EXPRESS ANALYSIS OF CHITOSAN AND ITS DERIVATIVES BY GEL ELECTROPHORESIS

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Abstract

Chitosan is one of the most promising polymers for biomedical applications due to its unique properties, such as its biocompatibility, low toxicity, biodegradation, and the presence of reactive amino and hydroxyl groups. Analysis of physicochemical properties of chitosan and its derivatives is a time-consuming process and requires expensive equipment and large amounts of the sample. This paper proposes a method for express analysis of the molecular weight (MW), the degree of deacetylation (DD), the substitution degree (SD), and the charge of chitosan and its derivatives using agarose gel electrophoresis under acidic and neutral conditions with Coomassie staining. Positively charged chitosan samples required acidic Tris Acetate-EDTA (TAE) buffer to move in the gel. The electrophoretic mobility of chitosan depended on MW, DD, SD, and the chitosan charge. Based on the dependences obtained by the proposed method, the MW and DD of commercial chitosan samples were determined. Express analysis of chitosan and its derivatives in agarose gel can be used to monitor the reactions of chitosan modification and to analyse samples with unknown characteristics.

Keywords: *chitosan, molecular weight, degree of deacetylation, chitosan derivatives, agarose gel electrophoresis*

Received: 10.04.2019
Accepted: 06.06.2019

1. Introduction

Chitosan is a derivative of the natural polymer chitin. The main source of chitin is crustaceans (crabs and shrimp shells). Chitosan is a linear polysaccharide consisting of glucosamine and N-acetylglucosamine units linked by 1,4-β-glycosidic bonds. The main characteristics responsible for its chemical and biological properties are its molecular weight (MW) and degree of deacetylation (DD). Depending on the source and method of preparation, the MW of chitosan may range from ≥10 to 1000 kDa, and the DD may range from 30% to 95% [1]. Chitosan is characterized by its unique properties, such as its biocompatibility, biodegradability, low toxicity, and the presence of reactive amino groups at the C-2 atom, as well as the primary and secondary hydroxyl groups at the C-3 and C-6 atoms in the units of the polymer chain, by which it is possible to synthesize derivatives with desired charge, solubility, and hydrophobicity.

Practical application of chitosan and its derivatives is limited by the problems of chitosan standardization due to the variety of polymer sources, its polydispersity, the presence of impurities, and the difficulty in determining the MW and DD [2]. The following three groups of different methods are used to determine the DD: spectral (NMR, UV, IR), electrochemical (conductometric and potentiometric titration), and destructive (elemental analysis, differential thermal analysis, complete hydrolysis of chitosan followed by colorimetric or HPLC analysis) [3]. Determination of chitosan MW is more difficult. This is due to the polymer polydispersity, its tendency to aggregate in aqueous solutions, and the lack of a direct method for determining the MW. All currently used methods (chromatography, viscometry, dynamic and static light scattering) measure not the actual MW, but the hydrodynamic diameter of the molecules, which depends on many factors (impurities, ionic strength of the solvent, etc.) [3]. All of these methods require a substantial period of time, a large amount of test sample, and some of them require expensive equipment, which do not allow for quick analysis of chitosan samples obtained in routine laboratory practices.

There are several works in which the authors used agarose and polyacrylamide gel electrophoresis to characterize the hyaluronan polysaccharides [4,5] and low molecular weight chitosan [6], respectively. To determine the DD of chitosan, some researchers have used capillary electrophoresis [7,8].

Determination of the main physicochemical characteristics of chitosan (MW, DD) is an important step since these characteristics directly affect its chemical and biological properties [9,10]. Knowledge of the main physical-chemical characteristics of chitosan is also of great importance for understanding the relationship between its structure, properties, and biological activity [11]. A decrease in the DD leads to a decrease in biocompatibility, solubility, and mucoadhesiveness. In turn, the decrease in MW leads to better biocompatibility and it increases antioxidant and antitumor properties. The purpose of this work was to select the conditions for the analysis of the physicochemical characteristics of chitosan and its derivatives by gel electrophoresis in agarose gel using neutral and acidic buffer.

