Effect of Pressure Increase on Macromolecules’ Adsorption in Ion Exchange Chromatography

Anja Kristl, Miha Lukšič, Matevž Pompe, and Aleš Podgornik*

ABSTRACT: In this study a new method for evaluating the pressure effect on separations of oligonucleotides and proteins on an anion exchange column was developed. The pressure rise of up to 500 bar was attained by coupling restriction capillaries to the column outlet to minimize differences in pressure over the column. Using pH transient measurements it was demonstrated that no shift in ion exchange equilibria occurs due to a pressure increase. Results from isocratic and gradient separations of oligonucleotides (model compounds) were evaluated by stoichiometric displacement and linear gradient elution model, respectively. Both elution modes demonstrated that for smaller oligonucleotides the number of binding sites remained unchanged with pressure rise while an increase for large oligonucleotides was observed, indicating their alignment over the stationary phase. From the obtained model parameters and their pressure dependencies, a thermodynamic description was made and compared between the elution modes. A complementary pattern of a linear increase of partial molar volume change with a pressure rise was established. Furthermore, estimation of the pressure effect was performed for bovine serum albumin and thyroglobulin that required gradient separations. Again, a raise in binding site number was found with pressure increase. The partial molar volume changes of BSA and Tg at the maximal investigated pressure and minimal salt concentration were −31.6 and −34.4 cm³/mol, respectively, indicating a higher rigidity of Tg. The proposed approach provides an insight into the molecule deformation over a surface at high pressures under nondenaturing conditions. The information enables a more comprehensive UHPLC method development.

INTRODUCTION

The effect of high pressure on biomolecules has been of an interest since Bridgman (1914) described the coagulation effect of an egg white at elevated pressures and room temperature that is similar to the appearance of a hard-boiled egg. Many have later described the influence of pressure on activity of enzymes, viruses, antigens, antibodies, and also studied the denaturation under elevated pressures (pressures above 1 kbar). The use of high pressure also found its applications in industrial processes, such as treatment of milk, production of vaccines, and many other possibilities in food science as well as medical and pharmaceutical applications.

The application of high pressure also contributed to the enhancement of purification or elaboration of bioactive molecules, due to its action on the forces governing inter- and intramolecular interactions. From analytical aspect, higher pressures were utilized to distinguish between isomers and to obtain greater resolutions between biopolymers of different length. Given the significant benefits of a cleaner separation modification with a pressure increase (no added reagents) and the tendency to develop faster and more efficient separation methods, many researchers focused on the description of the pressure effect on molecules during the separation. From the thermodynamic description, pressure affects the volume of the system, which changes the distribution of the analyte between the mobile and the stationary phase and thus the retention factor when the temperature and the flow rate remain constant. These changes under high pressure can result from variations of analyte and solvent interactions, solvent structure in the mobile and the stationary phase, pH value, and ionization of both phases. Other studies have shown that analyte molecular volume changes were the major contribution to the observed pressure effect, initially demonstrated with isocratic separations of small molecules on reversed phase columns. Partial molar volume changes (ΔV) of molecules such as phenolic compounds, methylene homologues, and...
tricyclic antidepressants at temperatures around 25–30 °C and a pressure increase of up to 500 bar ranged from −2 to −20 cm³/mol. Even greater partial molar volume changes were observed when separating peptides and proteins. From evaluating the separation of a homologues series of phenylalanine and a tryptic digest at 200 bar pressure rise, a partial molar volume change of −49 to −90 cm³/mol was observed and increased with the peptide length. The same study also determined around 100 cm³/mol partial molar volume decrease when separating lysozyme at such pressures. Similar values were obtained for the separation of insulin analogues at a pressure increase of 100 or 200 bar. With the developments of UHPLC separations that offer advanced resolving power and speed, pressures of more than 1000 bar are generated. The partial molar volume changes in this high pressure region remained unexplored until recently, when researchers demonstrated the effect of pressure on decapetides and glucagon. ΔV values of around −15 and −33 cm³/mol were reported. At entry level pressure of UHPLC separations, an effect of lipophilic mobile phase additives on partial molar volume changes of small molecules and proteins like myoglobin, cytochrome C, ribonuclease, lysozyme, and insulin was investigated. A pressure increase from 358 to 620 bar resulted in a volume change of around −30 cm³/mol for proteins and was greater when a weaker lipophilic additive was present. Few years later the same group investigated the conformational changes using differential deuterium exchange when separating a set of proteins and determined partial molar volume decreases of up to 100 cm³/mol at 1100 bar pressure increase. A less prominent but still considerable pressure effect was demonstrated even in gradient separations of peptides and proteins on reversed phase columns.

