Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 274–279
Bioorganic & Medicinal Chemistry Letters

# Specific volume and compressibility of human serum albumin–polyanion complexes

Tibor Hianik,[a,*] Slavomíra Poniková,[a] Jaroslava Bágel'ová[b] and Marián Antalík[b]

[a]*Faculty of Mathematics, Physics and Computer Sciences, Comenius University, 842 48 Bratislava, Slovakia*
[b]*Department of Biophysics, Institute of Experimental Physics, Slovak Academy of Sciences, 043 53 Košice, Slovakia*

Received 22 February 2005; revised 3 October 2005; accepted 5 October 2005
Available online 21 October 2005

**Abstract**—The ultrasound velocimetry, densitometry, and differential scanning calorimetry have been used to study the formation of the complexes between human serum albumin (HSA) and polyanions heparin (HEP) and/or dextran sulfate (DS). The values of the ultrasound velocity and specific volume allowed us to determine the specific adiabatic compressibility, $\varphi_K/\beta_0$, which reflects the degree of volume compressibility of the complexes. We showed that in the presence of HEP and DS the adiabatic compressibility of HSA decreases with increasing concentration of polyanions. HEP more strongly interacts with HSA than DS. pH of electrolyte in the range 4.7–8.5 weakly affects the adiabatic compressibility. Changes of compressibility of HSA can be caused by increase of the hydration due to the formation of the HSA–polyanion complexes and due to partial unfolding of HSA. The HSA–polyanion interaction resulted in decrease of phase transition temperature of the protein. This evidences about protein destabilization in the presence of polyanions.


The study of the mechanisms of interaction of polyanions with proteins has great significance for molecular biology and medicine. For example, the protein–DNA complexes are responsible for transcription of DNA, gene expression as well as for formation of specific structures of chromosomes and viruses.[1,2] On the other hand, several polyanions, such as, e.g., dextran sulfate (DS), heparin (HEP), and pentosan polysulfate reveal certain therapeutic action against development of transmissible spongiform encephalopaties (TSE). It has been shown that these polyanions bind to the prion proteins and prevent their accumulation in animals and cells.[3] Recent study revealed that DS blocked the synthesis of protease-resistant prion (PrPres).[4] Polyanions could also inhibit the entry of HIV-1 into the cells.[5] They play a substantial role in protein–protein interactions, protein folding and stabilization[6] as well as in cell–cell communication[7]. Polyanions are important in the functioning of human fluid-phase complement regulators[8] and may be used also in drug delivery.[9] Protein–polyanion interactions are in most cases not specific.[6] On the other hand, well over a hundred so-called heparin-binding proteins have been identified.[10] However, ability of these proteins to bind other polyanions (DNA, actin, tubulin, etc.) suggests that these interactions, although of a high affinity, are not so specific. In certain cases, however, the specific binding sites exist.[6] For example, antithrombin III has specific binding site to heparin.[10] High affinity of polyanions to the proteins is attributed to the presence of positively charged binding regions[11] at the protein surface and depends on electrolyte pH and ionic strength.[12] The interaction between strong polyanion—heparin and bovine serum albumin (BSA) has been studied by dynamic light scattering, capillary electrophoresis, and turbidimetry.[12,13] The interaction of BSA with heparin was observed even at pH well above the isoelectric point of BSA ($I_e \sim 4.9$).[14] This evidences about existence of positively charged domains at the protein surface, that is at physiological pH in general negatively charged.[14] Existence of positively charged domains has been confirmed also by computer visualization of the protein surface.[13] According to Hattori et al.[13] heparin is bound to BSA at heparin binding site. Strong interaction of heparin to the other protein—ferricytochrome *c* has been observed by differential scanning calorimetry. At low ionic strength heparin induced an important shift of the transition temperature $T_m$ from 84.1 to 59.8 °C.