2. Materials and Methods

2.1. Materials

Crab chitosan with a MW of 200 kDa and a DD of 96% (Bioprogress, Russia), succinic anhydride (Fluka, Germany), nitric acid, hydrochloric acid, sodium hydroxide, and sodium tetrahydride borate (ChimMed, Russia) were used in the work. Chitosan was purified by re-precipitation and dialysis. The other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of Chitosan Derivatives

2.2.1. Preparation of Low Molecular Weight Chitosan Samples

Chitosan samples with different MWs were obtained by the method of chemical hydrolysis based on chitosan with a MW of 200 kDa and a DD of 96%. Chitosan samples with MWs of 50 kDa and 90 kDa were obtained by nitrate hydrolysis. For this purpose,10 g of high-molecular weight chitosan was dispersed in 180 mL of water, and then 20 mL of concentrated nitric acid was added. The reaction mass was stirred at 70°C for 7 h and then kept for 12 h at room temperature. The precipitate was filtered, suspended in 180 mL of distilled water, and heated until complete dissolution (70–80°C). The solution was additionally passed through a Schott filter to remove mechanical impurities. Next, the solution was cooled to 6°C, and the precipitate was filtered, washed with isopropyl alcohol three times, and dried on a filter. The sample yield was 50% [12].

Samples with a MW of 25 kDa were obtained by hydrochloric acid hydrolysis. For this, 180 mL of 6 M hydrochloric acid solution was added to 30 g of chitosan at room temperature. The reaction mass was stirred at 90°C for 3 h and then the mixture was kept for 12 h at room temperature. Further processing was carried out similarly to hydrolysis with nitric acid. The yield was 45% [12,13].

2.2.2. Deacetylation of Chitosan

Deacetylation of chitosan was performed according to the method of Lim [14]. Crab chitosan with a MW of 200 kDa and a DD of 96% (20 g) was suspended in 200 mL of 10% (w/w) NaOH solution containing $NaBH_4$ (2 g) as a reducing agent. After 5 h of stirring at 110°C, the suspension was filtered over a glass filter and washed with distilled water until neutral pH. Next, the chitosan was washed with methanol and acetone and dried at 70°C under vacuum overnight. As a result, the MW of chitosan decreased to 150 kDa, and the DD increased to 98%. The product yield was 85%.

2.2.3. Reacetylation of Chitosan

The chitosan reacetylation reaction was carried out in a 1:1.7 (v/v) aqueousalcoholic medium. Chitosan purified by reprecipitation (1 g) was dissolved in 1% acetic acid and methanol with stirring for 30 min [15]. The acetylating agent was added in the required amount. The reaction was carried out for 5 min at 22°C. Then, the chitosan solution was dialyzed and lyophilized. The yield was 70–80%.

2.2.4. Succinylation of Chitosan

For the synthesis of succinyl chitosan, 2 g of chitosan was dissolved in 40 mL of 5% acetic acid, and then 160 mL of methanol was added. The amount of succinic anhydride was calculated based on the ratio of ν anhydride to ν amino groups (=20). Succinic anhydride was dissolved in a minimum volume of acetone and added to the chitosan solution with stirring. The mixture was incubated overnight. The resulting gel was dissolved in distilled water and the pH was adjusted to 10 with 2 M NaOH. The precipitate was washed with acetone (50 mL, 3 times each), then dialyzed against distilled water and lyophilized [16].

2.3. Characterization of Chitosan and Its Derivatives

The molecular weight characteristics of chitosan samples were determined using high performance gel permeation chromatography by an S 2100 Sykam chromatograph (Germany) with a column (7.8 × 300 mm) of Ultahydrogel-250 (Waters, USA) and a pre-column (4 × 3 mm) GFC-4000 (Phenomenex, USA) [17].

DOI: 10.15259/PCACD.24.007

The DD of the chitosan samples and the SD of succinyl chitosan derivatives were determined by proton magnetic resonance (^{1}H-NMR). The proton spectra were recorded on a Bruker AMX 400 spectrometer (USA). Samples were prepared in deuterated water, and 4,4-dimethyl-4-silapentane-sulfonic acid was used as a standard.

2.4. Agarose Gel Electrophoresis of Chitosan and Its Derivatives

The chitosan samples were added to wells of 0.8 or 2% agarose gel in an amount of 25 μg in 10 μL. The gel was prepared using neutral (pH 7.2) or acidic (pH 3.1) 0.04 M TAE-buffer. The gel thickness was 50 mm. Standard buffer containing 30% glycerol and Blue Dye (Bender Medsystems, Austria) was used to load the chitosan samples. Electrophoresis was carried out in neutral (pH 7.2) or acidic (pH 3.1) 0.04 M TAE-buffer for 1–4 h under a voltage of 60 V at room temperature. Gels were stained with 0.2% Coomassie R250 dye at room temperature for 30 min, and then washed three times with 10% acetic acid for 10 min or overnight. Electrophoregrams of gels were obtained using the gel-documentation system GelDoc EZ (Bio-Rad, USA). The length of the chitosan track in the gel was measured. A graph of the track length versus the variable parameter (DD, SD, MW) was plotted.

