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Cryostructuring of Polymer Systems.* Proteinaceous Wide-Pore Cryogels Generated by the Action of Denaturant/Reductant Mixtures on Bovine Serum Albumin in Moderately-Frozen Aqueous Media.

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Freeze-thaw processing of bovine serum albumin (**BSA**) aqueous solutions which contain also the additives of denaturant (urea in this case) and thiol-bearing reductant [cysteine (**Cys**) in this case] leads to the formation of wide-pore cryogels. The properties and porous morphology of these spongy gel matrices were demonstrated to depend on the initial concentration of all precursors and on the freezing/frozen storage temperature. The optimum conditions for preparing such BSA-based cryogels were found to be as follows: [BSA] = 3-5 g/dL, [urea] = 0.5-2.0 mol/L, [Cys] = 0.01 mol/L, freezing temperature in the range of -15 to -20°C. The size of gross pores in thus prepared cryogels is ~50-150 μm. The spatial network of the BSA-cryogels was shown to be cross-linked chemically *via* the interchain disulfide bridges. Significant role of hydrophobic interactions in the stabilization of 3D network of these cryogels is inferred, as well as the supposition about the relay-race sequence mechanism of the intermolecular disulfide cross-links formation is made.

1. Introduction

Polymeric cryogels are the macroporous gel matrices formed *via* consecutive freezing, frozen-storage and defrosting of solutions or colloidal dispersions of suitable (i.e., capable of gelling) monomeric or polymeric precursors.[1] Among the numerous cryogels reported up to date, there is a group of those prepared from such macromolecular precursors as proteins (for recent generalized data see Ref. **2**). Such cryogels were suggested for use in biotechnology, e.g., as carriers of immobilized biocatalysts (enzymes, organelles of plant cells, whole microbial cells),[3-5] in cell and tissue engineering as scaffolds for 3D cell culturing,[6-9] in medicine for the regenerative aims[10] or as the wound dressings,[11] etc.

In general, the following main approaches are employed for producing cryogels based on various polymeric precursors[12,13]: (i) covalent cross-linking of macromolecules with the aid of chemical (or enzymatic) crosslinking reagents or by irradiation; (ii) ionic crosslinking of polyelectrolytes through the formation of low-dissociating salt bridges; (iii) formation of spatial gel network *via* the physical interactions such as the H-bonds or hydrophobic linking. In all these cases the sol–to–cryogel transformation occurs within the so-called unfrozen liquid microphase (**UFLMP**)[12-14] of the macroscopically frozen system. The solutes are concentrated in the UFLMP as the main fraction of the solvent is crystallized, and such cryoconcentrating phenomenon facilitates the gel-formation processes.[12,13] In fact, each of the approaches (i)-(iii) was employed individually or in combinations upon the preparation of the above-mentioned protein-based cryogels.[2] The properties and macroporous morphology of the resultant gel matrices depended on the nature, chemical structure and conformation (globular or fibrillar) of the respective proteinaceous precursors, as well as on their initial concentration, on the reactivity and the amount of crosslinkers (if the latter were employed), and on the conditions of cryogenic processing.

The albumin-containing cryogels are of special interest, because different albumins (the class of water-soluble globular proteins[15]) are, first, widely-distributed in nature and thus readily available; and second, being biocompatible, are suitable for various biomedical applications. For instance, bovine serum albumin (**BSA**) was used for creating aldehyde-cross-linked spongy cryogel-type carriers of immobilized cells and organelles,[4,5,16,17] and ovalbumin (the albumin of the eggs' white) was the macromolecular precursor for producing wide-pore scaffolds for cell culturing.[8] In the latter case the cryogel preparation procedure was based on the approach[18] implying partial denaturation of protein globules induced by the additives of chaotropic substances, e.g. urea. This resulted in the formation of 3D polymeric network of ovalbumin cryogels with the nodes of their 3D networks stabilized by the intermolecular hydrophobic interactions.[19] However, such relatively mild influence on the spatial structure of protein macromolecules, i.e. slight changes of chain conformation, was insufficient for causing the cryotropic gelation of serum albumins. Thus, it was found that the denaturation-induced formation of BSA-cryogels could be achieved only when a certain amount of the substance capable of reducing the intramolecular disulfide bridges of this protein was introduced, in addition to urea, into the precursor solution.[18] However, the nature of intermolecular crosslinks in such serum albumin-based cryogels remained unclear up to date. Besides, the influence of the preparation conditions (concentrations of the protein and denaturant/reductant additives, the regimes of cryogenic processing) on the physico-chemical properties and macroporous morphology of the respective gel matrices was not studied. In this respect,

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clarifying these issues was the goal of the present research.

