 5% acetic acid, respectively. The dashed line indicates the same migration time of 0.05 and 0.5 mg/mL samples. CZE conditions: 30-cm total length (effective length: 20 cm) × 100-μm I.D. bare fused silica capillary, 100 mM Tris-phosphate buffer (pH 2.0), UV detection at 200 nm, applied voltage of +4.5 kV at 25 °C.

2.3.1. Four representative electropherograms are shown in Fig. 1. Chitosan samples at concentrations lower than 0.5 mg/mL exhibited the same electrophoretic migration times as indicated by the dashed line, whereas gradually increasing migration times were observed for the samples with higher concentrations. The distorted peak shape of the 5.0 mg/mL sample was mainly caused by sample overloading. To ensure the correct measurement of chitosan mobility without concentration influence, we chose the low sample concentration of 0.1 mg/mL as the injected concentration of chitosan sample for the CZE experiments.

3.1.4. Electric field strength

When measuring the electrophoretic mobilities of polyelectrolytes by CZE, very often the resulting mobilities would increase with increasing electric field strengths, especially at high applied voltages (Grossman & Soane, 1990; Hoagland et al., 1999; Stellwagen et al., 2001). This phenomenon could be attributed to the decrease in frictional coefficient caused by the Joule heating or to the molecular orientation of the rod-like polyelectrolytes induced by the applied voltage. A semi-rigid rod-to-rod conformation was postulated for chitosan chains dissolved in low pH environments due to the electrostatic repulsion between

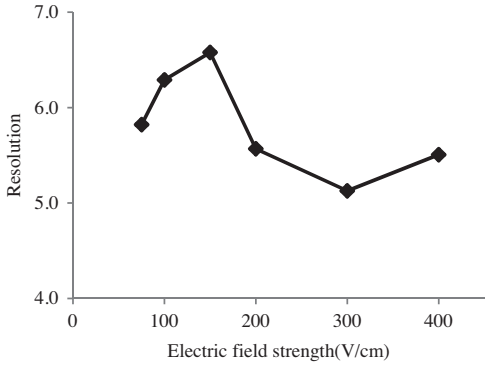

Fig. 2. Effect of electric field strengths (E = 75, 100, 150, 200, 300, 400 V/cm) on the separation resolution of RC01065 and MMW-reA0.4. The line only serves for guiding the eyes.

protonated amine groups (Novoa-Carballal, Fernandez-Megia, & Riguera, 2010; Pa & Yu, 2001). With the abovementioned optimal CZE conditions we investigated the effect of electric field strengths (E = 75–400 V/cm) on the electrophoretic mobilities of two chitosan samples, RC01065 and MMW-reA0.4, with the respective DDAs of 96.2% and 55.3% measured by ^{1}H NMR, covering the DDA range studied in this article. The measured electrophoretic mobilities of the two chitosans showed E-independence within the range of the applied electric field strengths, indicating no apparent occurrence of either Joule heating or molecular alignment with the applied electric field. Moreover, the current increases were proportional to the applied voltages for E = 75–300 V/cm, but a disproportionally higher current with relatively larger fluctuation was found at E = 400 V/cm, and consequently resulted in lower repeatability for the mobility measurement. Fig. 2 displays the separation resolutions of the two chitosan samples under the applied electric field strengths of 75–400 V/cm. Baseline separations of the two chitosans were achieved at all the six electric field strengths. The best separation resolution was obtained by applying electric field strength of 150 V/cm. Therefore, 150 V/cm was adopted for the DDA analysis of chitosan samples in all the CZE experiments.

