STUDY OF THE EFFECT OF THE PHYSICOCHEMICAL PROPERTIES OF CHITOSAN ON ITS HAEMOSTATIC ACTIVITY

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Abstract

Chitosan are biopolymers that are actively used for the production of local haemostatic agents. The physicochemical characteristics that determine its biological properties include the molecular weight and the deacetylation degree. However, there is no linear relationship between these parameters and haemostatic activity. The most reliable method of confirming the effectiveness is still in vivo experiments. The ability to initiate haemostasis depends on the conformational transition of chitosan macromolecules. The highest efficiency in vitro was for samples in which the transition of a significant part of the molecules from the 'rigid rod' state to the 'globule' occurred at physiological pH. It is proposed to expand the list of indicators of chitosan that can be controlled to evaluate the quality of raw materials, related to haemostatic activity, to include the definition of the conformational transition at physiological pH.

Keywords: *chitosan, bleeding, haemostasis, conformation, NMR, UV/Vis spectroscopy.*

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1. Introduction

Massive external bleeding remains one of the main causes of death at the pre-hospital stage in both military and civilian medicine. Many tools and methods for temporarily stopping external bleeding have been developed. Along with tourniquets and pressure dressings, local haemostatic agents (LHA) are becoming increasingly common [1], among which chitosan-based products occupy a special place. However, there is still no consensus on the mechanism by which chitosan provides its haemostatic effect, and there is not a complete understanding of the physicochemical properties of this biopolymer [2-5]. As a result, there are no uniform approaches and standards for quality control of chitosan raw materials and LHA obtained on its basis. The main indicators for quality control of raw materials are usually the deacetylation degree (DD), viscosity, humidity, microbiological purity, the content of heavy metals and pesticides and the granulometric composition [6, 7], but none of these indicators is crucial in determining its ability to initiate haemostasis. The most reliable test of effectiveness is still *in vivo* experiments on large animals.

Researchers agree that in chitosan samples with haemostatic activity, the DD is on average 68%-95%, while the molecular weight varies widely from 30 to 1,000 kDa [2, 8-10]. Indicators such as molecular weight distribution, surface absorption properties, optical activity and conformational state can also make a certain contribution to the realisation of the biological activity of chitosan. In addition, an increase in the rate of blood clot formation and aggregation of shaped blood elements is associated with an increase in the specific surface area and porosity of chitosan particles [11, 12]. In the course of studies [13, 14], researchers have found that the conformation of chitosan macromolecules in solution depends on the pH. By measuring the light transmission of solutions, conformational transitions were detected in the pH range 3.2-3.3 ('coil–rigid rod') and 4.8-6.0 ('rigid rod–globule'). The interaction in the 'rigid rod' conformation is stronger than in the 'globule' conformation. In the 'globule' conformation, the solubility of the macromolecule decreases, and chitosan precipitates. This phenomenon is associated with a decrease of its activity.

The aim of the study was to determine additional physicochemical characteristics, which in the future will allow us to predict the presence or absence of the haemostatic activity of chitosan and LHA at the stage of raw material selection and LHA creation.

2. Materials and Methods

2.1. Materials

The following chitosan samples were selected as the objects of the study: Natural Ingredients (Nat. Ingr.; Shanghai Medicines & Health Products Import & Export Corporation, China), Henan Tianfu (Henan Tianfu Chemical, China), Mezon (Orion Chemical Company Ltd., India) and Naturing (Tayga [Shanghai] Co., Ltd., China). In addition, the following LHA based on chitosan were used: Celox (MedTrade Products Ltd., Great Britain), Hemospas Bio (MDK Medica, Russia), Ellarga (New Biomedical Solutions, Russia) and Gepoglos (LUMI Ltd., Russia). A sample of Nat. Ingr. chitosan was selected to simulate a violation of storage conditions: storage for more than 3 years with free air access and a temperature > 40°C; it was labelled Chitosan2. For each sample, at least three parallel experiments were performed for each study method. Chitosan-free samples were used as a control.