2. Experimental

2.1. Materials

The following substances were used in the experiments without additional purification: bovine serum albumin (≥98%) and urea (Ultra grade; ≥99.5%) (both from Sigma, USA), cysteine (Ultra grade; ≥99.5%) (Fluka Chemie GmbH, Switzerland), 1,4-dithiotreitol (Panreac Quimica S.A.U., Spain), methylene blue (Merck GmbH, Germany), sodium dodecyl sulphate (>99%) (Serva, Gemany), guanidine hydrochloride (>99%; Helicon, Russian Federation). All aqueous solutions were prepared using water of Milli-Q quality.

2.2. Methods

Preparation of BSA-cryogels

BSA powder was dissolved in calculated volume of water for obtaining solutions with final protein concentration either 3, or 4, or 5 g/dL. Then the necessary amount of dry urea was added and quickly dissolved in the BSA solution. Urea concentration in the tested samples was varied over the range from 0.5 to 3.0 mol/L. The resultant solutions were cooled down in ice bath followed by the addition of the required volume of aqueous 0.9M cysteine stock solution. Further, the 3-mL-portions of the reaction systems thus prepared were put into 5-mL plastic syringes (BD Discardit™ II, Spain); the latter were stoppered and placed into the chamber of the precision programmable cryostat F-32 (Julabo, Germany) with a pre-set minus temperature, where the syringes were kept for 20 hrs. Then the samples were immersed in water bath for 5 min to defrost the contents of the syringes. The formed cryogels were rinsed from the sol-fraction by passing pure water (200 mL per sample) through the wide-pore cylindrical gel blocks inside the syringes. Prior to this operation each spongy cryogel was first gently removed from the syringe; then the thin branch piece formed in the outlet of the syringe was precisely cut off with a sharp scissors, and the cryogel cylinder was again placed into the syringe.

Characterization of BSA-cryogels

(i) Hydrodynamic properties of the cryogel columns: The water flow rates (mL/h) through the BSA-cryogel cylindrical samples swollen inside the plastic syringes were measured by passing the water at constant hydrostatic pressure of 900 mm water-column.

(ii) Swelling parameters of BSA-cryogels: The degree of swelling of albumin cryogels was measured gravimetrically as follows. Each equilibrium-water-swollen gel sample was placed onto a glass filter, and the free solvent was removed under vacuum (water pump) for 1 min with a 145-g load put on the top of this spongy cryogel and then keeping for 4 min also under vacuum but already without the load, the filter being sealed. The gel sample thus squeezed from free water was weighed and dried to a constant weight at 105°C in a SNOL 24/200 oven (AB Utenos Elektrotechnika, Lithuania). The swelling extent by weight ($S_{w/w}$) of the gel phase (the walls of macropores) of spongy BSA-cryogels was calculated with the formula:

$$S_{w/w} = (m_{ws} - m_d) : m_d \text{ (g } H_2O \text{ per 1 g of dry polymer)}, \quad (1)$$

where m_{ws} is the weight of the water-swollen and compressed cryogel after removal of free water, m_d is the weight of the dried sample.

(iii) Gel-fraction yield: The gel-fraction yield (Y) was calculated with a formula:

$$Y = (m_d : m_{th}) \cdot 100\%, \quad (2)$$

where m_d is the weight of the dried sample as in the formula (1), m_{th} is the 'theoretical' weight of the sample calculated by assuming that all the protein containing in the feed solution was incorporated in the 3D network of the resultant BSA-cryogel.