3. Results and Discussion

3.1. Chitosan Molecular Weight Analysis

Agarose gel electrophoresis is traditionally used to analyse the mobility of RNA and DNA molecules and is carried out in neutral current conducting TAE buffer. Previously, a method for the analysis of MW of hyaluronic acid derivative in a 0.5 % agarose gel was proposed and optimized by Cowman et al. [4]. The authors showed the dependence of the polymer mobility on MW. Since the structures of hyaluronic acid, a polysaccharide of the glycosaminoglycans family, and chitosan have significant similarities, it can be assumed that the mobility of chitosan samples will also vary depending on the MW during agarose gel electrophoresis. However, unmodified chitosan, unlike hyaluronic acid, is soluble only in dilute acids, most often in 0.1% acetic acid. Therefore, it was assumed that the mobility of chitosan using acidic TAE buffer and agarose gel, prepared in acidic buffer, will be higher than when using neutral buffer and gel.

Chitosan samples with different MWs and DDs, previously characterized by standard methods [2,18,19], were used to analyse their mobility in agarose gel (Table 1). Samples A_3–A_5, E_1–E_3, A_{C1}–A_{C3}, and E_{C1}–E_{C3} were obtained additionally. The DD of A_3–A_5 and E_1–E_3 samples and the SD of A_{C1}–A_{C3} and E_{C1}–E_{C3} samples by succinyl groups were determined by NMR (Fig. 1). Characteristics of the samples used in this work are shown in Table 1.

During the development of this method, we used different experimental conditions, such as different agarose concentrations (from 2 to 0.1%), different pH values of the gels and the buffers (from 7.2 to 2.5), different times (from 1 to 18 h), and different staining methods (Coomassie, fluorescent labelled samples, visualization in UV). We found a direct dependence on pH and time of electrophoresis but not on the method of staining. Agarose concentration was selected on the basis of a balance between the time of migration of chitosan samples and the hardness of the gel. Acidic gels of lower percentages were brittle. Finally, we selected the parameters described below.

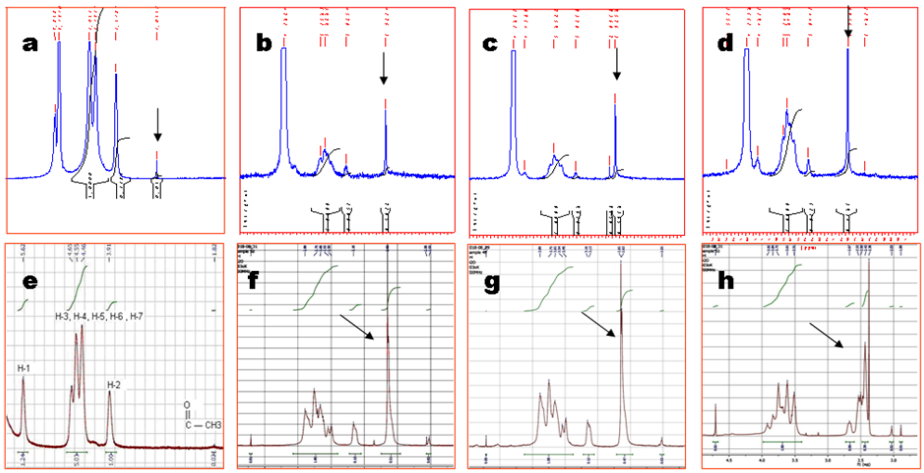

Figure 1. NMR spectra of chitosan derivatives.The spectra of unmodified chitosan with a MW of 200 kDa and a DD of 96% (a).Reacetylated chitosan with a MW of 200 kDa and DD of 48% (b), 36% (c), and 22% (d).The spectra of unmodified chitosan with a MW of 25 kDa and a DD of 98% (e).Succinyl chitosan with a MW of 25 kDa and a SD of 64% (f), 59% (g), and 49% (h). The arrows indicate the acetyl groups of N-acetylglucosamine (a–d) or the succinyl groups of N-acetylglucosamine (e–h)

