RESEARCH ON STABILITY OF BIOPOLYMERS BY MEANS OF LIGHT SCATTERING TECHNIQUES

KALINA Michal*, MICHALICOVÁ Petra+, SEDLAČEK Petr, KRATOCHVÍL Zdeněk, SMILEK Jiří,
KLUČÁKOVÁ Martina, PEKAŘ Miloslav

Brno University of Technology, Faculty of Chemistry, Materials Research Centre,
Purkyňova 118/464, 612 00, Brno.

**email: kalina-m@fch.vutbr.cz*
+*email: xcmichalicovap@fch.vutbr.cz*

Abstract

Nature and natural systems represent from the beginning of human being magnificent source of both basic raw materials as well as of an inspiration for majority of human driven processes and applications. Biopolymers, representing substances with indispensable role in natural ecosystems, can be listed as the proper example. One of the most important parameter of these substances is their stability, which nowadays still lacks for deeper research. The main aim of this work was to shed a new light on the area of natural substances ongoing aggregation and degradation processes especially in longer time scale. Even slight deviation of these natural substances, which are often used in industrial processes, as final products for distribution to customers or just in long-term research, can cause serious problems (e.g. different yield of product, bad purity or unfavorable property of obtained product, wrong directing of research…). All these effects are of course highly undesirable. This work is mainly focused on the optimization and deeper research on biopolymers stability using light scattering techniques. For purposes of experimental work the system of SEC-MALS was applied as crucial method providing the information about changes in molecular weight and particle size. Results were also compared and correlated with subsequent determination of zeta potential using electrophoretic light scattering. The obtained experimental results showed good correlation between individual light scattering techniques and proved suitability of both presented light scattering methods for the purposes of biopolymers stability research.

Keywords: biopolymers, light scattering, molecular weight, stability, zeta potential

1. INTRODUCTION

Nature provides broad variety of remarkable substances. Biopolymers must be listed as probably the most important example. These natural macromolecules are formed from large number of repetitive monomeric units linked together by covalent bonds. This backbone structure of covalently bound substances is subsequently associated mainly by non-covalent interactions to form supramolecular structures. The way of formation of these three dimensional structures is probably the most important phenomenon significantly influencing their proper functions in nature [1–3]. Another important feature of biopolymers comes from the prefix bio meaning that they produced by living organisms and thus are biodegradable [4].

Structure and conformation of biopolymers has been object of investigation since they were firstly discovered [5]. X-ray diffraction is used as the most accurate method for deeper research on the structure of biopolymers in a crystal state. On the other side, many methods have been successfully described and adopted for description of structure and properties of polymers and macromolecules in solution. Typically nuclear magnetic resonance, infrared spectroscopy or liquid chromatography is often used for these purposes [6]. The field of biopolymer chemistry was also for long decades connected with almost exclusive application of methods of light scattering, dilute solution viscosimetry, size exclusion chromatography or flow field fractionation. The most fundamental parameters obtained by these methods are molecular weight (and/or molecular weight distribution) and particle size (and/or particle size distribution) [7]. Typically in the case of proteins and peptides, these parameters are supplemented by the values of zeta potential and electrophoretic mobility. The values of zeta potential are also valuable as a basic characterization parameter for natural substances such as polysaccharides, oligonucleotides and antibodies. The common feature of biological macromolecules is their precisely defined value of molecular weight and extremely narrow particle size distribution, allowing almost perfect monodisperse solutions. These systems are perfect for characterization using dynamic light scattering (DLS). DLS is often described as an essential method mainly for the study on the size and conformation of proteins, nucleic acids, polysaccharides, lipids and their supramolecular assemblies [8,9]. Electrophoretic light scattering (ELS) can support the results obtained from particle size distribution analysis with the information about the electrostatic stability of studied system, which is often desirable.

Moreover in the case of samples with higher polydispersity, the classical light scattering methods are often coupled with initial chromatographic separation mainly using the size exclusion chromatography. Such a size separated material subsequently undergo the characterization using static light scattering (SLS) or even in better case with multiangle light scattering (MALS), dynamic light scattering, differential viscosimetric, differential refractive index or spectrometric (UV-VIS, fluorescence…) detection, which highly increase the precision of the analysis and also can provide additional information about analyzed material regarding the molecular weight, particle size and shape, conformation etc. [7].