In previous work we showed that a pressure variation shifts retention times of proteins, plasmid, and longer oligonucleotides even on an ion exchange column where gradient separations are conducted under nondenaturing conditions. An exact description of changes that result from a pressure rise are for such separations difficult, due to a constant change in the mobile phase composition. As ion exchange chromatography became one of the most used techniques for protein separation, and gradient separations became necessary to compensate for the steep adsorption isotherms, a model for linear gradient elution (LGE-IEX) prediction was established. We decided to test the application of the LGE model for the evaluation of the partial molar volume changes as well as changes in the number of binding sites of oligonucleotides and proteins at pressures up to 550 bar while keeping constant temperature and flow rate of the mobile phase. The oligonucleotides ΔV and the number of binding sites were compared to values obtained from description of isocratic separations by stoichiometric displacement model (SDM). Finally, the proposed method for evaluating gradient separations was applied to describe the adsorption of proteins thyroglobulin and bovine serum albumin under high pressures, of which isocratic separations were not possible.

**THEORY**

A complete development of equations is available in SI. To assist the result discussion the key steps are given in this section.

To describe the ion exchange equilibrium that governs the separation of molecules on an ion-exchange resin in isocratic and gradient elution mode, stoichiometric displacement model (SDM), and linear gradient elution model (LGE) are used. Both models enable the estimation of the number of binding sites (B) and the interaction parameter (A) through the experimentally governed retention factor (k) and/or the salt concentration (I) at elution:

**SDM:**

\[
 k = HK_A B I^{-B} - 1
\]

**LGE:**

\[
 K - K' \approx AI^{-B} = K_A B I^{-B}
\]

\[
 H = \frac{V_c - V_0}{V_0} = \frac{1 - \epsilon}{\epsilon} = \frac{V_0}{V_c}
\]

is the phase ratio and \( \epsilon = \frac{V_0}{V_c} \) is the bed void fraction (interstitial volume of the bed). The salt concentration on the stationary phase is approximated with

**MATERIALS AND METHODS**

**Chemicals.** Detailed description of the mobile phase and sample preparations are provided in the Supporting Information (SI). Briefly, the mobile phase/buffer A consisted of 20 mM Tris-HCl buffer with a pH value of 8.1. Mobile phase/buffer B was the solution A with 1 M NaCl and was set to the same pH value. Before injection, separate and mixed solutions of seven oligonucleotides with sequence \((GACT)_n\) (n ranges from 2 to 14) with concentrations from 2 to 7 μg/mL were prepared in buffer A. Bovine serum albumin (BSA) and thyroglobulin (Tg) in concentration of 2 and 4 mg/mL were prepared by dissolving the lyophilized powder in buffer A.

**Instrumentation and Measurements.** The instrumentation, restriction capillary positions, and dimensions as well as pressure drop determination are described in our previous work. Retention and model parameters described further on were calculated from each replicate measurement and an average value with a standard deviation was reported as a result.

**Separation of Biomolecules.** In this work, we mainly focused on the four longer oligonucleotides that previously showed a significant retention time shift with a pressure rise. For a reference purpose, the two shortest oligonucleotides were added in this study. Briefly, isocratic separations were performed at five different eluent compositions and column inlet pressures. Eluent compositions were set for each oligonucleotide individually to obtain retention factors between 2 and 25 for separations at the lowest pressure. The separations for the shortest two and the longest four oligonucleotides were repeated twice and three times, respectively.