3.1.5. Internal standard

To improve the precision of mobility measurement by CZE, an internal standard (IS) with known electrophoretic mobility was usually injected together with the analyte and separated in the same run. The resulting electropherogram was then corrected based on the IS migration time ratio (Mayer, 2001). To search for the adequate internal standard for the mobility measurements of chitosan samples, five compounds were tested with CZE experiments. The resulting electropherograms are listed in Fig. S3. In this study we selected hexammine cobalt(III) chloride as the internal standard for the CZE experiments. The $Co(NH_3)_6^{3+}$ peak showed sensitive UV signal and migrated without interfering the chitosan peaks, i.e. it did not comigrate with any of the chitosan peaks studied, nor did it influence the migration times of the chitosan samples. This can be proved by comparing the electropherograms of $Co(NH_3)_6^{3+}$, C-1000, and their mixture, as shown in Fig. S4. Measured by two analysts in the span of 10 days with the optimal CZE conditions, the average electrophoretic migration time of the trivalent cation $Co(NH_3)_6^{3+}$ was determined to be 3.72 ± 0.02 min in 110 replicate measurements. There was no observable difference in IS migration time measured alone or in mixture with the chitosan samples. Calculated according to the electropherograms of RC01065 and C-1000, the mobility RSDs (n = 5) of the two chitosan samples were lowered by more than 4 times when the migration times of the chitosan peaks were corrected by the respective IS migration time ratio R_{IS} (=3.72/T_{IS}, with T_{IS} being the migration time of the IS peak

in the corresponding electropherogram). Accordingly, we corrected all the electropherograms by multiplying the time scales by the respective R_{IS} values.

3.2. Establishment of the calibration curve of mobility versus DDA

At pH 2.0 the $GlcNH_2$ units are fully protonated and the mole fraction of the $GlcNH_3^+$ units determines the charge density of the polycationic chitosan chain. Therefore, the success of using CZE to determine the DDA of chitosan sample relies on the charge density dependent but molecular weight independent electrophoretic mobility measured by CZE under appropriate conditions. High molecular weight polyelectrolytes with the same charge density tend to have the same electrophoretic mobility. For examples, the electrophoretic mobility of DNA was independent of molecular weight when it was larger than 400 base pairs (Stellwagen, Gelfi, & Righetti, 1997). As the degree of polymerization of fully sulfonated PSS was bigger than 100, the CZE measured mobility was independent of N values (Cottet et al., 2000). Instead of determining the critical molecular weight of chitosans having the N-independent migrating behavior, we measured the electrophoretic migration times of two chitosan samples, C-10A and HMW, with similar DDAs but different molecular weights to confirm that all the CZE measured mobilities in this study were only functions of charge densities, and would not be influenced by the molecular weight differences. The ^{1}H NMR measured DDAs were 85.0% and 85.8% for C-10A and HMW, respectively. At the same concentration, C-10A had the lowest viscosity, while HMW was among the most viscous samples used in this study. The intrinsic viscosities ($[\eta]$) of chitosan samples in a 0.2 M CH_3COOH–0.1 M CH_3COONa buffer solution were measured by using a viscometer operating at 30 °C. The viscosity average molecular weights (M_v) were then calculated by using the Mark–Houwink equation $[\eta] = kM_v^{\alpha}$ and the DDA dependent k and α values according to the literature (Wang, Bo, Li, & Qin, 1991). The M_v values thus obtained for C-10A and HMW were 1.3×10^5 and 2.3×10^6, respectively. We carried out the electrophoretic experiments for the two chitosan samples by using the optimal CZE conditions and corrected the resulting electropherograms with the R_{IS} values. Shown in Fig. 3 are the two electropherograms, which revealed the same electrophoretic migration time for the two test samples in our CZE conditions. In spite of the more than 10-fold difference in molecular weight, C10-A and HMW possessed the same electrophoretic mobility, indicating that DDA should be the sole factor affecting the measured mobility.