*Keywords*: Human serum albumin; Dextran sulfate; Heparin; Polyanion–protein complexes; Compressibility; Specific volume; Differential scanning calorimetry.
* Corresponding author. Tel.: +421 2 60295683; fax: +421 2 65426774; e-mail: hianik@fmph.uniba.sk

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.10.010

This was accompanied by large cooperativity of thermal denaturation of heparin–cytochrome *c* complex, that evidences also on strong interactions between protein molecules.[15] The physical mechanisms how polyanions interacts with proteins are, however, not fully understood. In our recent work, we showed high sensitivity of the ultrasound velocimetry to study the compressibility of proteins and their changes caused by oxidation processes.[16] This method was also very sensitive to study the hybridization of DNA at the liposome surface.[17] In this work, we applied the method of measurement of ultrasound velocity and density to study the compressibility of globular protein—human serum albumin (HSA), polyanions DS and HEP, as well as their complexes. We also used high sensitive differential scanning calorimetry to study the influence of DS and HEP on thermal denaturation of HSA. HEP is mucopolysaccharide, that has been found in extracellular space of certain tissues. The polymeric chain of HEP is composed of repeating disaccharide unit of D-glucosamine and uronic acid linked by $1 \rightarrow 4$ interglycosidic bond. This is a negatively charged polymer with an average charge of ≈2.3 per individual saccharide residue (Fig. 1a). DS is also a negatively charged polysaccharide composed of anhydroglucose. At low ionic strength it is fully extended due to repulsion of negatively charged groups (Fig. 1b).

Human serum albumin (molecular weight 68.5 kDa), heparin (molecular weight 14 kDa), and dextran sulfate (molecular weight 500 kDa) have been purchased from Sigma and used as received. The properties of these molecules were studied in a 10 mM NaCl + 10 mM Tris–HCl, pH 7.6, or in 10 mM NaCl of various pH (4.7; 7.0 and 8.5). pH was adjusted by NaOH or HCl. All solutions were prepared with Milli-Q water (Millipore, El Paso, USA).

The measurement of the velocity of ultrasound allowed us to evaluate the elastic properties of aqueous media. This is based on a simple relationship: $\beta = 1/(\rho u^2)$, where $\beta$ is the coefficient of adiabatic compressibility and $\rho$ is the density. The so-called concentration increment of ultrasound velocity $[u]$ is a convenient parameter that characterizes changes in compressibility of polymer suspension and can be easily determined experimentally.[17] This value is defined by the equation

$$[u] = (u - u_0)/u_0 c, \tag{1}$$

where $u$ and $u_0$ are the sound velocities of protein suspension and buffer, respectively, $c$ is the molar concentration of colloid particles, e.g., proteins. The determination of the $[u]$ value is based on the measurement of changes of resonance frequency of the acoustic wave propagated in a small cavity (0.7 ml) of the cell containing piezoelectric transducers. It has been shown that changes of resonance frequency are proportional to changes of sound velocity.[18] In the experiments, we used a two resonance cell configuration.[19] One cell was filled with sample—HSA, HEP or DS dissolved in buffer, while the second only with buffer. In the study of the mechanical properties of the complexes, the measuring cell was filled with HSA (concentration 5 mg/ml), while HEP or DS was added both to the measuring and to the reference cells. The ultrasound velocity was measured at frequency ~7.2 MHz. Since the intensity of the sonic signal in the sample was very small (the pressure amplitude in the ultrasonic wave being less than $10^3$ Pa), any effect of the sound wave on the properties of biocolloids was avoided. The resonance frequencies have been measured by a network analyzer (USAT, USA).

A high precision densitometric system (DMA 60 with two DMA 602 M cells, Anton Paar KG, Graz, Austria) operating according to the vibrating tube principle[20] has been used to determine the density $\rho$ of the vesicle solution. Specific volume $\varphi_V$ was calculated from the density data using the equation

$$\varphi_V = [1 - (\rho - \rho_0)/c]/\rho_0 = 1/\rho_0 - [\rho], \tag{2}$$

whereby the subscript 0 again refers to the buffer and $[\rho] = (\rho - \rho_0)/(\rho_0 c)$ denotes the concentration increment of density.