Gradient separation methods consisted of a linear gradient of salt concentration, a wash (100% buffer B) and an equilibration step. The specific conditions for the separations of oligonucleotides and proteins are available in the SI. The separation results at the lowest gradient slope of BSA and Tg were omitted from evaluation, due to an excessive peak broadening, and less accurate peak position determination.

On account of the greatest sensitivity the UV detector was set to measure absorbance at 220 nm for separations of proteins and at 260 nm for separations of oligonucleotides. To ensure the elution strength of the mobile phase was reproducible between runs, the concentration of the salt and the mobile phase pH value were monitored by a conductivity detector and a pH meter, respectively.

The pH transition profiles were measured at the lowest and the highest pressure as described in the SI.

---

4528 https://dx.doi.org/10.1021/acs.analchem.9b05729
Anal. Chem. 2020, 92, 4527–4534
the total ion exchange capacity ($\Lambda$), $K_e$ denotes the equilibrium constant and $K'$ represents the distribution coefficient of the salt.

Retention behavior in chromatography is directly related to the equilibrium thermodynamics, more specifically, to the change in Gibbs free energy ($\Delta G$) of the system

$$\Delta G(T, p, I) = \Delta H(T, p, I) - T\Delta S(T, p, I) = -RT\ln K_e(T, p, I)$$

(3)

$\Delta H(T, p, I)$, $\Delta S(T, p, I)$ are changes in enthalpy and entropy, respectively, and $R$ is the universal gas constant. $\Delta G$, $\Delta H$, and $\Delta S$ are functions of temperature ($T$), pressure ($p$) and concentration of the salt ($I$). By deriving the total differential of the Gibbs free energy and obtaining its partial derivative with respect to the pressure at constant temperature and salt concentration, we obtain the partial molar volume change $\Delta V(T, p, I)$:

$$\left[\frac{d\Delta G(T, p, I)}{dp}\right]_{T, I} = -RT\left[\frac{dK_e(T, p, I)}{dp}\right]_{T, I} = \Delta V(T, p, I)$$

(4)

$\Delta V(T, p, I)$ is defined as the difference between partial molar volumes of solutes in the stationary ($V_e$) and the mobile ($V_m$) phase (SI eq S9). By expressing the $K_e$ as defined by eq 2, eq 4 becomes

$$\left[\frac{d\ln(\Lambda^e + K')}{dp}\right]_{T, I} + \left[\frac{d\ln(A^pB)}{dp}\right]_{T, I} = \frac{-\Delta V_e + \Delta V_{sl}}{RT}$$

(5)

where $\Delta V_e$ and $\Delta V_{sl}$ represent partial molar volumes changes of a biomolecule and salt, respectively. Given the recent research on $\Delta V$ of macromolecules compared to small molecules,28,30,31 the contribution of $\Delta V_{sl}$ to the total partial molar volume change is considered to be negligible.

A lot of research on partial molar volume changes of macromolecules has been conducted on reversed phase column using eq 4.23,31,42,45 Results of isocratic separations in some cases show a constant partial molar volume change with pressure,23,28,31,42,45 signifying that

$$\ln K(T, p, I) = a'(T, I)p + b'(T, I)$$

(6)

whereas others indicate a linear relationship between $\Delta V(T, p, I)$ and $p$,24,27 or in other terms

$$\ln K(T, p, I) = a'(T, I)p^2 + b'(T, I)p + c'(T, I)$$

(7)

Parameters $a'(T, I)$, $b'(T, I)$, and $c'(T, I)$ are temperature and ionic strength dependent values, attained by fitting the linear or quadratic curve to a pressure dependent $\ln K(T, p, I)$ function.