(iv) High-Sensitivity Differential Scanning Calorimetry measurements: Stock suspensions of the BSA-cryogels in water were prepared using a manual homogenizer of the Potter type. The protein concentration in the stock suspension was determined by weight after drying of the suspension at 105°C to a constant weight (~20 h). The suspension for calorimetric measurements was prepared by mixing equal volumes of the stock suspension with required concentration and the buffer solution (40 mM Na-phosphate buffer, 0.3M NaCl, pH 7.4). The obtained suspension of the BSA-cryogel with concentration of 5 mg/mL in 20 mM phosphate buffer (0.15M NaCl, pH 7.4) was incubated for 20 h at 5°C under continuous stirring, then placed into the calorimeter. The thermogram of the BSA solution in 20 mM phosphate buffer (0.15 M NaCl, pH 7.4) was used as reference. Calorimetric measurements were carried out with a differential adiabatic scanning microcalorimeter DASM-4 ("Biopribor", Pushchino, Russian Federation) at the heating rate of 2 K/min within the temperature range of 10-110°C and at the excess pressure of 0.25 MPa. The data acquisition and the conversion of thermograms to the apparent heat capacity functions were performed using COMPORT and NAIRTA software, respectively (Institute of Organoelement Compounds of the RAS, Moscow).

(v) Microstructure of BSA-cryogels: The cryogel sample for the microscopy studies were formed as 1-mm-thick discs. The reaction solution containing the required concentration of BSA, urea and cysteine was poured into a plastic Petri dish (45 mm inner diameter), which was quickly installed onto a flat pallet immersed in the coolant of the cryostat to freeze the system of interest. After the process completion, the disc gel sample thus prepared was rinsed 15 times with an excess of deionised water and stained by treatment for 1 min with 0.125 mM aqueous solution of methylene blue dye followed by exhaustive rinsing with water. Porous morphology of BSA-cryogels was investigated using optical stereomicroscope SMZ1000 (Nikon, Japan) equipped with a MMC-50C-M system (MMCSoft, Russian Federation) for digital image recording. The images obtained have been treated with the 'MMCatalog Multimeter v.2.2' program supplied with this digital camera.

The dimensions of the gross pores of BSA-cryogels were calculated similar to the approach employed earlier for spongy chitosan cryogels,[23] using the following formulas:[24,25]

$$D_n = \left(\frac{\sum (N_i \cdot D_i^3)}{\sum N_i} \right)^{1/3} \quad \text{and} \quad D_w = \left(\frac{\sum (N_i \cdot D_i^6)}{\sum (N_i \cdot D_i^3)} \right)^{1/3} \quad (3)$$

where D_n and D_w are the numerical root-mean-cube and the weighted root-mean-cube values of pore diameters, respectively, and $\sum N_i \geq 100$. The polydispersity index, which characterizes the width of values variation, was determined as $k = D_w/D_n$.

The values of gel-fraction yield, swelling extent and water flow rates through the BSA-cryogel columns were measured for 3 parallel samples; the samples were obtained in 2-4 independent experiments (this was also done in case of morphological studies). The results obtained were averaged.

3. Results and discussion

3.1. Phenomenology of BSA cryotropic gel-formation in moderately frozen aqueous systems

As it was indicated in the 'Introduction', cryogenic treatment, i.e. moderate freezing, then storing frozen and, finally, thawing of the simple aqueous solutions of various serum albumins, BSA in particular, does not result in the formation of gel matter with the typical cryogel-like properties and behaviour. Also, the feed-inserted denaturant like urea (with concentrations capable of causing the formation of ovalbumin cryogels[19]) did not work in BSA case, i.e. BSA solutions with urea additives did not yield cryogels when treated in the same way as the respective ovalbumin-based systems. This obviously meant that the urea-induced denaturation changes of globular conformation alone were insufficient in the case of BSA to result in the formation of stable enough interchain hydrophobic contacts similar to the ovalbumin case. In other words, a "stronger" unfolding of BSA globules was evidently required in order to cause the formation of BSA cryogels. This effect was achieved by the additional action of reductants like the low-molecular thiols [e.g., cysteine (**Cys**)] that were able to split the intramolecular disulfide bridges in serum albumin thus increasing the potential of polypeptide chains for unfolding. At the same time, no gelation took place at room temperatures in the feed solutions of the same composition. This shows that besides the correlated action of denaturant and reductant the cryogel formation also requires the cryoconcentrating effect, which, as was noted above, takes place upon the solvent crystallization when the system is non-deeply frozen, and the solutes are concentrated in the UFLMP.[12-14]

The albumin cryogels formed *via* freeze-thaw treatment of the mixed aqueous solutions BSA-urea-Cys were the white-coloured spongy gel matrices capable of releasing free liquid upon slight compression and swelling again with complete restoration of its initial shape after being immersed in water. These proteinaceous cryogels were not dissolved in an excess of water either upon prolonged storing at room temperature, or upon heating in boiling water bath for 2-3 hrs.