Generally the area of basic biopolymer characterization with different experimental methods is well described and adopted in literature [1,6,10]. Moreover the knowledge on the effect of different parameters (e.g. temperature, ionic strength, pH) and modifications on protein and polysaccharides stability is sufficient [9–13]. Contrary to this, almost nothing can be found in the area of biopolymer time stability, kinetic of ongoing aggregation/degradation processes and aging of the biopolymers itself. Most of the published work from this area is limited only on the study of hyaluronic acid [14]. The main aim of this publication is to enhance our previous pilot research [15] focused on the optimization of the application of simple, non-destructive, relatively fast and low cost methods of light scattering for purposes of the study of aggregation/degradation processes of biopolymers and biocolloids. The present work is aimed mainly on the area of utilization of light scattering techniques for purposes of long-scale research of the behavior of biopolymers and also on finding of kinetic models, which would help to describe the behavior of these substances in time.

2. MATERIALS

All the biomaterials described in this paper can be divided into two groups – polysaccharide and proteins. The group of polysaccharides was represented by hyaluronic acid, chitosan and sodium alginate. Bovine serum albumin (BSA) was selected as an example of proteins. Moreover, these samples were supplemented by carboxymethyl cellulose (CMC) – a representative of semisynthetic substance with broad industrial applications. All studied materials used in this work were purchased from Sigma-Aldrich in p.a. purity grade.

The aqueous samples for consequent analysis using SEC-MALS and ELS were prepared by dissolving of the corresponding weights of solid materials in Millipore distilled water to reach their final concentration in the sample 2 $g \cdot dm^{-3}$. This value was selected according to our previous study [15]. After overnight stirring the samples were filtered using 0.45 μm syringe filters (nylon membrane).

3. METHODS

The experimental works described in this paper were focused on research on the aggregation/degradation processes of biopolymers by means of the innovative and simple approach using light scattering techniques. The work was mainly oriented on deeper investigation of these processes in longer time scale (up to 30 days). The main experimental method applied for these purposes in this paper was size exclusion chromatography (SEC) coupled with multiangle static light scattering (MALS) and differential refractive index (dRI) detection (SEC chromatographic system from Agilent Technologies, detectors from Wyatt Technology). The separation step was performed using size exclusion chromatographic column (PL aquagel–OH MIXED–H 8 μm). The mobile phase utilized in the system was 0.1 M $NaNO_3$, applied flow rate 0.6 $ml \cdot min^{-1}$ and injected volume of each analyzed biopolymer into the system was 100 μl. Subsequently after the separation step, the size separated fractions of analyzed biopolymers were transferred into the detectors (MALS, dRI). All these measurements were performed at 25 °C and were duplicated.

As the supplementary method to SEC-MALS in this work, electrophoretic light scattering (Zetasizer Nano ZS instrument) was also utilized. The measurements were performed in classical UV-VIS glass cuvettes with inserted zeta potential dip cell. Again all these measurements were run at 25 °C. Zeta potential analysis of each sample was made in 4 repetitions. The determined parameter – zeta potential – represents valuable tool for deeper investigation of colloidal stability of studied materials. According to the values of zeta potential the stability can be directly determined. If the value of zeta potential of sample lies between –30 and 30 mV, the sample is non-stable. Oppositely in the case of either highly negative or highly positive value of zeta potential the sample can be assumed to be stable.

Time development of both molecular weight and zeta potential for all biopolymers was subsequently analyzed using the first order kinetics. This model was presented in literature for description of hyaluronic acid time degradation [14,16]. In this paper we have generalized this approach on all studied biopolymers. Using this mathematical model more described in details in [16] the exponential dependence of both molecular weight (1) and zeta potential (2) on time can be stated as follows:

$$\ln M_{\mathrm{w}} = \ln M_{\mathrm{w}0} - k_{\mathrm{M}} t \tag{1}$$

$$\ln \zeta = \ln \zeta_0 - k_{\zeta} t \tag{2}$$

In these equations M_w and ζ represent the values of molecular weight and zeta potential of the sample at time t from its preparation, M_{w0} and ζ_0 describe the initial values of both parameters at the moment of sample preparation, k_M and k_ζ characterize the slope of the decay of molecular weight and zeta potential, respectively.