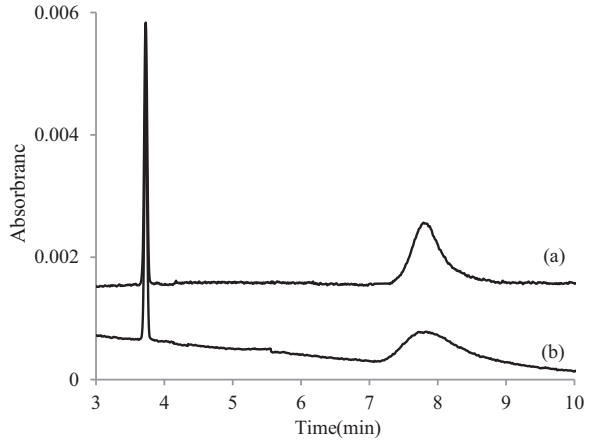

Fig. 3. Electropherograms of 10 μM $Co(NH_3)_6Cl_3$ and 0.1 mg/mL chitosan samples, (a) HMW and (b) C-10A. The other CZE conditions were the same as in Fig. 1.

In principle, the electrophoretic mobility of polyelectrolyte would increase with increasing charge density, providing that the charge density has not yet reached the threshold of the occurrence of counterion condensation, which would, according to Manning's prediction (Manning, 1981), result in a charge density independent electrophoretic mobility. To further verify the electrophoretic mobility dependence on the charge density for chitosan samples with various DDAs, we purchased four chitosan samples from three different companies and prepared another four chitosan standards through reacetylation reaction or deacetylation followed by reacetylation reaction. The DDAs of the eight chitosan standards were measured by ^{1}H NMR, while the corresponding

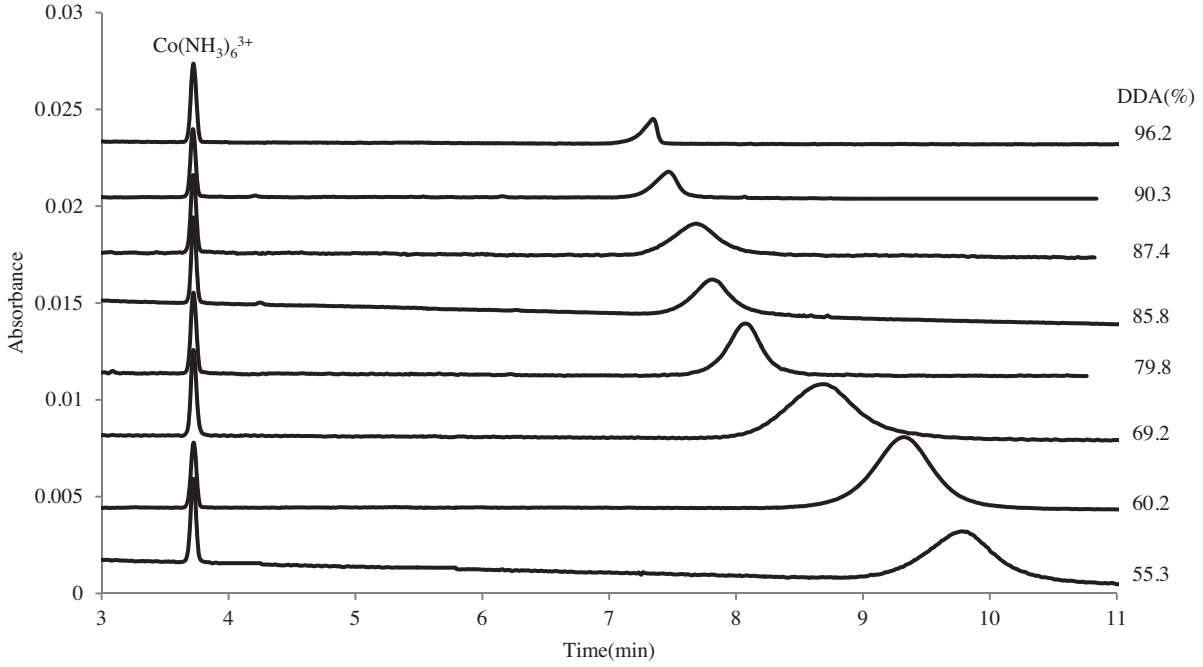

Fig. 4. Electropherograms of the IS and the eight chitosan standards with DDAs ranging from 55.3% to 96.2%, as indicated at the end of each trace. CZE conditions were described in Section 2.3.1.