In order to examine the stability of the prepared BSA-cryogels in different media, the following tests have been carried out:

The freshly prepared identical samples were rinsed from the sol-fraction by water passing through the gel-containing syringes (see 'Experimental'), then the gel cylinders were removed from the syringes and put into an excess of water. After incubation for 48 hrs the samples were transferred into the flasks filled with, respectively, either 8 mol/L aqueous solution of urea (1A, Fig. 1), or 5 mol/L aqueous solution of guanidine hydrochloride (**Gu·HCl**) (1B, Fig. 1), or 1 % (w/v) aqueous solution of sodium dodecyl sulfate (**SDS**) (1C, Fig. 1), where the cryogels were kept also for 48 hrs at room temperature. It turned out that such gel samples were not dissolved in the indicated solutions, and only swelled therein up to different levels with visible distortion of the "native" shape (Fig. 1, row 2, samples A, B and C, correspondingly). The smallest swelling degree was found for the sample immersed in the concentrated urea solution, a well-known agent[20] for disrupting of hydrogen bonds. A somewhat higher swelling extent of the BSA-cryogel was observed in the concentrated Gu·HCl solution capable of destabilizing both hydrogen and ionic bonding. And last, the most pronounced swelling was found for the sample incubated in the SDS solution, thus indicating that intermolecular hydrophobic interactions play a certain role in the stabilization of BSA-cryogel's 3D network, and that these interactions were obviously affected by the surfactant. After this swelling step, an aliquot of concentrated dithiothreitol (**DTT**) aqueous solution, the disulfide-reducing reagent,[21] was added into each flask with swollen cryogel so that the final DTT concentration became equal to 0.05 mol/L. This additive caused complete dissolution of all three samples (Fig. 1, row 3), thus pointing to the destruction of SS-bridges in the nodes of spatial polymeric network in these BSA-cryogels.

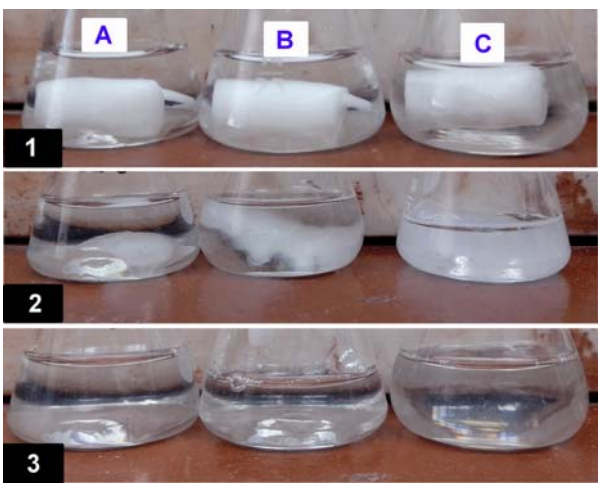

Fig. 1. The swelling-dissolution behaviour of the BSA-cryogel samples prepared from the feed solution with [BSA] = 4 g/dL, [urea] = 1 mol/L and [Cys] = 0.01 mol/L.

Row **1**: The water-rinsed samples just after their immersion in 8 mol/L urea solution (**A**), 5 mol/L Gu·HCl solution (**B**) and 1% (w/v) SDS solution (**C**).

Row **2**: The same samples after the 48-hrs-incubation in the solutions of these denaturants.

Row **3**: The same samples 1 h after the addition of DTT (0.05 mol/L).