Electrophoresis of chitosan samples was carried out in three versions: neutral–neutral conditions were used for both gel formation and tank filling in TAE buffer (pH 7.2); acidic–neutral conditions were used to form gel in the acidic TAE buffer (pH 3.1) and neutral tank buffer; or acidic–acidic conditions when both gel and tank buffer were based on acidic TAE buffer (pH 3.1). For the staining of chitosan and its derivatives in the gel, Coomassie R250 was used, which is an acidic dye bearing sulfonic acid residues that interact with the amino groups of proteins. The chitosan molecule binds Coomassie R250 dye due to the availability of glucosamine groups in its structure. The estimated parameter was the length of Coomassie stained chitosan migration.

Table 1. Physicochemical characteristics of chitosan and its derivatives

Chitosan sample	Name	MW(kDa)	DD(%)	SD(%)
Chitosan A	A	200	96	–
Chitosan A	A_1-A_5	200	70/60/48/36/22	–
Chitosan B	B	150	98	–
Chitosan C	C	90	98	–
Chitosan D	D	50	98	–
Chitosan E	E	25	98	–
Chitosan E reacetylated	$E_1/E_2/E_3$	25	84/65/52	
Chitosan F	F	1000	98	–
Succinyl chitosan A	$A_{C1}/A_{C2}/A_{C3}$	200	–	49/60/70
Succinyl chitosan E	$E_{C1}/E_{C2}/E_{C3}$	25	–	49/59/64

DOI: 10.15259/PCACD.24.007

Analysis of unmodified chitosan samples with a DD of 96–98% and a MW from 50 to 200 kDa under neutral conditions showed low polymer mobility over 4 h, but the migration track was inversely proportional to the MW (Fig. 2a,d).

Under acidic conditions, the migration track of all samples increased (Fig. 2b,c) and was also was inversely proportional to the MW. The differences between the acid/neutral and acid/acid gel/buffer conditions led to an increase in the slope of the migration track curve versus MW, as well as a decrease in the electrophoresis time. So, for acid/neutral gel/buffer conditions the average analysis time was 2–3 h. For acid/acid gel/buffer conditions, 1.5 h was enough for the significant migration sufficient for a reliable analysis (Fig. 2b,c). Fig. 2d shows the dependence of the migration track length on MW and the conditions of electrophoresis. The data in all three cases were approximated by linear dependencies inversely proportional to the MW. Accordingly, in the case of analysing samples with an unknown MW, chitosan standards can be used and the MW can be determined from the dependence curve of the migration track on the MW.