Table 1
^{1}H NMR measured DDAs, CZE measured mobilities, and the corresponding RSDs of the chitosan standards.

Chitosan standard[a]	^{1}H NMR		CZE	
	DDA (%)	RSD (%, n = 3)	Mobility (10^{-4} cm^2 V^{-1} s^{-1})	RSD (%, n = 5)
RC01065	96.2	0.6	3.00	0.4
MMW-deA-reA0.05	90.3	0.2	2.93	0.6
MMW	87.4	0.05	2.86	0.5
HMW	85.8	0.4	2.83	0.5
C-1000	79.8	0.5	2.72	0.3
MMW-reA0.2	69.2	0.4	2.53	0.6
MMW-deA-reA0.4	60.2	0.1	2.37	0.4
MMW-reA0.4	55.3	0.3	2.25	0.5

[a] All the chitosan samples were described in detail in Sections 2.1 and 2.2.

electrophoretic mobilities were determined by CZE with the aforementioned optimal conditions. Fig. 4 displays the electropherograms of the eight chitosan standards with DDAs ranging from 55.3% to 96.2%. The electrophoretic migration times of the chitosans decreased with increasing DDAs, revealing that the electrophoretic mobilities of the chitosans would increase with increasing charge densities, and thus the proposed CZE method would be applicable to the DDA determination if the calibration curve between mobility and DDA was established. The resulting average DDAs and mobilities of the eight chitosan standards along with their relative standard deviations (RSDs) are listed in Table 1. Good repeatabilities were achieved for both DDA and mobility measurements with RSDs less than 1% for all the eight chitosan standards. Also revealed in Table 1 is the slow increase of the electrophoretic mobilities with the DDAs rising from 55.3% to 96.2%. Accordingly, we plotted the calibration curve of the CZE measured electrophoretic mobility (μ, cm^2 V^{-1} s^{-1}) against the ^{1}H NMR determined DDA (%), and obtained a linear regression equation of $\mu = 1.83 \times 10^{-6} \times DDA + 1.26 \times 10^{-4}$, with the coefficient of determination $r^2 = 0.9981$, indicating very good linearity between the electrophoretic mobilities and the DDAs in the range of 55.3–96.2% studied in this work (as shown in Fig. 5). It has been reported that polyelectrolytes at low charge density regime exhibited linear increases of electrophoretic mobilities with increasing charge densities (Gao et al., 1997; Hoagland et al., 1996). Nevertheless, the linearity would tend to level off in the high charge density regime where counterion condensation occurred. Notwithstanding the theoretic prediction regarding the independence of electrophoretic mobility on charge density due to the counterion condensation effect, the experimental results usually showed non-linearity (Mnatsakanyan et al., 2013; Oudhoff et al., 2004) or gentle linearity (Gao et al., 1997; Hoagland et al., 1996) between μ and charge density, or good electrophoretic separations of polyelectrolytes with charge densities in the counterion condensation regime (Cottet et al., 2000). With the optimal CZE conditions we were able to construct the linear equation, though with small slope, for the measurement of DDAs covering the medium to high charge density regime for chitosan samples. According to the literature and our results, it was found that as the counterions condensed on the polyelectrolytes, the extents of charge density dependences of the electrophoretic mobilities would vary with the natures of the charged functional groups as well as the hydrophobicity of the polyelectrolyte backbones.