The determination of the specific volume in addition to the sound velocity concentration increment allows us to estimate the specific adiabatic compressibility, $\varphi_K/\beta_0$, of

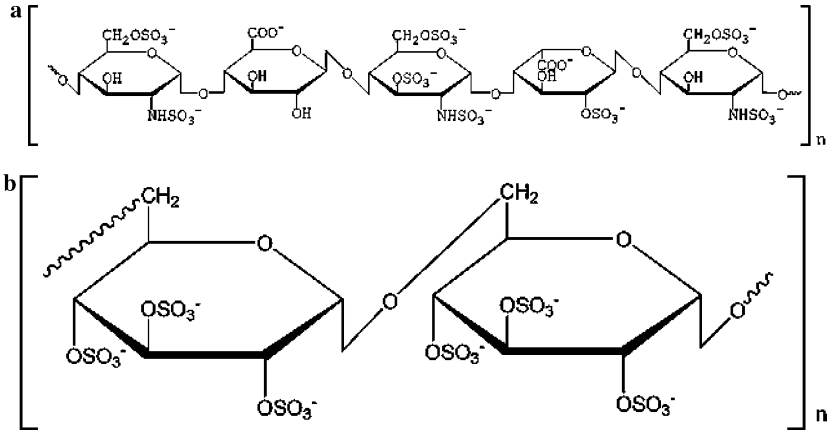


**Figure 1.** Structural formulas of (a) heparin and (b) dextran sulfate.

the suspension, which is based on the following equation:

$$\varphi_K/\beta_0 = -2[u] - 1/\rho_0 + 2\varphi_V, \quad (3)$$

where $\varphi_K$ is specific adiabatic compressibility, $\beta_0$ is the coefficient of adiabatic compressibility of the buffer, and $\rho_0$ is the density of the buffer.[17] The value of $\varphi_K/\beta_0$ indicates the volume compressibility of the macromolecules relative to the buffer. The cells used both in ultrasound velocity and density measurements were thermostated at $T = 25 \pm 0.05$ °C with a Lauda RK 8 CS or Lauda E 200 ultrathermostat, respectively. The accuracy of determination of the sound velocity increment, $[u]$, and the specific partial volume, $\varphi_v$, was better than $10^{-3}$ ml/g. The accuracy of the determination of the density was better than $10^{-3}$ g/ml.

Differential scanning calorimetry (DSC) measurements were performed on high sensitive DASM-4 microcalorimeter (Pustchino, Russia) with a cell volume 0.47 ml, under a constant pressure of 2 atm. The heating rate was 1 K/min. HSA was dissolved in a buffer: 10 mM NaCl + 10 mM Tris–HCl, pH 7.6, the concentration of protein being 1 mg/ml. We studied the thermodynamic properties of the pure HSA as well as their complexes with HEP and DS. The concentrations of polyanions at these complexes were 0.1 and 1 mg/ml. The accuracy of determination of the excess heat capacity was 0.1 mJ/K and the accuracy of determination of the phase transition temperature was 0.1 K. All heating curves were corrected using an instrument baseline obtained by heating of buffer.

Each series of measurements was performed at least three times.

In first series of experiments, we determined the values of $[u]$, $\varphi_V$, and $\varphi_K/\beta_0$ for HSA, HEP, and DS in a concentration range 1–10 mg/ml (for HSA) and 5–25 mg/ml (for HEP and DS). We showed that these values do not depend on the concentration of individual macromolecules, i.e., HSA, HEP or DS in the above mentioned concentration range. This means that at the used concentration range individual compounds HSA, HEP or DS do not form aggregates. (Please note that the situation is different for HSA and polyanion complexes, see below). The measured values are shown in Table 1.

The values obtained for HSA are comparable with those reported earlier for bovine serum albumin (BSA),[21] which evidence about similar mechanical properties of these globular proteins. The substantial difference between HSA and polyanions (DS and HEP) consists in different specific volume and in the $\varphi_K/\beta_0$ value. Moreover, the apparent specific compressibility of DS and HEP is negative, while that for HSA is positive.

Overall compressibility of the colloid is composed of the compressibility of the molecule itself + compressibility of the hydrated shell

$$\varphi_K/\beta_0 = (\varphi_K/\beta_0)_P + (\varphi_K/\beta_0)_H, \quad (4)$$

where indexes P and H correspond to the polymer and hydrated shell, respectively.[18,21] The hydration term is negative at relatively low temperatures, while the term related to the polymer itself is positive. Therefore, the overall value of the apparent adiabatic compressibility could be positive or negative depending on the degree of hydration and temperature. For example, at 20 °C the compressibility of globular proteins is in the range 0.05–0.15 ml/g, while for more hydrated fibrilar proteins the values of apparent compressibility are in the range −0.1 to −0.75 ml/g.[21] The value of overall apparent compressibility is therefore a sensitive indicator of the structural and conformational states of the biopolymer. From Table 1 it is also seen that the value of $\varphi_K/\beta_0$ is more negative for DS in comparison with HEP, i.e., DS is less compressible than HEP. These values differ significantly according to Student's $t$ test with $p < 0.01$. The differences between compressibility of DS and HEP are consistent with higher length of DS and consequently with higher degree of hydration.