4. RESULTS AND DISCUSSION

The main aim of this work was to enhance our previous work [15] dealing with the optimization of application of light scattering techniques for purposes of basic characterization of ongoing aggregation/degradation processes of biopolymers and biocolloids. The biggest efforts were subjected mainly on deeper investigation of time stability of biopolymers and also finding the proper kinetic models describing this phenomenon. For these purposes two methods were selected – SEC-MALS-dRI and electrophoretic light scattering.

Firstly, by using the method of SEC-MALS all the samples were characterized to obtain their molecular weights. These experiments were repeated in longer time scale (up to 30 days), to get the information regarding the ongoing processes in the samples. The obtained results of this measurement can be found in Fig. 1A. All displayed time development of measured values of molecular weight for the samples of polysaccharides displays the similar shape with the maximal measured molecular weight in the case of initial fresh samples and subsequent decrease of the value of molecular weight in time. These samples obviously underwent degradation in time. The process of degradation of polysaccharides was the most significant in the case of fresh samples (at low observation time) and with increasing time started to decelerate. Oppositely, in the case of studied protein BSA the molecular weight was increasing with increasing time of observation indicating the aggregation of the molecules. Firstly at low observation time, the whole process of BSA aggregation was not so significant, but subsequently after 5 days from sample preparation it starts to be more significant. More details about the kinetic description of both aggregation and degradation processes in all studied samples can be found at the end of this section.

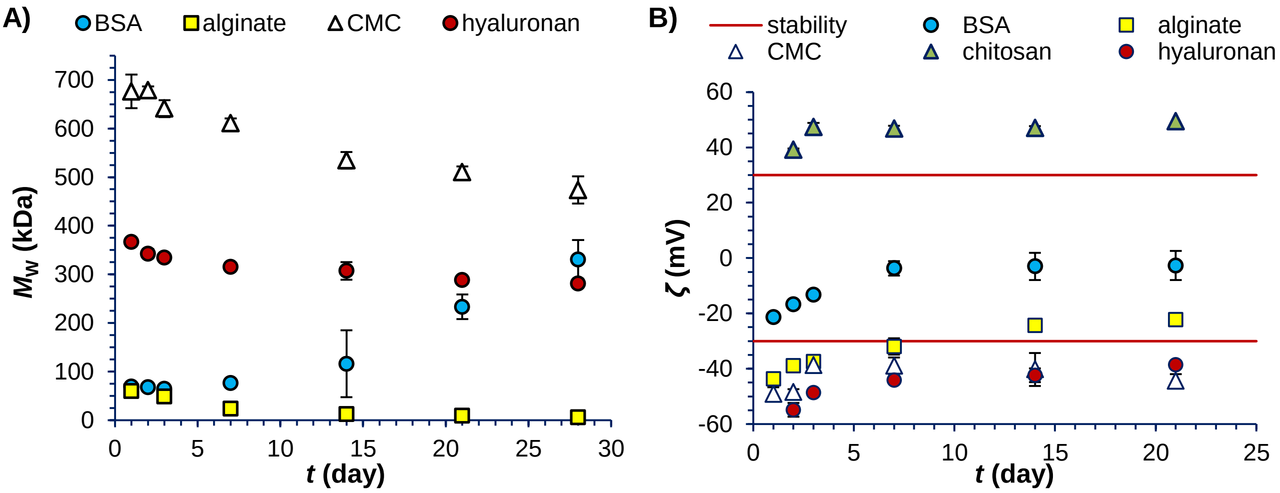

Fig. 1 Time development of A) molecular weights and B) zeta potential values for all studied biopolymers