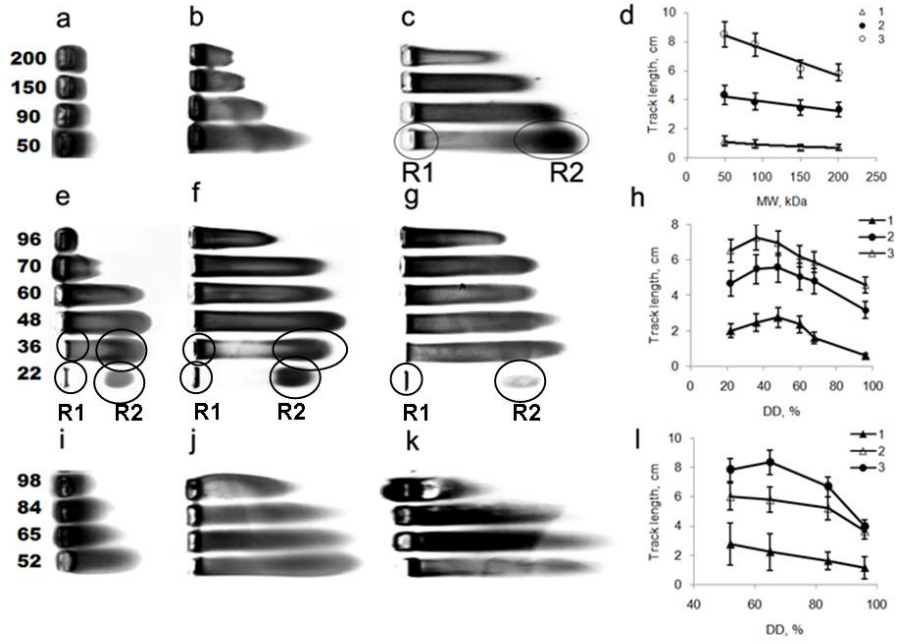

Figure 2. Analysis of chitosan mobility by agarose gel electrophoresis. Migration of chitosan samples with different MW and DD 98% (a–c); different DD and MW 200 kDa (e–g); and different DD and MW 25 kDa (i–k) in neutral 0.8% gel/ neutral buffer (a, e, i); acidic 2% gel/ neutral buffer (b, f, j); and acidic 2% gel/ acidic buffer (c, g, k). Dependences of the chitosan migration track on MW and DD (d, h, l): 1–3 correspond to the electrophoresis data, located from left to right. Soluble and insoluble fractions were found in some samples (R1 and R2 regions).

It should be noted that chitosan was distributed over the entire length of the track (Fig. 2) on the electrophoregrams, in contrast to the data obtained by Cowman et al. for hyaluronan, where individual bands corresponding to different MWs were shown [4]. It can be assumed that a significant fraction of chitosan in the agarose gel remains at the start (Fig. 2, region R1), and only the low molecular weight fraction moves in the

electric field, as evidenced by the uneven staining of the polymer in the tracks. When analysing under acidic/acidic gel/buffer conditions, the separation of low molecular weight samples with MM 50 kDa into high and low molecular weight fractions is clearly seen (**Fig. 2c**, regions R1 and R2).

3.2. Chitosan Deacetylation Degree Analysis

In some cases, the DD of chitosan samples is not determined or samples with different DD are required. To analyse the dependence of electrophoretic mobility on the DD, chitosan samples with a molecular weight of 25 and 200 kDa with different DD values were obtained. Analysis of electrophoretic mobility was also performed in neutral, half-acid, and acidic conditions (4 h, 2.5 h, and 1.5 h in 0.8%, 2%, and 2% gels, respectively). The mobility of chitosan samples with the same MW and different DD values in neutral conditions was maximal for samples with a DD of about 50% (Fig. 2e,i). Increased mobility was associated with a greater solubility of these samples. The high solubility of chitosans with approximately a 50% DD in aqueous solutions with a neutral pH was confirmed by N. Kubota et al. [20]. This effect was observed both for chitosan with a MW of 200 kDa (Fig. 2e) and for chitosan with a MW of 25 kDa (Fig. 2i). In semi-acidic and acidic conditions, the mobility of chitosan with different DD values increased, as was observed for the samples with a high DD (Fig. 2f,g,j,k). The dependence of the migration track on the DD was non-linear and was characterized by the same type of curves for all phoresis conditions and for chitosan of any MW (Fig. 2h,l). The decrease in the solubility in the samples with a DD less than 50% led to a decrease in the migration track of chitosan samples in the gel. Thus, for the chitosan samples with a MW o f200 kDa and DD of 22%, two fractions are observed—insoluble at the start and soluble with a migration track lower than for the sample with a DD of 36% (Fig. 2e,f,g). Fractionation in this case identified the compositional heterogeneity of the sample with a DD of 22%.

The obtained data showed that the DD assessment was the highest under neutral conditions. A decrease the DD to 70-80% was enough to register a change in the migration track when analysing in a neutral 0.8% gel and in neutral buffer for 4 h (**Fig. 2e,i**). In semi-acidic and acidic conditions, there were no significant differences in the migration track of chitosan with a DD in the range of 80% to 40%, but there was a significant difference between the samples with a DD that was >90%. Samples with very low DD can be differed by the presence of two separable fractions in the migration track.

3.3. Molecular Weight and Deacetylation Degree Analysis of Commercial Chitosan Samples

The results obtained were used to estimate the MW and DD of eight commercial chitosan samples characterized by a wide range of parameters (**Table 2**).

To analyse the MW of unknown chitosan samples, reliable in-lab standards are needed. All the samples used in this work were characterized by us earlier using several methods [2]. Determination of chitosan MW still presents a problem, resulting in the limitation of chitosan use in medicine. Different methods used to estimate chitosan MW (HPLC, viscometry, dynamic or static light scattering) can produce significantly different results [2]. These reasons can explain the discrepancy between the MW declared by some companies and defined by electrophoresis (Table 2).