The following reagents and materials were also used: glacial acetic acid (99.5%, ChimMed, Russia), sodium acetate trihydrate (≥99.0%, Sigma-Aldrich), hydrochloric acid (35.0%-38.0%, Reachim, Russia), sodium hydroxide (≥ 97.0%, Sigma-Aldrich Co.), deuterium oxide (99.9%, Cambridge Isotope Laboratories, Inc., USA), calcium chloride (93%, Sigma-Aldrich Co.) and the GPC standard dextran kit (Pharmacosmos, Denmark).

In vitro haemostatic activity was examined by using whole blood samples obtained from 15 healthy male donors aged 25-40 years. Blood samples were collected in Vacuette® coagulation tubes (containing anticoagulant sodium citrate in a ratio of 1:9). All participants provided written consent in compliance with the rules of asepsis and antiseptics.

2.2. Methods

The DA was determined by using a Bruker Avance III HD 600 MHz nuclear magnetic resonance spectrometer at 50°C, according to Lavertu *et al*. [15].

The viscosity average molecular weight (MWv) of chitosan was determined by capillary viscometry [16] with a glass capillary viscometer (Labtex, Russia; inside diameter of 0.54 mm) at 25.0 ± 0.5°C, with use an acetate buffer solution (pH 4.6) as the solvent containing 0.3 M acetic acid and 0.2 M sodium acetate trihydrate. The molecular weight was calculated using the Mark-Houwink equation, with constants corresponding to the temperature and the given polymer-solvent system [17, 18].

The average molecular weight (Mw), the numeric average molecular weight (Mn) and the polydispersity index (PI) of chitosan was determined by diffusion-ordered spectroscopy (DOSY) according to a method described previously [19-21], with some modifications. The spectrum of samples with 1 mg/ml of deuterium oxide and 0.02 ml of a 20% solution of hydrochloric acid were recorded with a Bruker Avance III HD 600 MHz nuclear magnetic resonance (NMR) spectrometer using the legbpgp2s pulse sequence with the following settings: 25°C; diffusion time Δ = 250 ms; gradient pulse duration δ = 2.0 ms; and d1 = 5 s. Solutions of standard dextran samples with 1 mg/ml of deuterium oxide were used to construct a calibration graph of the dependence of the self-diffusion coefficient on the molecular weight. The molecular weight of chitosan was determined according to the calibration graph. To determine the contribution of each fraction to the average molecular weight, the peak of the distribution of self-diffusion coefficients on the obtained spectrum was integrated. Mn and Mw were calculated using Eq. (1) and (2), respectively:

$$\mathrm{Mn} = \frac{\sum \mathrm{niMi}}{\sum \mathrm{ni}}, \tag{1}$$

$$\mathrm{Mw} = \frac{\sum_{\mathrm{niMi}} 2}{\sum \mathrm{niMi}}, \tag{2}$$

where is the relative proportion of resonating nucleus in the sample, characterised by the value of the molecular weight ().

The PI of the polymer – used to estimate the spread of macromolecules by molecular weight – was determined by the ratio .

The conformational transitions of macromolecules depending on the pH of the medium was determined by using an Agilent 8453 spectrophotometer at 650 nm, with a cuvette length of 10 mm, according to the method presented by Apryatina *et al*. [13] with a few modifications. Aqueous solutions of chitosan samples with a concentration of 0.3 mg/ml, pH 3.5-4.0, were prepared with the addition of a 20% (v/v) hydrochloric acid solution. As 2.5% (w/v) sodium hydroxide solution was added, the pH was measured using a Fisher Scientific Accumet excel XL60 pH meter and the light transmission of the solutions was recorded by a spectrophotometer. A stepwise decrease in the light transmission of the chitosan solution by at least 30% from the initial value was taken as a conformational transition with a pH change of 0.3-0.4 units, which indicated the transition of a significant part of the macromolecules to a different conformational state.

The haemostatic activity of the samples in *in vitro* experiments was studied using donated blood. The method described by Wu *et al*. [22] was used to determine the coagulation time (CT).

The results of the experiments were statistically analysed using Microsoft Excel 2010. The average value and standard deviation of the indicators in each study group were calculated. Statistical significance was assigned at a greater than 95% confidence level ($p < 0.05$).