We studied the changes of the compressibility of the HSA in the presence of HEP or DS and as a function of pH of the electrolyte. Like above, the studies were performed at a relatively low ionic strength, at which the shielding effect of cations is not remarkable.[12] Figure 2 shows the changes of concentration increment, $[u]$ of the solution of HSA as a function of HEP concentration at different pH. We can see that $[u]$ values increase with increasing heparin concentration for all pH. Higher value of $[u]$ at pH 4.7 may be connected with starting of unfolding of the HSA at low electrolyte pH.[22] Interaction of HEP with HSA resulted in a decrease of specific volume similarly for all pH studied (Fig. 3). Having $[u]$

**Table 1.** The concentration increment of ultrasound velocity $[u]$, specific volume, $\varphi_V$, and apparent specific adiabatic compressibility, $\varphi_K/\beta_0$, for buffer solutions (10 mM NaCl + 10 mM Tris–HCl, pH 7.6) of HSA, HEP, and DS

| System | $[u]$ (ml/g) | $\varphi_V$ (ml/g) | $\varphi_K/\beta_0$ (ml/g) |
|---|---|---|---|
| HSA | 0.156 ± 0.001 | 0.754 ± 0.001 | 0.198 ± 0.001 |
| HEP | 0.147 ± 0.002 | 0.535 ± 0.004 | −0.227 ± 0.010 |
| DS | 0.148 ± 0.005 | 0.489 ± 0.002 | −0.323 ± 0.003 |

The results represent means (±SD) obtained in three independent experiments.

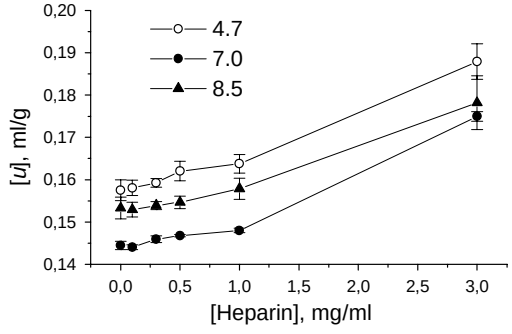


**Figure 2.** Plot of the concentration increment of ultrasound velocity, $[u]$, of the HSA solution (5 mg/ml) as a function of heparin concentration at different pH: 4.7 (○); 7.0 (●); 8.5 (▲). The results represent means (±SD) obtained in three independent experiments.

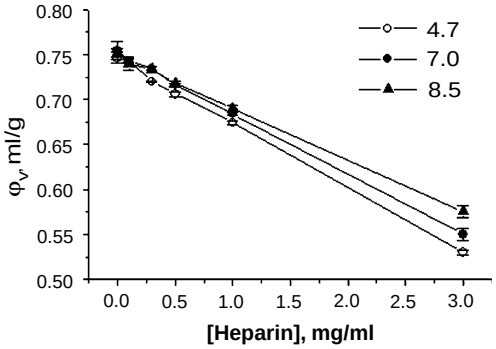


**Figure 3.** Plot of the specific volume, $\varphi_V$, of the HSA solution (5 mg/ml) as a function of heparin concentration at different pH: 4.7 (○); 7.0 (●); 8.5 (▲). The results represent means (±SD) obtained in three independent experiments.

and $\varphi_V$, and using Eq. 3, it was possible to determine the adiabatic compressibility of HSA as a function of HEP concentration. The plot of this dependence is shown in Figure. 4. We can see that with increasing of the HEP concentration the value $\varphi_K/\beta_0$ decreases and at higher concentrations of HEP it becomes even negative. More remarkable differences in compressibility of the HSA–HEP complexes at pH 4.7 may be connected with additional positive charge at HSA, that causes a stronger interaction of the polyanions with protein.