Summing up the results of such qualitative swelling/solubility tests we can conclude that in the course of freeze-thaw treatment of aqueous BSA solutions containing definite amounts of denaturant (urea) and reductant (Cys) the formation of the chemically-linked albumin cryogels occurs. The intermolecular covalent crosslinks in such cryogels are the SS-bonds, and hydrophobic interactions, ionic and hydrogen bonding also contribute in a certain degree to

stabilization of the spatial structure of polymeric network.

3.2. Influence of the preparation conditions on the properties of the resultant BSA-cryogels

The physico-chemical properties and porous morphology of various polymeric cryogels are known to be strongly dependent on the preparation conditions that include the concentrations and ratio of the solutes in the initial feeds, as well as the regimes of freeze-thaw processing.[1,12,22] Therefore we studied how the concentration of the gel precursor and process parameters effect the BSA cryotropic gelation itself and the properties/structure of the resultant cryogels.

In the respective experiments the variables under consideration were as follows:

(i) initial BSA concentration;
(ii) urea concentration in the feed solution;
(iii) concentration of Cys in the feed solution;
(vi) freezing temperature.

In turn, the following parameters characterizing the gelation process efficiency and the properties of the resulting cryogels have been evaluated:

(i) the gel-fraction yield showing the fraction of the protein precursor which was incorporated in the 3D network of particular BSA-cryogel;

(ii) the swelling degree of the gel phase of heterophase wide-pore BSA-cryogel (this parameter characterizes, in fact, the density of crosslinks in the pore walls of cryogel sample);

(iii) the flow rate of water passed under constant hydrostatic pressure through the syringe with BSA-cryogel (this parameter is known to be useful for the sake of comparison of different cryogels with respect of their porosity, since the larger the pore cross-section, the greater volume of liquid per unit of time can flow through the column of porous material[23,26,27]).

The results of the experiments on the influence of feed composition and freezing conditions on the properties of BSA-cryogels (with the exseption of flow rates) are summarized, respectively, in Tables I and II that also contain the qualitative description of the cryogel samples formed under the particular set of processing parameters.

First of all, preliminary tests showed that at initial BSA concentration lower than 3 g/dL the final gel-fraction yield essentially decreased, and at initial protein concentration higher than 5 g/dL the resultant cryogels were so dense as to cause significant experimental difficulties for rinsing the samples in the flow through mode and for the subsequent examination of their properties. Therefore, the initial BSA concentration range of 3 to 5 g/dL was chosen for the subsequent experiments. The data from section "A" in Table I demonstrate that within this diapason of the precursor's initial concentration the denaturant/reductant-induced cryotropic gel-formation of BSA occurred with high efficiency. The gel-fraction yield was of around 90% not only at the exemplified urea and Cys concentrations of 1 mol/L and 0.01 mol/L, respectively (nos. 1*b*, 1*d* and 1*g*; Table I), but at other values as well, whereas in the absence of such 'inductors' the system remained liquid after completion of the freeze-thaw cycle (nos. 1*a*, 1*c* and 1*e*; Table I). In parallel with increase in the initial BSA concentration, the swelling degree of the polymeric network of the pore walls of the resulting heterophase sponge-like cryogels somewhat decreased (*cf.* $S_{w/w}$ values for nos. 1*b*, 1*d* and 1*g*; Table I). This shows that larger amount of interchain crosslinks was formed in the gel phase of BSA-cryogels prepared from the more concentrated solutions of the polymeric precursor. Such a trend is observed, as a rule, for numerous other cryogels being produced by chemical cross-linking of macromolecules in the moderately-frozen media.[12,13,22] Also, the higher was the initial BSA concentration, the "organoleptically" (i.e., when examined by compression with fingers) stronger were the resulting cryogels (nos. 1*b*, 1*d* and 1*g*; Table I). However, since many of sponge-like samples prepared under the used conditions were rather weak, it was difficult to measure their mechanical characteristics, therefore we were limited to just a qualitative description of these properties in Table I.