3. Results and Discussion

The haemostatic activity of chitosan is associated with the presence of positively charged amino groups in the macromolecule, which initiate the aggregation of negatively charged red blood cells, the number of which is associated with the DA [5]. Another parameter that has a significant impact on the properties of chitosan is molecular weight, which serves as a measure of the length of the polymer chain and depends not only on the type of natural objects used, the method of obtaining chitin and the method of its deacetylation, but also on the storage conditions and the time of collection of chitosan-containing raw materials. Chitosan samples could have significant differences in these parameters but still have similar haemostatic activity, and vice versa, with similar physical and chemical parameters, and could show a variable ability to initiate haemostasis. This phenomenon is due to the fact that many factors affect the relationship between the structure and activity of polymers of biological origin.

The physicochemical properties and haemostatic activity of chitosan samples *in vitro* are presented in Table 1. The graphical dependence of light transmission on pH is shown in Figure 1.

Table 1. Physicochemical parameters and haemostatic activity of chitosan and local haemostatic agents

Sample	DA (%)	MWv (kDa)	Mw (kDa)	Mn (kDa)	PI	CT (min)	Light transmission at pH 7.4*
Nat. Ingr.	91.2	373.0	396.8	372.0	1.07	10.9 ± 0.6	38.1 ± 4.9
Chitosan2	91.0	361.0	415.1	370.5	1.12	No haemostasis	85.2 ± 8.4
Henan Tianfu	92.3	160.0	165.2	160.6	1.03	12.4 ± 0.7	43.2 ± 5.1
Mezon	76.9	658.0	838.6	693.4	1.21	15.8 ± 0.7	72.4 ± 5.6
Naturing	89.1	120.0	106.4	69.9	1.52	15.6 ± 1.3	42.5 ± 3.9
Celox	76.0	Not determined for local haemostatic agents				20.9 ± 0.7	66.1 ± 7.1
Hemospas Bio	77.6					21.8 ± 1.2	64.2 ± 7.7
Ellarga	94.1					19.7 ± 1.1	55.1 ± 6.4
Gepoglos	96.0					No haemostasis	95.0 ± 7.9

*Determined by the graph in Figure 1

Abbreviations: MWv, viscosity average molecular weight; Mw, average molecular weight; Mn, numeric average molecular weight; PI, polydispersity index; CT, coagulation time

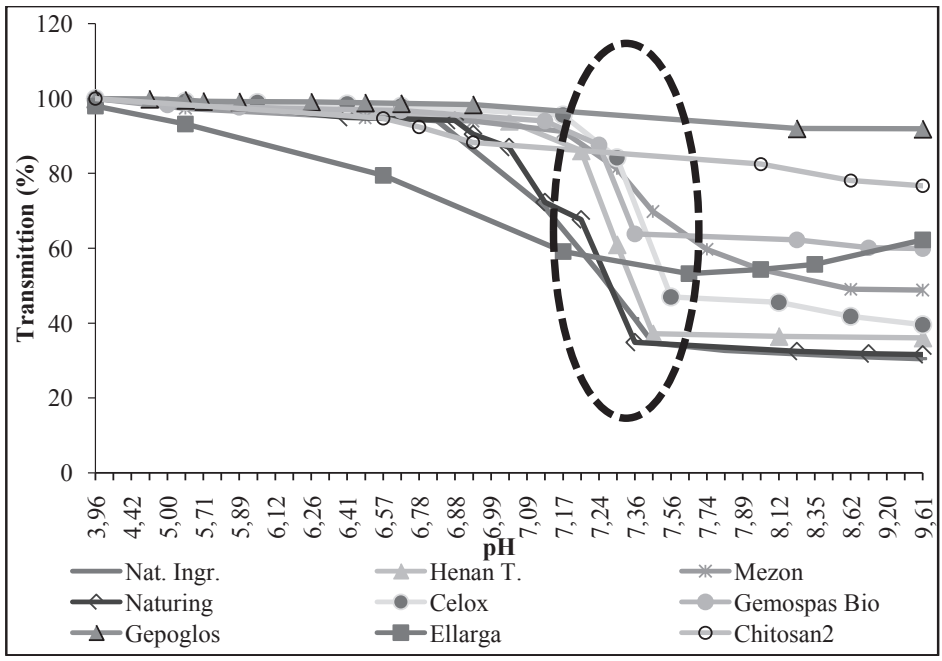

Figure 1. The dependence of the light transmission of 0.3 mg/ml solutions of samples on the pH. There is a conformation transition in the area presented in the oval