## RESULTS AND DISCUSSION

### The Effect of Pressure on Ion Exchange Capacity

It is well-known that variations in pressure cause changes in the mobile (density, viscosity, flow velocity, dielectric, and dissociation constants) and the stationary phase (size, shape, porosity of the packing material) as well as difference in frictional heating of both phases.46,47 In our previous research we have shown a stationary phase compression and greater solute dispersion with pressure.15 However, to evaluate conformational or partial molar volume changes of the selected biomolecules, the pressure effect on ion exchange equilibrium of the mobile and the stationary phase must be investigated. To minimize the complexity of the investigated system we chose Tris-HCl buffer, which is reported to have a minimum volume change in the reaction of protonation with a pressure change.7 This allowed us to independently investigate changes in the column ionic capacity with the pressure increase by measuring p$\Delta$ transition profiles. This nondestructive method was shown to be linearly proportional to the total ionic capacity.48 Figure 1 shows average profiles of two replicate measurements at 43 and 520 bar, when switching from buffer A (20 mM Tris, pH 8.1) to buffer B (20 mM Tris with 1 M NaCl, pH 8.1) (depicted with a dashed line). The mobile phase flow rate was set to 0.3 mL/min, resulting in column inlet pressures of 43 bar (black) and 520 bar (red). Each curve represents average values of two replicate measurements.

### Binding Sites and Volume Changes in Isocratic Separations of Oligonucleotides

To get an insight into the pressure effect on the separations of biomolecules we initially performed isocratic separations of oligonucleotides with a sequence of bases (GACT)$_n$ where $n$ ranged from 2 to 14. The $\ln(\kappa)-\ln(I)$ curves were plotted for all the oligonucleotides (SI Figure S1) and a linear curve was fitted to obtain the number of binding sites $B$ and the interaction parameter $A$ that defines the intercept. From eq 1 the intercept is also a function of the phase ratio $H$, which changes with pressure. Therefore, to evaluate whether the product $K_eA^B$ (denoted as $A$ in eq 2) shows any pressure dependency, the antilogarithm values of the intercept were divided by $H$ values at each pressure. The pressure dependencies of $A$ for all oligonucleotides are depicted in SI Figure S2. To elucidate a possible trend, a dashed line of a linear regression was plotted. With the exception of the longest oligonucleotides where a slight decrease can be noticed, scattering around a constant value can be concluded.

To further elaborate the pressure dependency of the ion exchange interaction, we also evaluated the numbers of binding sites of all the oligonucleotides. The average $B$ values of each oligonucleotide at the lowest pressure (45 bar) as oppose to the total charge are gathered in Table 1. The first and the
second oligonucleotide show a good agreement with the total number of charges, indicating that an oligonucleotide molecule has to be spread over the stationary phase surface during adsorption. On the other hand, partially coiled structures seem to occur for longer oligonucleotides as the deviation between the number of binding sites and total charge increases with the biopolymer’s length. Similar relation was shown by other researchers when performing separations of polyA and polyT larger than 11-mer \((n > 2)\) in isocratic and 20-mer \((n > 5)\) in gradient elution mode.

The standard deviations and the correlations of models of two (shortest two oligonucleotides) and three sets \((n \text{ from 8 to 14})\) of isocratic measurements and three sets of gradient separations for all oligonucleotides are given. The correlation coefficients \((R^2)\) of the linear fit ranged from 0.998 to 1.000 for isocratic and from 0.996 to 0.999 for gradient separations.

Figure 2a depicts \(B\) values at different column inlet pressures for oligonucleotides with 2, 4, 8, and 14 sets of \((\text{GACT})\) bases (other two are provided in SI Figure S3). Linear lines (dashed black) are fitted to elucidate the trends of \(B\) change with the pressure increase. Oligonucleotides with \(n\) equaling 2 and 4 show a constant value with some scattering of \(B\) values from 45 to 554 bar. Such trend is expected due to a complete spreading of the molecule over the surface already at low pressure. On the other hand, oligonucleotides with \(n \geq 8\) seem to have a more complex structure when adsorbed on the stationary phase, which can change when a higher pressure is applied, resulting in a binding number increase.