The next key factor affecting significantly both the possibility of BSA-cryogel formation and the properties of such gel materials is the urea concentration in the initial protein solutions. The influence of this denaturant is given in section "B" of Table I for the case of feeds containing BSA in amount of 4 g/dL. For the systems with other protein concentrations the observed tendencies were very close. It was found that increasing the initial urea concentration from 0.5 to 2.5 mol/L resulted in a systematic decrease in the gel-fraction yield (nos. 2*a*-2*e*, Table I). That is, the higher was the amount of the denaturant in the feed solution, the lower fraction of the feed-dissolved BSA macromolecules was incorporated in the gel phase of the respective spongy sample. At the same time, the dependence of $S_{w/w}$ values on urea feed concentration in this series passed through a maximum, when the swelling degree first grew with increasing the initial urea concentration from 0.5 to 2.0 mol/L (nos. 2*a*-2*d*, Table 1), but then unexpectedly felt down at [urea] = 2.5 mol/L (no. 2e, Table I). Whereas the former effect is easy to explain (the lower is the gel-fraction yield, the smaller amount of the polymer constitutes the 3D network and, hence, the higher is the swelling ability of this network), the causes of the decrease in $S_{w/w}$ in the case of BSA-cryogel prepared from the equi-concentrated protein solution, but at slightly higher urea amount (i.e., 2.0 and 2.5 mol/L), are remained unclear so far. Note that the textural morphology of these wide-pore cryogels formed in the presence of increasing concentrations of urea (examples #2, Table I) also changed in a bell-like fashion: from a rather soft spongy matter (no. 2*a*, Table I) to elastic sponges (nos. 2*b* and 2*c*, Table I), and then to a weak macroporous material (no. 2*e*, Table I). This was apparently due to the diffences in the polymer content within the gel phase which, in turn, influenced the swelling properties of the corresponding samples.

The last examined constituent of the feeds used in this study for preparing BSA-cryogls was the thiol-bearing reagent cysteine capable of reducing the intramolecular disulphide bonds in proteins, especially in the presence of denaturants.[28] The influence of different Cys concentrations (from 0.001 to 0.7 mol/L) was traced for the cases of feed solutions with [BSA] = 5 g/dL (section "C", Table I). It was found that Cys influenced the efficiency of BSA incorporation into the spatial

Table I. Influence of the feed composition on the morphology and physico-chemical characteristics of BSA-cryogels [a)]

Example number	Concentration of feed components [b)]			Freezing temperature [b)] (°C)	The samples' qualitative morphology after completion of the freeze-thaw cycle	Characteristics of the resultant BSA-cryogels	
	[BSA] (g/dL)	[Urea] (mol/L)	[Cys] (mol/L)			Gel-fraction yield (%)	Swelling extent of the gel phase ($S_{w/w}$; g H_2O/g dry polymer)
A) Influence of initial BSA concentration							
1 ***a***	**3.0**	0	0	-15	Solution	–	–
b		1.0	0.01		Weak spongy cryogel	90.0 ± 0.9	1.92 ± 0.07
c	**4.0**	0	0		Solution	–	–
d		1.0	0.01		Elastic spongy cryogel	89.8 ± 0.9	1.87 ± 0.10
e	**5.0**	0	0		Solution	–	–
g		1.0	0.01		Elastic spongy cryogel	90.2 ± 0.7	1.79 ± 0.07
B) Influence of initial urea concentration							
2 ***a***	4.0	**0.5**	0.01	-15	Soft spongy cryogel	91.0 ± 0.6	1.58 ± 0.07
b		**1.0**			Elastic spongy cryogel	89.8 ± 0.9	1.87 ± 0.10
c		**1.5**			Elastic spongy cryogel	86.2 ± 2.0	1.88 ± 0.20
d		**2.0**			Spongy cryogel	82.0 ± 0.7	1.98 ± 0.19
e		**2.5**			Weak spongy cryogel	78.7 ± 2.2	1.73 ± 0.16
C) Influence of initial Cys concentration							
3 ***a***	5.0	1.0	**0.001**	-15	Weak spongy cryogel	85.9 ± 0.8	1.68 ± 0.06
b			**0.010**		Elastic spongy cryogel	90.2 ± 0.7	1.79 ± 0.07
c			**0.100**		Rubber-like gel matter	77.4 ± 2.0	2.66 ± 0.03
d			**0.700**		Loose brittle cryogel	67.1 ± 11.9	2.25 ± 0.48

[a)] Freezing/frozen storage duration in all the cases was 20 hrs.
[b)] The main parameters varying within the series are shown in bold type.