Based on the data, we concluded that there is no pronounced pattern between the molecular mass distribution parameters, in particular, the PI and the ability of chitosan to initiate haemostasis. If we consider the effect of the MWv and DD on the CT, it is obvious that for a number of samples – Naturing, Henan Tianfu and Nat. Ingr. with a relatively constant DD – the CT decreased as the MWv increased. At the same time, taking into account the standard deviation, the light transmission decreased at pH 7.4. In the case of high-molecular chitosan Mezon, it can be assumed that a large number of acetyl residues in the molecule (i.e. a low DD), alongside a large MW, sterically prevent it from 'folding' into a 'globule'. *In vitro*, the Chitosan2 sample showed no haemostatic activity, despite having parameters close to the active chitosan samples.

The LHA samples also demonstrated the dependence of the haemostatic activity on the conformational state of the macromolecules. A low ability to initiate haemostasis was observed in those samples for which there was no abrupt change in the light transmission of the solution (Gepoglos) in the physiological pH range; this finding could indicate that a significant part of the macromolecules did not transition from the 'rigid rod' state to the 'globule' state. For inefficient samples, the light transmission value at pH 7.4 was > 85%. For effective samples, this indicator was 38.1%-72.4%. When examining the conformational transitions of LHA (Figure 1), the curves were not completely similar to the curves of chitosan samples, a discrepancy that could be due to modifications of the raw materials used in the production of LHA (the use of chitosan salts with various substituents, crosslinking agents, etc.) and auxiliary substances in the compositions of commercial samples of haemostatic agents (antibacterial components, preservatives, etc.). This conclusion was made on the basis of additional experiments. Specifically, light transmission was measured for chitosan solutions Nat. Ingr. with concentrations

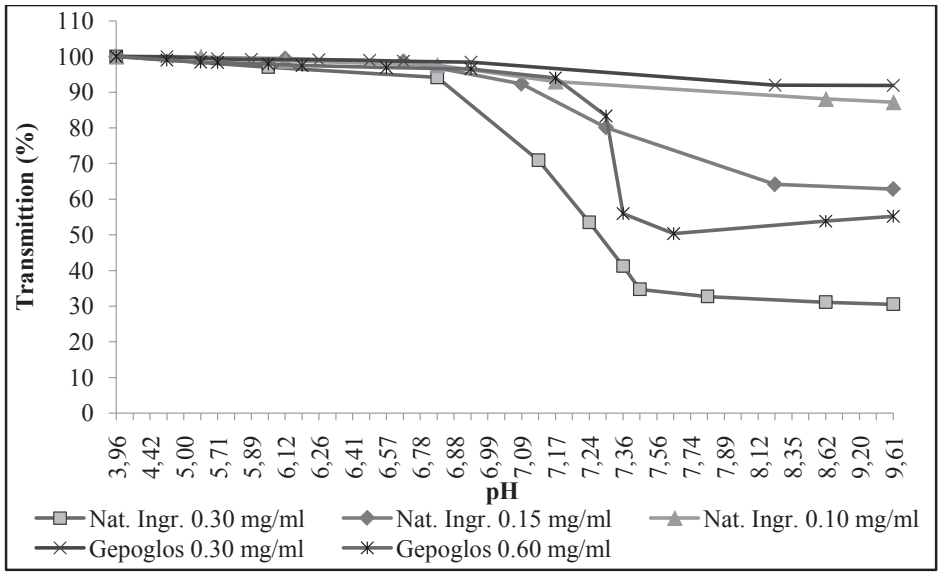

Figure 2. The dependence of the light transmission of samples solutions with different concentration on the pH

of 0.30, 0.15 and 0.10 mg/ml and for the Gepoglos sample with concentrations of 0.30 and 0.60 mg/ml, which showed low haemostatic activity during *in vitro* experiments (Figure 2).