Presented \(B\) values were determined for changing \(A\) values. However, since these changes were small, especially taking into account standard deviations of measurement, we reviewed if the same trend of \(B\) would be obtained for constant \(A\) value. Therefore, the \(A\) value at 45 bar for each oligonucleotide was fixed and new regressions of ln\((k)\)-ln\((l)\) curves were obtained. The correlations of the new ln-ln relationships changed for less than 0.5% \((\text{determined as} \Delta R^2 = [(R_{\text{old}}^2 - R_{\text{new}}^2)/R_{\text{old}}^2] \times 100\%)\). The newly obtained values of \(B\) are depicted in blue on Figure 2a (also SI Figure S3) and fall in the interval of standard deviations of measurements. Generally, the slope of each function with new \(B\) values became slightly shallower and the intercept value a bit higher. The small deviation of values preserving the original trend allows the assumption that the strength of interactions \((A)\) can be considered as constant within tested experimental conditions and was used as such for further analysis.

With the determined \(B\) and \(A\) values and eq 2 the distribution coefficients \(K\) of oligonucleotides were determined for salt concentration \(I\) at which isocratic separations were performed. The value of \(K'\) was approximated as 0.78 and can

| \((\text{GACT})_n\) | tot. available charge (−) | isocratic sep. | gradient sep. |
|------------|-----------------|----------------|--------------|
| 2          | 6               | 6.57 ± 0.07    | 6.8 ± 0.3    |
| 4          | 12              | 12.7 ± 0.1     | 12.9 ± 0.5   |
| 8          | 24              | 21.8 ± 0.2     | 24.7 ± 0.5   |
| 10         | 30              | 24.6 ± 0.4     | 27.5 ± 0.4   |
| 12         | 36              | 28.4 ± 0.2     | 30.8 ± 0.6   |
| 14         | 42              | 32.6 ± 0.2     | 33.9 ± 0.7   |

Figure 2. Numbers of binding sites \((B)\) and the standard deviations of two (top plots) and three (remaining plots) replicate determinations at different column inlet pressures, obtained by evaluating the isocratic (a) and gradient (b) separation results of oligonucleotides with 2, 4, 8, and 14 \((\text{GACT})\) units by SDM and LGE model, respectively. Linear regressions of the original data (dashed black) and corrected data at fixed \(A\) value (a) dashed blue and both (b) plots are depicted to display the \(B\) increase.

Figure 3. (a) ln\((K)\) at different concentrations of salt \((\text{NaCl})\) and different column inlet pressures with an example of a fitted quadratic function to the data points. (b, c, d) Salt concentration dependence of ln\((a)\), ln\((b)\), and ln\((c)\), described by the logarithm of a power function with the corresponding equations (blue). All the data is calculated from isocratic separations of the \((\text{GACT})_{14}\) oligonucleotide.

be assumed as a constant for all the calculated data in this work.33–36,43 The calculated ln\((K)\) values vs pressure for the longest oligonucleotide are depicted in Figure 3a. For all salt

4530 https://dx.doi.org/10.1021/acs.analchem.9b05729 Anal. Chem. 2020, 92, 4527–4534
concentrations a quadratic pressure dependence of \( \ln(K) \) can be seen. The pressure dependence was further evaluated by fitting quadratic equation with parameters \( \ln(a) \), \( \ln(b) \) and \( \ln(c) \) at each concentration of salt. Likewise, plots of the obtained parameters against the concentration of salt (Figure 3b–d) gave functions of salt concentration that were independent of the pressure. As defined by eq 2, \( K \) is proportional to the power of salt concentration. Therefore, it seems reasonable to describe the concentration dependence of parameters \( a \), \( b \), and \( c \) also by a power function, such as

\[
f(I) = f_1 I^n + f_0
\]  

(8)