The second method used for the research on the time stability of biopolymers was electrophoretic light scattering providing the values of zeta potential. This parameter can be used as a simple tool for direct investigation of the colloidal stability of measured sample. If its absolute value is higher than 30 mV the particles in the sample have sufficiently high charge to repel each other and such a sample is assumed to be stable. Data gained from development of zeta potentials of all studied biopolymer samples can be found in Fig. 1B. Taking into account the definition of zeta potential, from Fig. 1B, it is obvious that in the case of fresh initial biopolymer solutions, the only unstable sample is BSA. All the remaining samples are at the beginning of their observation in colloidal stability. Subsequently, the time development shown in this figure is again exposing the decrease of the absolute values of zeta potential for all samples. Generally, with increasing observation time the zeta potential values are more or less (depends on the sample) approaching value of zero zeta potential. It means that the charge of the colloidal particle, which is stabilization element is decreasing, causing the collapsing of the structure, which would be followed by consequent aggregation or even degradation. These findings are in good correlation with the data obtained from SEC-MALS analysis.

The shape of the dependences in Fig.1 exhibits slightly exponential decay. This led us to an idea to describe this phenomenon of degradation/aggregation processes with the first order kinetics. More details about the mathematic approach can be found in our previous paper [16] dealing with the degradation processes of hyaluronic acid. This mathematic model was in present paper generalized on all studied biopolymers. The obtained dependences of both M_w / M_{w0} and ζ / ζ_0 on time can be seen on Fig. 2A, B and Fig. 3 respectively.

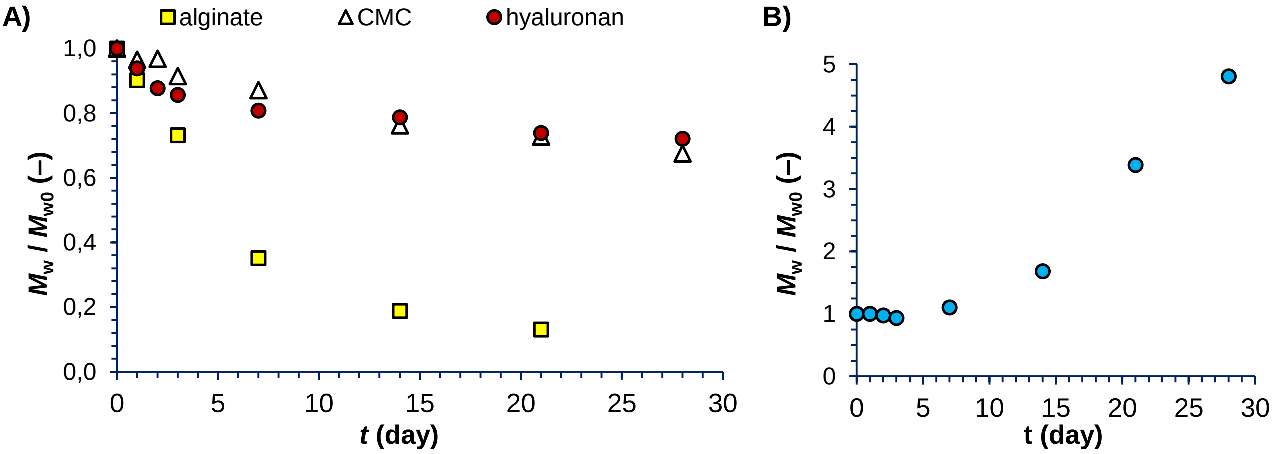

Fig. 2 Results after the application of the model of first order kinetic on the time development of A) molecular weights of polysaccharides, B) the same data for BSA

Fig. 2A shows similar behavior of all studied polysaccharides indicating the ongoing degradation processes. From the comparison of individual curves is obvious that the rate of degradation of sodium alginate is significantly higher than in the case of hyaluronic acid and CMC. Completely different behavior was obtained in the case of BSA, which underwent aggregation (Fig. 2B). Again similar behavior was observed after application of first order kinetic on the values of zeta potential (Fig. 3). In this case the biggest decrease of the ratio ζ / ζ_0 is responding to the biggest difference in zeta potential, which means the highest destabilization of the system. According to this condition the highest destabilization of the sample was observed for BSA, followed by sodium alginate, hyaluronic aids and CMC. In the case of chitosan there was observed almost constant value of zeta potential lying highly above the upper border of colloidal stability.