With a decrease in the concentration of Nat. Ingr., the curve became smoother, and there was no conformational transition. For the LHA Gepoglos, when the concentration increased to 0.60 mg/ml, there was an abrupt change in light transmission. The low efficiency of this product *in vitro* could be due to insufficient chitosan content. The data obtained are in full agreement with the results of our previous experiments on the haemostatic activity of LHA *in vivo* and *in vitro* [23]. Taken together, our findings confirm the possibility of using this research method to assess the haemostatic activity of chitosan and LHA samples based on it without conducting a biological *in vivo* experiment.

The possibility of using ^{1}H NMR spectroscopy to assess the quality of chitosan raw materials and LHA based on them was studied. Violation of the storage conditions of the Chitosan2 sample altered the structure (Figure 3). Considering region **A**, the spectrum of Chitosan2 contains additional unidentified signals compared with the spectrum of Nat. Ingr. These changes in the structure of the sample during storage explain its low activity *in vitro*. When comparing the spectrum of Gepoglos with other LHA samples, there is a low intensity of proton signals is observed in the **B** region, which confirms the assumption of a lower concentration of chitosan in the sample.

Thus, the analysis of ^{1}H NMR spectra can be used to assess the quality and to predict the activity of haemostatic agents based on chitosan. However, based on the data obtained, we could not judge what concentration of chitosan in LHA would be sufficient to initiate haemostasis.

4. Conclusions

As a result of the study, we determined the main and additional physicochemical properties of chitosan samples and products based on it. The controlled parameters that

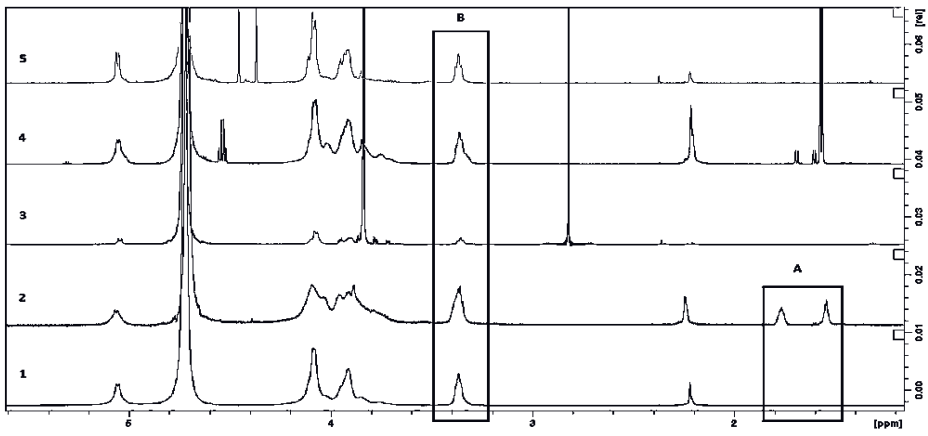

Figure 3. Comparison of ^{1}H nuclear magnetic resonance spectra of chitosan and local haemostatic samples. Identities: 1 – Nat. Ingr.; 2 – Chitosan2; 3 – Gepoglos; 4 – Celox; 5 – Ellarga. **A** and **B** indicate spectrum areas of 1.5-1.9 ppm and 3.2-3.5 ppm, respectively, for comparison

are most often associated with the haemostatic activity of chitosan are molecular weight and DD. Obviously, the control of these parameters does not allow us to fully assess the presence/absence of haemostatic properties. In addition, we selected the parameters of the molecular mass distribution and the conformational state of macromolecules at the physiological pH value. The results were supplemented with ^{1}H NMR spectra. The obtained data showed that the ability to initiate haemostasis depends on the conformational transition of chitosan macromolecules, which, in turn, depends on the molecular weight, the DD and the concentration of chitosan, although the dependence cannot be considered linear. The greatest efficiency in the *in vitro* experiment was for samples in which, at pH values of the solution close to physiological pH, a significant part of the molecules passed from the 'rigid rod' state to the 'globule' state, which was accompanied by a sudden change in the light transmission of the solution. Thus, the analysis of ^{1}H NMR spectra, conformational transition and the presence of available active centres in the molecule can be considered indicators related to the haemostatic activity of chitosan. In this regard, we propose expanding the list of chitosan indicators that can be controlled to assess the quality of raw materials, including the determination of the conformational state in which the chitosan macromolecule is located in a solution with a concentration of 0.30 mg/ml at a physiological pH.

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