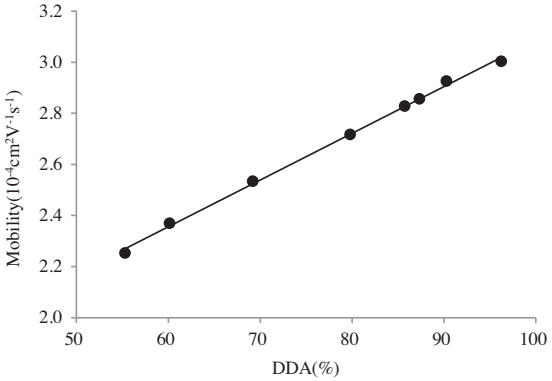

Fig. 5. Calibration curve of electrophoretic mobility versus DDA.

3.3. Validation of CZE method for DDA analysis

To validate the effectiveness of the calibration curve and the applicability of the proposed CZE method, we analyzed two chitosan samples, LMW and its reacetylated derivative LMW-reA0.4, alone or in mixtures of different compositions (with concentration ratios of 2:1, 1:1, and 1:2) by using both the CZE and the ^{1}H NMR methods, and subsequently compared the results of the two analytical techniques. Fig. 6 shows the resulting electropherograms, each with the IS peak and one or two chitosan peaks as labeled in the figure. The two chitosan samples were baseline separated in all the three mixtures. Moreover, within measurement errors, their respective electrophoretic migration times kept unchanged no matter they were injected as a single sample or in the mixtures. It was reported that polyelectrolytes having different charge densities could exhibit strong interaction with each other when injected together as a mixture, and consequently ruined the electrophoretic separation. To improve the separation, buffer compositions were adjusted to lower the interaction (Cottet et al., 2000). With our CZE conditions, however, not only were the two chitosan samples separated, but also no observable influences of the intermolecular interactions on the measured mobilities were found. By using the linear equation of the calibration curve between mobility and DDA we were able to calculate the DDAs of the chitosan peaks in the electropherograms shown in Fig. 6. The DDAs thus obtained along with the corresponding NMR data are summarized in Table 2. The expected average DDAs of the NMR measurements for the three

Table 2
Comparison of DDAs measured by ^{1}H NMR and CZE for individual and mixed chitosan samples.

Chitosan sample	DDA (%)		
	^{1}H NMR	CZE	
LMW	86.1	86.6	
LMW-reA0.4	54.9	55.0	
Ratio[a]		1st[c]	2nd[c]
2:1	76.5 (76.2)[b]	86.4	54.9
1:1	71.6 (71.1)[b]	87.2	55.1
1:2	66.5 (65.8)[b]	86.6	55.2

[a] Concentration (mg/mL) ratio of LMW: LMW-reA0.4 in the mixture.
[b] The expected average DDA of the mixture.
[c] The respective DDAs of LMW and LMW-reA0.4 separated in the electropherograms (Fig. 6(c)–(e)).