Table II. Influence of the freezing/frozen storage temperature on the morphology and physico-chemical characteristics of BSA-cryogels [a)]

Example number	Concentration of feed components [b)]			Freezing temperature [b)] (°C)	The samples' qualitative morphology after completion of the freeze-thaw cycle	Characteristics of the resultant BSA-cryogels	
	[BSA] (g/dL)	[Urea] (mol/L)	[Cys] (mol/L)			Gel-fraction yield (%)	Swelling extent of the gel phase ($S_{w/w}$; g H_2O/g dry polymer)
1 ***a***	4.0	0.5	0.01	**-15**	Soft spongy cryogel	91.0 ± 0.6	1.58 ± 0.07
b		1.0			Elastic spongy cryogel	89.8 ± 0.9	1.87 ± 0.10
c		1.5			Elastic spongy cryogel	86.2 ± 2.0	1.88 ± 0.20
d		2.0			Spongy cryogel	82.0 ± 0.7	1.98 ± 0.19
e		2.5			Weak spongy cryogel	78.7 ± 2.2	1.73 ± 0.16
2 ***a***	4.0	0.5	0.01	**-20**	Weak spongy cryogel	78.0 ± 1.5	1.95 ± 0.06
b		1.0			Spongy cryogel	83.5 ± 1.9	2.73 ± 0.29
c		1.5			Spongy cryogel	79.5 ± 2.9	3.23 ± 0.68
d		2.0			Weak cryogel with jelly-like inclusions	57.8 ± 8.9	3.89 ± 0.62
e		2.5			Weak cryogel with jelly-like inclusions	39.4 ± 5.4	2.87 ± 0.53
3 ***a***	4.0	0.5	0.01	**-25**	Weak cryogel poorly-maintaining its shape	75.1 ± 4.3	3.13 ± 0.69
b		1.0			Jelly-like cryogel	37.7 ± 7.2	7.84 ± 3.67
c		1.5			Mobile fluid	–	–
d		2.0			Mobile fluid	–	–
e		2.5			Mobile fluid	–	–

[a)] Freezing/frozen storage duration in all the cases was 20 hrs.
[b)] The main parameters varying within the series are shown in bold type.

network in the same way as urea did, namely, with increase in the initial reductant concentration, the gel-fraction yield decreased (nos. 3*a*-3*d*, Table 1). In the case of [Cys] = 0.7 mol/L, the resulting gel samples were very heterogeneous in their texture, brittle and difficult to handle, which was the main reason of the rather high experimental error in determining the yield in this case (no. 3*d*, Table I).

BSA is the protein consisting of 582 amino acid residues, 35 of them are the cysteine ones, and 34 thereof form 17 intramolecular disulfide bridges while one Cys residue is free (i.e., it carries free thiol group); the molecular weight of BSA is ~69 kDa.[14] Hence, the molar concentrations of BSA in its 5 g/dL solution is ~ 0.72 mmol/L, the total concentration of Cys residues therein is about 25.2 mmol/L, the concentration of SS-bridges is ~12.6 mmol/L. Consequently, over the concentration range (1-700 mmol/L) of the molecular cysteine added to the feed solutions used for the preparation of BSA-cryogels whose properties are shown in section "C" of Table I, the molar ratios of SS-bridges and dissolved Cys varied from 12.6 : 1 (more than the 10-fold deficiency of the reductant) up to 12.6 : 700 (i.e., more than 55-fold excess of the disulphide-reducing reagent). The samples prepared from the feeds with [Cys] = 0.01 mol/L (nos. 1*g* and 3*b*; Table I), when such ratio was close to 1 : 1, had the best characteristics (cryogel morphology, the gel-fraction yield and swelling behaviour). Thus, this condition appears to be the optimum one in terms of the reductant amount. Certainly, in the feed solutions with BSA concentrations of 3 and 4 g/dL such ratio somewhat differed (by the factors of 1.67 and 1.25, respectively, in favour of Cys). Based on the data from section "C" of Table I, the Cys concentration of 0.01 mol/L was selected for all subsequent experiments including the study of the freezing conditions influence on the outcome of (urea+Cys)-induced BSA cryotropic gelation. The results obtained in such tests with the feeds of 4 g/dL BSA concentration are given in Table II.