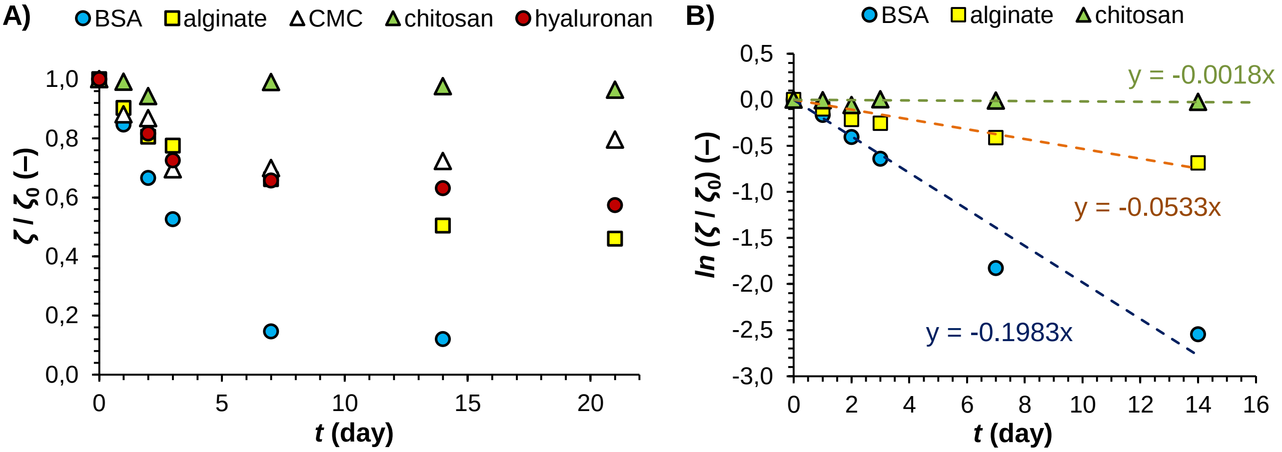

Fig. 3 Results zeta potential measurements after the application of first order kinetic A) the time development of zeta potential values, B) linearization of selected time developments of zeta potential values

Experimental data shown in Fig. 2 and Fig. 3 were linearized according to the mathematical model of first order kinetic (equation (1) and (2)) to obtain values of rate constant of destabilization k_M and k_ζ in dependence on the applied measuring method. Both the parameters were calculated from the slope of these dependences. The summarization of these parameters (k_M and k_ζ) for all studied samples is given in Table 1.

Table 1 Determined values of k_M and k_ζ for all studied biopolymers

Biopolymer	k_M	k_ζ
bovine serum albumin	–0.0521	0.1983
carboxymethyl cellulose	0.0153	0.0636
hyaluronic acid	0.0143	0.0417
chitosan	–	0.0018
sodium alginate	0.0910	0.0533

The calculated rate constant of degradation obtained by both experimental methods showed for individual samples different values. This was expected because of the fact that both of them were computed from different parameters (either molecular weight or zeta potential) describing the situation in the samples from a different view. First one (molecular weight) is characterizing the distribution of the mass, the second one (zeta potential) displays the situation in surface layer of colloidal particles and the tendency of the sample to aggregate. On the other side, similar behavior can be seen for both k_M and k_ζ. The biggest absolute value of rate constant was obtained from both methods for BSA. In the case of k_M for BSA the negative value was observed. This is connected with the fact, that this sample underwent aggregation contrary to the other samples of polysaccharides, which underwent degradation of their structures. Similar values of rate constant were determined for group of polysaccharides (hyaluronic acid, sodium alginate and CMC). Enormously low value of k_{ζ} was observed for chitosan indicating almost no changes in colloidal stability in time. Unfortunately there is no comparison with k_{M} for this sample.

5. CONCLUSION

The main aim of present paper was to study the time stability of biopolymers, which is very important parameter nowadays still lacking for deeper research. The presented combination of SEC-MALS analysis and zeta potential measurements proved to be suitable for this purpose. Results of both methods were in good correlation indicating aggregation of protein BSA in time contrary to behavior of remaining polysaccharides (carboxymethyl cellulose, hyaluronic acid, chitosan and sodium alginate), which underwent degradation. Calculated rate constants of destabilization can also provide valuable tool for prediction of behavior of biopolymers in longer time scale, which is often desirable both in industrial and scientific area.

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