The power function approximation is shown with blue curves and equations with correlation coefficients on Figure 3b–d. By inserting eq 8 into the quadratic function of pressure dependence of \( \ln(K) \), the oligonucleotide distribution coefficient is derived

\[
\ln(K) = \ln(1 + a_1 I^n + a_0) + \ln(1 + b_1 I^h + b_0) + \ln(c_1 I^c + c_0)
\]  

(9)

The last parameter in eq 9 is pressure independent and has values \( c_0 \), \( c_1 \), and \( c_2 \) that resemble the \( A \), \( B \), and \( K' \) term in eq 2. In fact, \( c_2 \) represents the fixed \( A \) value, \( c_1 \) estimates the \( B \) value at 45 bar, and \( c_0 \) is the approximated value of \( K' \) (0.78). This seems to hold for all the oligonucleotides as seen in Figures 3d and SI Figures S4d, S5d, and S6d.

To determine the partial molar volume changes of oligonucleotides a partial derivative of eq 9 with respect to the pressure at constant temperature and salt concentration was obtained

\[
\left[ \frac{d \ln K(T, p, I)}{dp} \right]_{T,} = 2 \ln(1 + a_2 f^n + a_0) + \ln(1 + b_2 f^h + b_0) = -\frac{\Delta V(T, p, I)}{RT}
\]  

(10)

The studies that utilized trifluoroacetic acid (TFA) and ACN as the mobile phase constituents also report a decrease of the partial molar volume change of a protein with pressure increase,24,27 which is opposite to our findings. On the other hand, when performing isocratic separations with a mix of ACN and phosphoric acid, a constant to minor increase in the change of partial molar volume of the same solutes was reported.24 Studies of fluorescence showed that proteins’ inner hydrophobic amino acids were not exposed when using phosphoric acid as the modifier as oppose to TFA. TFA is also reported to be more hydrophobic and can thus in combination with ACN promote greater denaturation. In our experiments, the hydrophobic domains of macromolecules do not contribute to the retention of oligonucleotides. As indicated by Figure 2 the increase in the number of electrostatic interactions is the cause of greater retention under elevated pressures. If we assume that the charge is evenly distributed on the surface of an oligonucleotide, the results on Figure 4 indicate a greater longitudinal spreading of the structure upon a perpendicular compression to the stationary phase with higher pressure. The results of the pressure effect in ion exchange separations thus resemble the effect on separations in reversed phase chromatography under minor to nondenaturing conditions (the use of phosphoric acid). This allows us to study adsorption processes of a biomolecule’s native form.

**Volume Changes in Gradient Separations.** With the development of the LGE-IEX model33–36 that enables the determination of \( K = f(I) \), the study of partial molar volume changes of solutes during separation becomes possible. Gradient separations of oligonucleotides were performed with the same buffer A (20 mM Tris buffer, pH 8.1) and B (buffer A with 1 M NaCl, pH 8.1); however, the v/v % of buffer B increased linearly with time. The \( \ln(GH) \)–\( I \) plots obtained for each oligonucleotide are shown in SI Figure S10. As on plots of isocratic separations (SI Figure S1), a similar increase in order among lines at different pressures of longer oligonucleotides is observed. The B and A values were calculated from the slope and the intercept, respectively (eq 6). In Table 1 the number of binding sites at the lowest pressure of gradient separations are reported. Similar to the results of isocratic separations, the value of B equals the total available charge for shorter and is lesser for longer oligonucleotides.
However, it seems that the gradient separations slightly overestimate the number of binding sites. The discrepancy starts at the B of the oligonucleotide with 10 (GACT) units. As concluded from isocratic separations, A values at different pressures are equal within the experimental error. Therefore, we kept A constant at the value attained at 47 bar when evaluating B at different pressures. The correlation coefficients of LGE model for oligonucleotides changed for less than 0.5%. Figure 2b depicts the pressure dependencies of the obtained B values for the oligonucleotides with 8 and 14 repetitive units (remaining in SI Figure S11). The resemblance to the plots of isocratic separations (Figure 2a and SI Figure S3) is indisputable. The pressure independence is seen on the plots of the shortest two oligonucleotides (SI Figure S11), whereas an increase of B with pressure (Figure 2 and SI Figure S3, blue line) is observed for oligonucleotides that showed a significant pressure effect on the retention. The only significant deviation is evident from higher B values at all pressures of longest oligonucleotides as a result of a slight overestimation with gradient separations as seen in Table 1. This further confirms that higher pressure in ion exchange separations increases the number of binding sites of macromolecules.