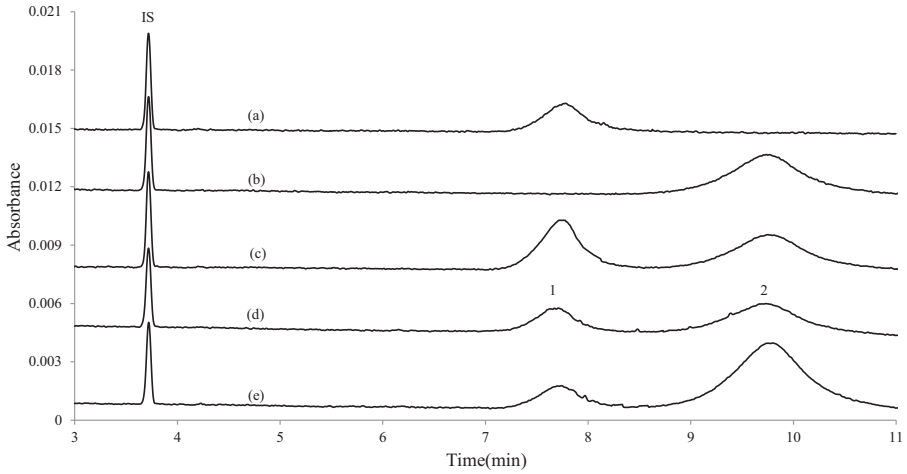

Fig. 6. Electropherograms of (a) 0.1 mg/mL LMW, (b) 0.1 mg/mL LMW-reA0.4, (c) mixture of 0.2 mg/mL LMW and 0.1 mg/mL LMW-reA0.4, (d) mixture of 0.1 mg/mL LMW and 0.1 mg/mL LMW-reA0.4, and (e) mixture of 0.1 mg/mL LMW and 0.2 mg/mL LMW-reA0.4. Peak identification: IS, $Co(NH_3)_6^{3+}$; (1) LMW; (2) LMW-reA0.4. The other CZE conditions were the same as in Fig. 1.

mixtures were calculated by computing the mole fractions of the $GlcNH_2$ monomers in the mixtures according to the respective concentrations and average molecular weights of the monomers in the two chitosan samples (Lavertu et al., 2003). The data listed in Table 2 showed very good agreement between the NMR and the CZE measured DDAs for the two single component samples. However, for the three mixtures, the NMR measurements could only provide the average DDAs of the two mixed chitosans, whereas the CZE method, due to its separation power, was able to correctly reveal two DDAs for the mixed samples. The NMR method could accurately determine the average DDAs of the three mixtures, as validated by the closeness between the experimental results and the expected values shown in Table 2. Averaging the four CZE measured DDAs listed in Table 2, one from the individual and three from the mixed samples, we arrived at the respective DDAs of 86.7 ± 0.3 and 55.0 ± 0.2 for the LMW and LMW-reA0.4 samples, which were consistent with the NMR results of the individual samples, and furthermore, demonstrated the high precision DDA measurements of the CZE method. In regard to the measurements of average DDAs for chitosan samples, the CZE results were as precise and reliable as the NMR results. Nevertheless, it was more advantageous to use CZE method to analyze chitosan mixtures due to its high resolving power for the separation of chitosans with different DDAs.

3.4. Detection of DDA variability in different lots of chitosan products

Chitosan is the deacetylated derivative of chitin which can be extracted from different natural sources and exists in various crystal forms. The manufacture of chitosan samples usually involves multi-step treatments by concentrated acid and base in heterogeneous phases at high temperature. Therefore, in order to maintain the quality consistency, it requires constant checking of the important properties such as DDA, molecular weight, and their dispersities for chitosan products. Quality control on both average DDA and DDA distribution is important for a better characterization of the chitosan products, especially when evaluating the effectiveness of chitosan samples on various applications. Two C-10 samples, C-10A and C-10D, with different lot numbers were purchased at different times and analyzed by using the developed CZE method to examine the DDA consistency of the products. The resulting electropherogram was first normalized by multiplying the migration time axis by R_{IS}, converted to the electrophoretic mobility scale, and then translated into DDA value by using the linear calibration curve between mobility and DDA. The DDA distribution profiles of the two C-10 samples are plotted in Fig. 7(a) and (b), and their average DDAs along with the peak widths at half height w_h obtained from the DDA distribution plots are listed in Table 3. The w_h values can be used to estimate and compare the DDA dispersities between chitosan samples. The two lots of C-10 samples were very similar

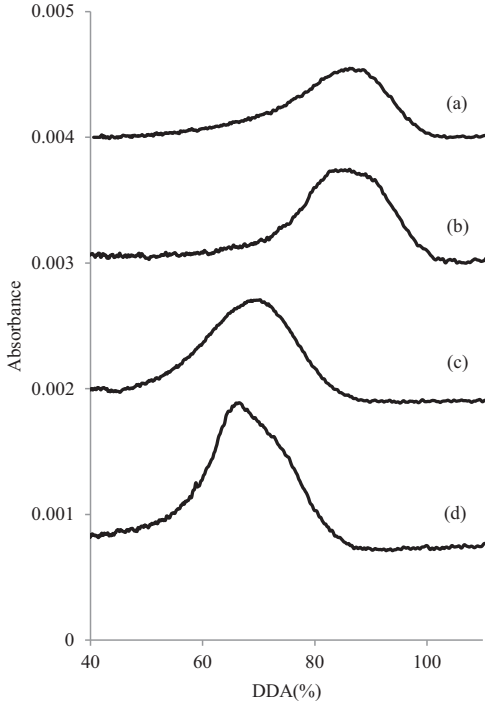

Fig. 7. DDA distribution profiles of (a) C-10A, (b) C-10D, (c) C-10A-reA0.2, and (d) C-10D-reA0.2.