Table 2. Physicochemical characteristics of commercial chitosan samples

	Chitosan sample	**Declared parameters**		**Defined parameters**	
		MW(kDa)	DD(%)	MW(kDa)	DD(%)
1	Heppe LMW	30	>88	>250	>70
2	Sigma LMW1	50-190	75-85	>300	>70
3	Protasan UP B 80/20	110 [21]	82	>400	>70
4	Heppe HMW	300	>88	>600	>70
5	Chitopharm M	100–2000 [22]	>70	>1300	>70
6	Protasan UP G 213	200–600	75-90	>1650	>70
7	Sigma LMW2	–	75-85	>3500	>70
8	Chitopharm L	500–5000 [22]	>70	>3500	>70
*Pearson's correlation coefficient of declared and defined data.				r=0.931, p<0.001	

*Pearson's correlation coefficient is calculated without the Sigma LMW2 sample. For the correlation analysis, the average value of the declared MW used.

For example, Protasan UP B 80/20 was characterized by a MW 110 kDa and DD 82% [21], Chitopharm M chitosan was characterized by a MW that ranged from 100 to 2000 kDa and a DD that was >70% [22]. For the commercial samples, the analysis was performed under neutral (0.8% gel, 4 h) and acidic (2% gel, 4 h) conditions using the reference chitosan samples with different MW values and a DD of 96–98% as standards. Additionally, sample F with a MW of 1000 kDa and DD of 95% was used. All commercial samples under neutral conditions remained at the start (**Fig. 3a**), but they migrated differently under acidic conditions (**Fig. 3b**).

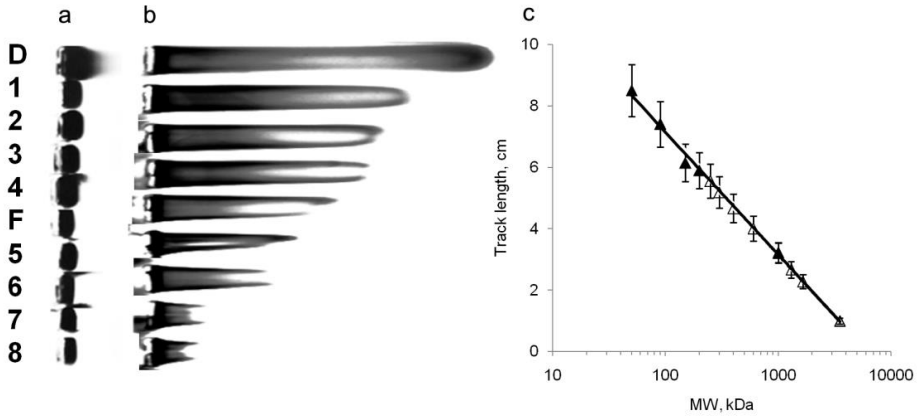

Figure 3. Determination of commercial chitosan sample MW by gel electrophoresis**.** a, b - Electrophoregrams of chitosan samples with different MW and DD 75–85% in 0.8% neutral gel (a) and 2% acidic gel (b). Samples D and F correspond to MW 50 and 1000 kDa.; c - Estimation of MW of commercial samples using chitosan standards with MW 1000, 200, 150, 90 and 50 kDa (black symbols).

Accordingly, with a high probability, the DD of all samples was above 80%. Indeed, manufacturing companies indicated that the DD was >75% (Table 2). The approximate

MW was determined according to the graph of the migration track length on the MW using the available standards (Fig. 3c). The MWaveragedataforallsamples (Table 2) wereobtained. Comparison of obtained and declared MW was carried out using the average value of the MW, declared by the manufacturer. As a result, the declared characteristics significantly correlated ($p<0.001$) with the data determined using the express analysis, while the average declared and defined MW in several samples differed significantly (Table 2). Thus, by analysing the migration of chitosan in a gel, a rough estimation of the MW and the DD of chitosan samples can be determined.

3.4. Chitosan Charge and Substitution Degree Analysis

Chitosan has a large number of amino groups, which allows for the development of derivatives with different properties, including those with a negative total charge. To analyse the migration of succinylated chitosan derivatives into an agarose gel, samples with different SD values by succinyl residues were obtained (**Table 1**, **Fig. 4a–d**).