Interaction of HSA with DS revealed a similar behavior like HEP–HSA interaction. This is demonstrated in Figure 5, where the plot of adiabatic compressibility of HSA as a function of DS concentration is presented at three different electrolyte pH. Similar effect of pH in the case of HEP and DS at pH 7 and 8.5 could be connected with the fact that both HEP and DS are strong acids with p$K$ below 3.[23] Therefore, at used pH both polyanions are negatively charged and pH has no significant effect on this charge. However, at pH 4.7 due to an additional positive charge at HSA the interaction of this protein with polyanions is stronger, which is reflected also in compressibility. The comparison of adiabatic compressibility for HEP and DS for pH 7 is shown in Figure 6. It is seen that HEP revealed a stronger effect

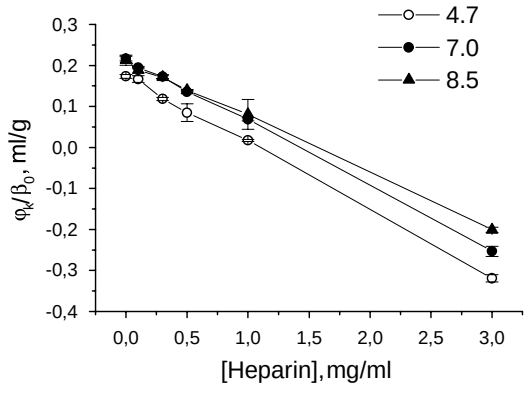


**Figure 4.** Plot of the specific adiabatic compressibility, $\varphi_K/\beta_0$, of the HSA solution (5 mg/ml) as a function of heparin concentration at different pH: 4.7 (○); 7.0 (●); 8.5 (▲). The results represent means (±SD) obtained in three independent experiments.

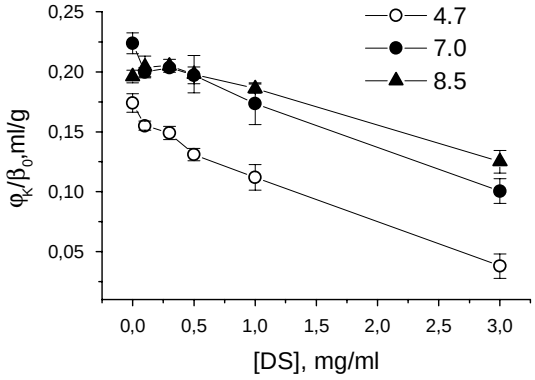


**Figure 5.** Plot of the specific adiabatic compressibility, $\varphi_K/\beta_0$, of the HSA solution (5 mg/ml) as a function of DS concentration at different pH: 4.7 (○); 7.0 (●); 8.5 (▲). The results represent means (±SD) obtained in three independent experiments.

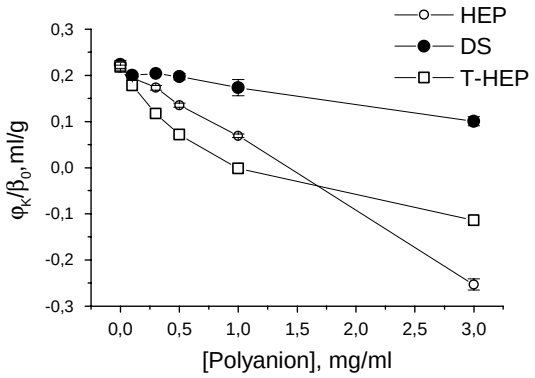


**Figure 6.** Plot of the specific adiabatic compressibility, $\varphi_K/\beta_0$, of the HSA solution (5 mg/ml) as a function of heparin (○) or DS (●) concentration at pH 7.0. The results represent means (±SD) obtained in three independent experiments. T-HEP (□) is the theoretical curve for heparin calculated according to Eq. 5.

on the compressibility of HSA in comparison with DS, which may indicate a stronger interaction of HEP with HSA probably due to the existence of a HEP binding site at the protein surface.