Table 3
CZE measured DDAs and w_h values of the C-10 series.

Chitosan sample	DDA (%, $n = 3$)	w_h (%, $n = 3$)
C-10A	85.0 ± 0.7	19.7 ± 0.7
C-10D	85.3 ± 0.9	19.3 ± 1.3
C-10A-reA0.2	67.9 ± 0.6	19.3 ± 0.5
C-10D-reA0.2	68.1 ± 0.9	18.8 ± 0.4

to each other in average DDA and w_h. However, the DDA distribution profiles of the two C-10 samples were apparently different from each other. It is not surprising to find the asymmetric profile for C-10D. Compared with the rest of the chitosan samples used in this study, both C-10 samples had relatively broader DDA distributions. This heterogeneity in DDA distribution might arise from the particular manufacture process or the certain chitin sources used to produce the samples. To explore the CZE method for the direct analysis of reaction products without sample purification, C-10A and C-10D were subjected to reacetylation reactions with acetic anhydride by using 0.2 molar ratio of acetic anhydride/amine. After the reaction, 1.0 μL of the product solution was mixed with IS, diluted with 5% acetic acid to the final concentration of 0.1 mg/mL, and then directly injected into the capillary for CZE analysis. Fig. 7(c) and (d) display the DDA distribution profiles of the reacetylation products C-10A-reA0.2 and C-10D-reA0.2. Their respective average DDAs and w_h values are listed in Table 3. Comparing the DDA distribution profiles of C-10A and C-10D with those of their respective reacetylated derivatives, we found that both derivatives inherited similar characteristic shapes of their original samples, and the w_h values became slightly narrower. Assuming that the reacetylation reaction proceeded as the designed molar ratio of the reactants, i.e. acetic anhydride/amine = 0.2, then the expected average DDAs of C-10A-reA0.2 and C-10D-reA0.2 could be estimated to be 68.0% and 68.2%, respectively. The expected average DDAs were calculated by multiplying the CZE measured DDAs of C-10A and C-10D by 0.8 because 20% of the original amine groups would be reacetylated by the added acetic anhydride. The experimental DDAs of the derivatives shown in Table 3 were in agreement with the expected values. These CZE results suggest that the reacetylation reaction of the amine groups occurred randomly along the polysaccharide chains, and that all the added acetic anhydride reacetylated the amine groups as designed in the homogeneous reaction conditions.

4. Conclusion

In this study, the accurate DDA values of chitosan standards were measured by using ^{1}H NMR method, while the precise mobility data were obtained by employing the optimal CZE conditions and corrected by the internal standard, $Co(NH_3)_6Cl_3$. Consequently a mobility-versus-DDA calibration curve with very good linearity was attained. Obtained with the linear calibration curve constructed by using the current chitosan standards, the CZE results were proved to be valid for the DDA determination of chitosan samples with DDA higher than 55.3%, molecular weight larger than 1.3×10^5, and concentration lower than 0.5 mg/mL. By measuring the DDAs of the mixed chitosan samples and comparing with the corresponding ^{1}H NMR results, the newly developed CZE method was validated to be efficient and reliable. With the developed CZE method, we have demonstrated that the average DDAs and the DDA distribution profiles of various chitosan samples, alone, in mixtures, or with a complicated matrix, could be obtained successfully and efficiently due to the advantageous separation capability of the CZE technique.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.086.

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