Similar to the analysis of isocratic separation results, plots of ln(K) at different column inlet pressures were obtained also for gradient separation. For better comparison, the ln(K) values at investigated pressures were calculated for the same concentrations of salt. SI Figure S15 depicts the identical set of results as Figure 3, but for gradient separations of oligonucleotide (GACT)_{14}. All the data retain the same shape and trend with only minor differences in the coefficient value. The similarity between the results of shorter biopolymers regardless of the elution mode is also evident from plots in SI Figures S12−S14.

The partial molar volume changes of oligonucleotides for gradient separations at different column inlet pressures were calculated from eq 10. The trends of the partial molar volume change of (GACT)_{14} between gradient (Figure 4b) and isocratic separations (Figure 4a) are the same. The absolute value of ΔV increases with a decrease of the displacer and an increase of the column inlet pressure. However, the estimated change is slightly greater for isocratic separations. For example, ΔV at 47 bar and I = 0.50 M equals −19.9 cm^3/mol and −13.9 cm^3/mol for isocratic and gradient separations, respectively. At 554 bar and the same salt concentration the agreement between results of separation modes is much better, with values −36.4 and −32.6 cm^3/mol. This can be explained by the lesser sensitivity of gradient separations to pressure change and thus accuracy at lower pressures, indicating a bigger pressure increase is necessary to get an accurate estimation of ΔV. A better agreement between ΔV vs p and ΔV vs I is evident on plots of the remaining oligonucleotides in SI Figures S8 and S9. Due to a less pronounced pressure effect on the separations of (GACT)_{8} (SI Figure S7), the lesser accuracy of gradient separations predicts a slightly greater ΔV decrease.

Overall the LGE model and SDM deliver comparable results, allowing interpretation of the pressure effect in gradient separations of proteins with steeper adsorption isotherm. Bovine serum albumin (BSA) and Thyroglobulin (Tg) were separated with linear gradients of salt concentration, produced by the same buffers. From ln(GH)-ln(I) plots (SI Figure S16) A and B parameters were determined at different column inlet pressures. The number of binding sites for BSA and Tg at the lowest pressure, obtained from three sets of replicate measurements equaled 13.4 (±0.9) and 19 (±2), respectively.

The estimated negative charge of BSA is in agreement with the titration curves at similar salt concentrations. To determine the function of K(p, I) we adopted the same protocol as for evaluating the retention data of oligonucleotides. By fixing the parameter A to the value obtained at the lowest pressure and recalculating B, a pressure dependency of B was obtained (Figure 5). Because there is a lesser pressure effect on the separation of BSA,^{15} the first pressure increase does not yet result in the rise of B. A linear trend is shown only at pressures that significantly affect the separation, which meant regression of four points for BSA and all five for Tg data. Also depicted in Figure 5 are the pressure dependencies of ln(K) at different salt concentrations for both proteins. The data points follow a quadratic equation, similar to the one that describes ln(K) of oligonucleotides. The parameters ln(a), ln(b), and ln(c) are obtained by fitting a power function (eq 8) to the distribution of parameters at different salt concentration (SI Figure S17). The trends of the parameters that describe the function of Tg show the same trend as the larger three oligonucleotides, whereas the ln(b) of BSA shows a minor increase with pressure. This might be due to a stable structure at low pressures and a steeper change at pressures above 200 bar. For both proteins, the K^′ , B at approximately 50 bar and A (fixed) define the coefficients of ln(c). After attaining the full descriptive functions of lnK(p, I) for both proteins, the changes of partial molar volumes at the lowest concentration of salt were calculated (Table 2). Also added are the ΔV values of the four oligonucleotides at the corresponding salt concentrations. As expected, the absolute ΔV values at the highest pressure (ΔV_{max}) increase with the length of the oligonucleotide or size of the protein. Given the previous research on the partial molar volume change in reversed phase chromatography, greater ΔV_{max} values for proteins were expected (around 100 cm^3/mol or more).^{26,28,30} However, those separations used ACN and TFA or formic acid as the mobile phase modifiers that promote protein denaturation (combination of organic solvent with acidic and/or ion pairing conditions) and therefore
increased the conformational changes under higher pressure. Expectedly, protein structures proved to be much more stable under our separation conditions as we used nondenaturing buffers.