We determined also the values of $[u]$, $\varphi_V$, and $\varphi_K/\beta_0$, for HSA, HEP, and DS for three different pH of the electrolyte. While for HSA these parameters depend on pH, in the case of HEP and DS there was no significant pH dependence on the pH range of 4.7–8.5, that is probably connected with rather low p$K$ of polyanions (see above). Table 2 shows therefore only the parameters for HSA.

**Table 2.** The concentration increment of ultrasound velocity $[u]$, specific volume, $\varphi_V$, and apparent specific adiabatic compressibility, $\varphi_K/\beta_0$, of HSA at three pH of electrolyte (10 mM NaCl) and that for buffer (pH 7.6)

| pH | $[u]$ (ml/g) | $\varphi_V$ (ml/g) | $\varphi_K/\beta_0$ (ml/g) |
|---|---|---|---|
| 4.7 | 0.158 ± 0.002 | 0.746 ± 0.001 | 0.174 ± 0.005 |
| 7.0 | 0.145 ± 0.001 | 0.754 ± 0.002 | 0.218 ± 0.006 |
| 8.5 | 0.153 ± 0.003 | 0.753 ± 0.012 | 0.196 ± 0.012 |

The results represent means (±SD) obtained in three independent experiments.

We can see that there were no significant differences for specific volume as a function of pH. However, the adiabatic compressibility for pH 4.7 and 8.5 was lower than that for pH 7. This may be connected with partial unfolding of HSA at these pH, which resulted in a higher hydration of the protein.

Obtained results evidence that both HEP and DS induced decrease of adiabatic compressibility of HSA. In turbidimetric and light-scattering study of the formation of the complexes between HEP and bovine serum albumin (BSA), it has been shown that this formation depends on electrolyte pH. At pH > 7.2, no aggregates were formed, while at pH < 5.0 considerable increase in turbidity and light scattering was observed, that evidences on strong aggregation process. It is interesting that aggregation has been observed also at pH well above the isoelectric point of the protein (4.7), where BSA is dominantly negatively charged. This has been explained by the existence of positively charged patches at the protein surface.[24] The commercial HSA usually consists of two components with isoelectric points at 4.8 and 5.6, respectively. The 5.6 component comprises from 30 to 60% of the total protein.[25] However, the strong interaction of HSA we observed also at pH above 5.6, i.e., when HSA is mostly negatively charged. This confirms the above-mentioned results of the study of the properties of BSA–polyanion complexes by turbidimetry, where the aggregation process at the pH higher than the isolelectric point of the protein was explained by the existence of positively charged patches at the protein surface. Thus, a strong interaction of polyanions with HSA takes place in the wide range of electrolyte pH. This evidences about the high sensitivity of ultrasound velocimetry and densitometry methods. The decrease of the adiabatic compressibility of the HSA with increasing of the concentration of polyanions evidences on the formation of HSA–polyanion complexes, that are characterized by high degree of hydration. Certainly, due to the additive nature of adiabatic compressibility, this value can be expressed as a sum of the compressibilities of individual compounds multiplied by their relative concentrations in a solution

$$\beta = \beta_{HEP}X + (1 - X)\beta_{HSA}, \quad (5)$$

where $\beta = \varphi_K/\beta_0$ is the overall compressibility of the complex HSA–HEP, $\beta_{HEP}$, $\beta_{HSA}$ are compressibilities of HEP or HSA, respectively, and $X$ is the molar ratio HEP/HSA. According to Eq. 5 we calculated the $\beta$ value for various molar ratios X and plotted this value as a function of HEP concentration. This plot is shown in Figure 6 (curve T-HEP, see legend of the figure). We can see that at high concentration of heparin (3 mg/ml, i.e., complex of three molecules of heparin per one molecule of HSA) the experimental point is considerably more negative than that calculated by means of Eq. 5. We can assume that due to interaction of HEP with HSA additional hydration of HSA takes place, which can be presumably connected with unfolding of the HSA.

Earlier the hydration phenomena of another polyanions—nucleic acids have been studied in detail by means of ultrasound velocimetry.[26] It has been shown that the hydration contribution to the [$u$] value of $-NH_2$ atomic group of adenine is about 2.5 ml/mol. If all changes of the value of [$u$] in the concentration range 1–3 mg/ml of HEP are related to the changes of hydration of HSA due to unfolding, then $\delta[u] = 0.027$ ml/g = (0.027 ml/g) × (68.5 g/mol) = 1.8 ml/mol (at pH 7.0). Thus, the deviation from additivity in the formation of the complexes between HSA and HEP can be explained by increase of the hydration of the protein due to unfolding that corresponds to the hydration of ≈1 amino group per one HSA molecule.