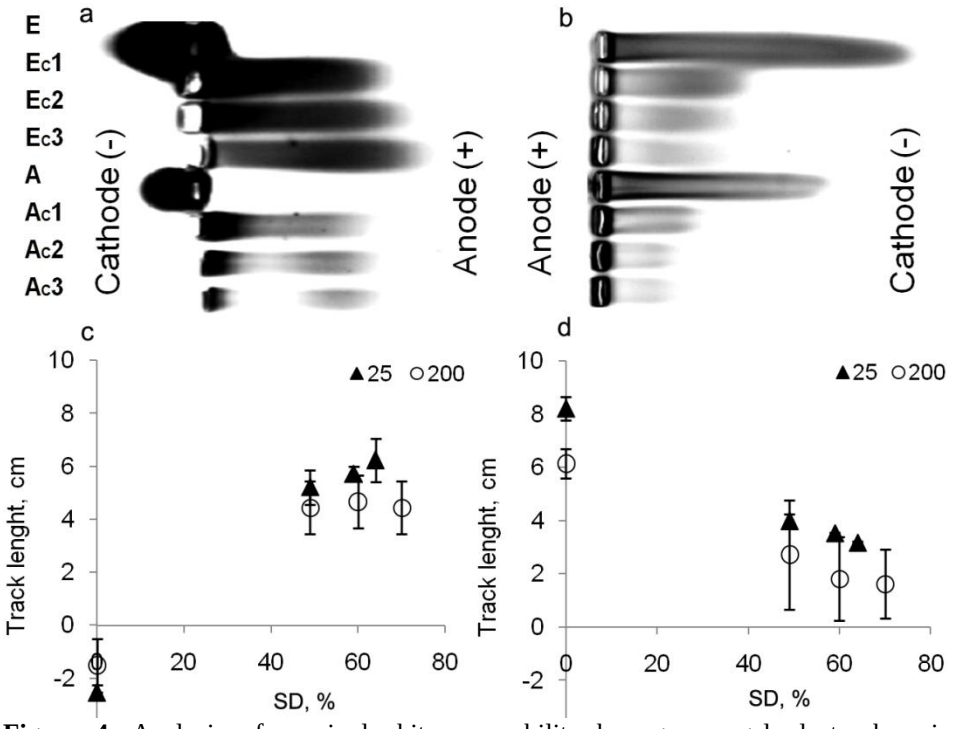

Figure 4. Analysis of succinyl chitosan mobility by agarose gel electrophoresis**.** Migration of the unmodified chitosan samples with a MW of 25 kDa (E) and 200 kDa (A) and their succinyl derivatives with different SD values (Ec1–Ec3 49/59/64% and Ac1–Ac3 49/60/70% accordingly) in neutral gel and buffer (a) and acid gel and buffer (b). The graphs show the dependences of the unmodified chitosans and their succinyl derivative migration length on the degree of substitution. In a neutral gel, chitosan moves to the cathode, while succinyl chitosans move towards the anode.In the acid gels, succinyl chitosans recharge and migrate to the cathode.

Electrophoresis was carried out in both the neutral and acidic conditions. As expected, in the neutral gel/buffer, the initial unmodified positively charged chitosan samples migrated to the cathode, while the succinyl derivatives migrated to the anode

(Fig. 4a). The migration rate of negatively charged derivatives was higher than the velocity of unmodified positively charged chitosan and depended on both the MW and SD (Fig. 4a,c). During electrophoresis of the samples under acidic conditions, it turned out that all derivatives migrate to the cathode (Fig. 4b). The migration velocity of succinyl derivatives was significantly lower than that of the unmodified samples and was inversely proportional to the SD (Fig. 4d). Obviously, with an increase in SD, the number of amino groups in the chitosan molecule decreases and the proportion of carboxyl groups increases. Accordingly, under neutral conditions migration is determined by the number of carboxyl groups, and under acidic conditions it is determined by the number of amino groups. Gel electrophoresis under acidic conditions can be recommended for evaluating the SD in the case of succinyl derivatives.

4. Conclusions

This paper proposes a simple method for a comparative analysis of molecular weight, degree of deacetylation, charge, polydispersity, and compositional heterogeneity of chitosan and its derivatives using gel electrophoresis in a 0.8–2% agarose gel with Coomassie R250 staining. The optimal conditions for the analysis (concentration of agarose gel, pH buffer solution, time, method of staining) were found. This method does not require expensive equipment or a large amount of the test sample and it is simple in reproduction.The result can be obtained within a few hours. It can be recommended for the express analysis of chitosan and its derivatives in the laboratory.

5. Acknowledgments

This work was supported by the Fundamental Research Program of the Presidium of the Russian Academy of Sciences "Nanostructures: physics, chemistry, biology, fundamentals of technology".

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