Greater vicinity of charged groups to the stationary phase upon compression increases the $B$ values of both proteins. Oligonucleotides do not have as restricted secondary structures as of proteins, even though the molecules are smaller (the highest $M = 17.239$ kDa).

When comparing the sensitivity of the volume change to the pressure effect ($\Delta V(p)/\Delta p$) among macromolecules, oligonucleotides seem to have the same conformation change with a rise of pressure. On the other hand, after reaching the mentioned compression threshold pressure ($p > 200$ bar), BSA shows a greater pressure sensitivity than Tg, resulting in a very similar $\Delta V_{\text{max}}$. The magnitude of the volume change can be compared by assessing the size difference. Before the adsorption, both molecules are fully hydrated in the mobile phase, having Stokes radius of 3.6 and 8.9 nm for BSA and Tg, respectively. Considering it to be a rough approximation of the size difference (Tg being larger for approximately 2.5-times), one would still expect a greater $\Delta V_{\text{max}}$ distinction between the proteins. Therefore, the compression of BSA above 200 bar represents a much greater structure compromise as for Tg.

### CONCLUSIONS

To investigate the pressure effect on biomolecules’ adsorption during ion exchange separations, isocratic and linear salt concentration gradient runs of oligonucleotides and proteins under different pressures were performed. Similar results between the modes demonstrate that both can be used to evaluate the adsorption phenomena under elevated pressure. For large oligonucleotides and proteins, an increase of binding sites with a pressure rise was evident while all molecules expectedly exhibited a decrease in partial molar volume with a pressure increase. Proposed analysis therefore facilitates transfer of chromatographic methods from HPLC to UPLC but also provides an insight into interactions of macro-molecules with a stationary phase at elevated pressures. This widens the use of ion exchange chromatography to study the adsorption processes under nondenaturing conditions, regardless of the elution mode.

### SUPPORTING INFORMATION

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b05729.

Detailed experimental protocols, development of equations, and additional figures (PDF)

### AUTHOR INFORMATION

**Corresponding Author**

Aleš Podgornik — Faculty for Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia; COBIK, S270 Ajdovščina, Slovenia; orcid.org/0000-0001-7308-0222; Phone: +386 1 479 8584; Email: ales.podgornik@fkkt.uni-lj.si

**Authors**

Anja Kristl — Faculty for Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia

Miha Lukšič — Faculty for Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia; orcid.org/0000-0001-7190-4013

Matevž Pompe — Faculty for Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.9b05729

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The financial support is gratefully acknowledged from the Slovenian Research Agency (ARRS) through programme P1-0153, P1-0201, and project J2-9440. The study was also supported by European Regional Development Fund and Slovenian Ministry of Education, Science and Sport (project BioPharm.Si).

**REFERENCES**

(1) Bridgman, P. W. J. Biol. Chem. 1914, 19, 511–512.

(2) Gross, M.; Jaenicke, R. Eur. J. Biochem. 1994, 221 (2), 617–630.

(3) Frye, K. J.; Royer, C. A. Protein Sci. 1998, 7 (10), 2217–2222.

(4) Heremans, K.; Smeller, L. Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1998, 1386 (2), 353–370.

(5) Balny, C.; Masson, P.; Heremans, K. Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 2002, 1595 (1), 3–10.