In order to analyze whether polyanions induce the unfolding of HSA, we used the DSC method. We determined excess heat capacity as a function of temperature for pure HSA (concentration 1 mg/ml) and for two concentrations of HEP and/or DS: 0.1 and 1 mg/ml. The plot of excess heat capacity for the system of HSA–HEP is shown in Figure 7. We can see that for pure HSA there is a well-resolved peak with a maximum at 64.0 ± 0.3 °C, as revealed from three independent experiments. This temperature corresponds to the transition temperature of the protein.

The transition temperature is in good agreement with that reported in the literature (65 °C)[27]. The presence of HEP resulted in broadening of the transition peak and in a shift of transition temperature toward lower values. At the concentration of HEP 1 mg/ml, the transition peak is poorly determined and the temperature of phase transition is ≈60 °C. Similar results were observed also in the presence of DS in a suspension of HSA however, the influence of DS was weaker (Table 3). At a con-

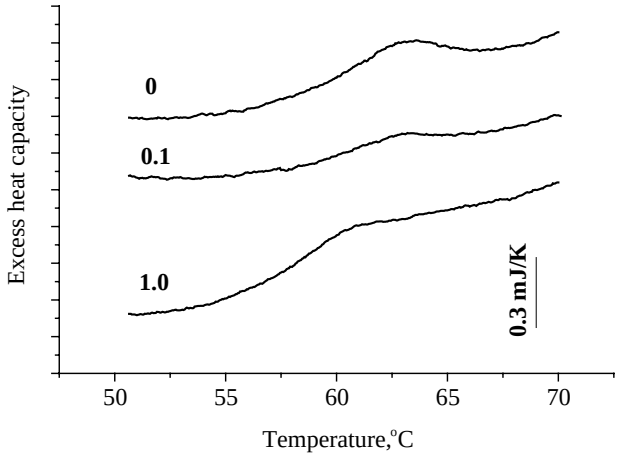


**Figure 7.** Differential scanning calorimetry scans of HSA in a buffer with increasing concentration of HEP. The HAS concentration was 1 mg/ml. The concentration of HEP in mg/ml is shown at the curves.

**Table 3.** Phase transition temperature of HSA in the presence of HEP and/or DS

| HEP (mg/ml) | $T$ (°C) | DS (mg/ml) | $T$ (°C) |
|---|---|---|---|
| 0 | 64 ± 0.3 | 0 | 64 ± 0.3 |
| 0.1 | 62 ± 0.4 | 0.1 | 63.1 ± 0.6 |
| 1.0 | 60 ± 0.6 | 1.0 | — |

centration of DS 1 mg/ml, the shift of phase transition temperature was only ≈1 °C. However, at the concentration of 1.0 mg/ml the peak also disappeared like for HEP. The shift of phase transition temperature to lower values in the presence of HEP or DS evidences about destabilization of the structure of HSA and correlates well with the influence of the HEP to cytochrome *c*.[15] The results of the DSC study are also in agreement with ultrasound velocity and density measurements.

The influence of polyanions on the compressibility of HSA and on its thermodynamic properties through possible changes of the polypeptide chain folding supports the assumption that polyanions could mimics the action of chaperons in living cells.[28] Most recently, the formation of multilayer assemblies of HEP with HSA was shown also using FTIR multiple internal reflection spectroscopy.[29]

Thus, the ultrasound velocimetry, densitometry, and DSC studies showed that polyanions could create complexes with HSA. The formation of complexes is accompanied by a decrease of specific volume and apparent specific adiabatic compressibility of the protein as well as by a decrease of its phase transition temperature. This can be connected with increasing of the hydration of the protein–polyanion complexes and particularly also with increasing the hydration of the protein due to its unfolding caused by destabilization of the protein structure in the presence of polyanions.

## Acknowledgments

This work was supported by European Commission 6 FP Program NoE NeuroPrion (Contract No. FOOD-CT-2004-506579), by the Slovak Grant Agency (Project Nos. 1/1015/04 to T.H. and 2/4068/25 to M.A.) and Ministry of Education of Slovak Republic.

## References and notes

1. Ptashne, M.; Gann, A. *Genes and Signals*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2002.
2. Neidle, S. *Nucleic Acid and Recognition*; Oxford University Press: New York, 2002.
3. Gauczynski, S.; Hundt, C.; Leucht, C.; Weiss, S. *Adv. Protein Chem.* **2001**, *57*, 229.
4. Beringue, V.; Adjou, K. T.; Lamoury, F.; Maignien, T.; Deslys, J. P.; Race, R.; Dormont, D. *J. Virol.* **2000**, *74*, 5432.
5. Bobardt, M. D.; Armand-Ugón, M.; Clotet, I.; Zhang, Z.; David, G.; Este, J. A.; Gallay, P. A. *Virology* **2004**, *325*, 389.
6. Jones, L. S.; Yazzie, B.; Middaugh, C. R. *Mol. Cell Proteomics* **2004**, *3*, 746.
7. Linhardt, R. J.; Toida, T. *Acc. Chem. Res.* **2004**, *37*, 431.
8. Ganesh, V. K.; Smith, S. A.; Kotwal, G. J.; Murthy, K. H. M. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8924.
9. Yessine, M.-A.; Leroux, J.-C. *Adv. Drug. Del. Rev.* **2004**, *56*, 999.
10. Conrad, H. E. *Heparin-Binding Proteins*; Academic Press: San Diego, CA, 1998.
11. Li, W.; Johnson, D. J. D.; Esmon, C. T.; Hungtington, J. A. *Struct. Mol. Biol.* **2004**, *11*, 857.
12. Seyrek, E.; Dubin, P. L.; Tribet, C.; Gamble, E. A. *Biomacromolecules* **2003**, *4*, 273.
13. Hattori, T.; Kimura, K.; Seyrek, E.; Dubin, P. L. *Anal. Biochem.* **2001**, *295*, 158.
14. Park, J. M.; Muhoberac, B. B.; Dubin, P. L.; Xia, J. *Macromolecules* **1992**, *25*, 290.
15. Bagel'ova, J.; Antalik, M.; Bona, M. *Biochem. J.* **1994**, *297*, 99.
16. Hianik, T.; Rybár, P.; Benediktyová, Z.; Svobodová, L.; Hermetter, A. *Gen. Physiol. Biophys.* **2003**, *22*, 467.
17. Hianik, T.; Rybar, P.; Andreev, S. Yu; Oretskaya, T. S.; Vadgama, P. *Bioorg. Med. Chem. Lett.* **2004**, *14*/*15*, 3897.
18. Sarvazyan, A. P. *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 321.
19. Halstenberg, S.; Heimburg, T.; Hianik, T.; Kaatze, U.; Krivanek, R. *Biophys. J.* **1998**, *75*, 264.
20. Kratky, O.; Leopold, H.; Stabinger, H. *Methods Enzymol.* **1973**, *27*, 98.
21. Sarvazyan, A. P.; Kharakoz, D. P. In *Molecular and Cell Biophysics*; Frank, G. M., Ed.; Nauka: Moscow, 1977; p 93.
22. Peters, T. *Adv. Protein Chem.* **1985**, *37*, 161.
23. Katayama, H.; Ishihama, Y.; Asakawa, N. *Anal. Sci.* **1998**, *14*, 407.
24. De Vries, R. *J. Chem. Phys.* **2004**, *120*, 3475.
25. Evenson, M. A.; Deutsch, H. F. *Clin. Chim. Acta* **1978**, *89*, 341.
26. Buckin, V. A. *Biophys. Chem.* **1988**, *29*, 283.
27. Wetzel, P. B. *Thermochim. Acta* **2000**, *1473*, 141.
28. Muronetz, V. I.; Kazakov, S. V.; Dainiak, M. B.; Izumrudov, V. A.; Galaev, I. Y.; Mattiasson, B. *Biochim. Biophys. Acta* **2000**, *1475*, 141.
29. Houska, M.; Brynda, E.; Bohatá, K. *J. Colloid Interface. Sci.* **2004**, *273*, 140.