The first significant observation was that the temperature range of freezing/frozen-storage, over which the 'good quality' spongy BSA-cryogels could be fabricated, turned out to be rather narrow. Particularly, the feeds, especially those with high urea content, did not freeze at above -15°C because of the evident cryoscopic effect. In turn, after cryogenic processing at -25°C or lower, we obtained either very weak cryogels, whose characteristics were impossible to measure with reasonable precision (nos. 3*a* and 3*b*, Table II), or the cryotropic gelation did not occur at all (nos. 3*c*-3*e*, Table II). Therefore, the temperature interval suitable for the formation of such BSA-cryogels was from -15 down to -25°C, and the best cryogels were obtained when the gel-formation occurred within the range still narrow, namely, from -15 to -20°C. Further, comparing the results of BSA cryostructuring at -15 and -20°C showed that the highest gel-fraction yield was attained upon cryogenic processing at the former temperature (nos. 1*a*-1*c*, Table II). Also, the resultant cryogels possessed the better morphological qualities in terms of stability and mechanical properties of the gel samples (*cf.* nos. 1*a*-1*c* and 2*a*-2*c*, Table II) when the samples were compressed to remove free liquid upon the measurements of $S_{w/w}$ values (see 'Experimental'). The swelling extent of BSA-cryogels prepared from the equi-concentrated solutions of the precursors was smaller for cross-linked networks formed at -15°C than for those synthesized at -20°C (samples ## 1 and 2, respectively; Table II). This indicates that polymeric network in pore walls of the former cryogels was cross-linked in a greater degree as compared to the latter case.

Thus, the studies of the influence of feed composition and freezing/frozen-storage temperature conditions on the efficiency of the (urea+Cys)-induced BSA cryotropic gel-formation showed that the possibility of obtaining such BSA-cryogels *per se*, as well as the properties of these gels, were controlled by the initial albumin concentration, mandatory presence of both denaturing and SS-reducing agents, their concentrations and the temperature of cryogenic processing. However, the integral properties of different spongy cryogels, in general, usually depend not only on the characteristics of the gel phase proper, but also on the wide-pore morphology of such polymeric matrices.[1,12,13,22] Therefore, the effects of the preparation conditions on the structural features of the resultant BSA-cryogels had been studied as well.

3.3. Wide-pore morphology of the prepared BSA-cryogels

The micrographs in Fig. 2 show the images recorded with an optical stereomicroscope for the wide porous texture of the methylene-blue-stained 1-mm-thick discs of BSA-cryogels that were prepared by freezing at -15 and -20°C of 4 g/dL-BSA-solutions containing varied concentration of urea and constant (0.01 mol/L) concentration of Cys. In the images of Fig. 2 the gel phase is seen as dark areas (dark strands), and large pores of the spongy matter are the light regions. In addition to the micrographs, Table III summarizes the data on the pore dimensions for the samples, as well as on the hydrodynamic properties of the similar BSA-cryogels formed inside the plastic syringes (Fig. 3). The flow through rate values can also be considered, as it was pointed out above (section 3.2), as the indicators of the samples' porosity.

The web-like morphology of BSA-cryogels seen in Fig. 2 is typical for most of the spongy cryogels prepared either by cross-linking of polymeric precursors, or *via* the cross-linking polymerization of monomeric precursors.[1,12,13] As a rule, the size and shape of gross pores in such heterophase gel matrices, as well as the thickness of pore walls, are controlled by many factors, including the chemical nature and initial concentration of the solutes, the type of solvent used, and, certainly, the regimes of cryogenic processing. In the case investigated in the present work, the most evident trend observed for the texture of BSA-cryogels was the decrease in size of macropores of the samples with lowering of the gel-formation temperature from -15 to -20°C (*cf.* the respective images in the columns -15 and -20°C in Fig. 2, and also the data in Table III on pore diameters for the samples 1 and 2 equi-concentrated with respect of urea). This effect is well-known for various other cryogels,[12,13,22,27-30]

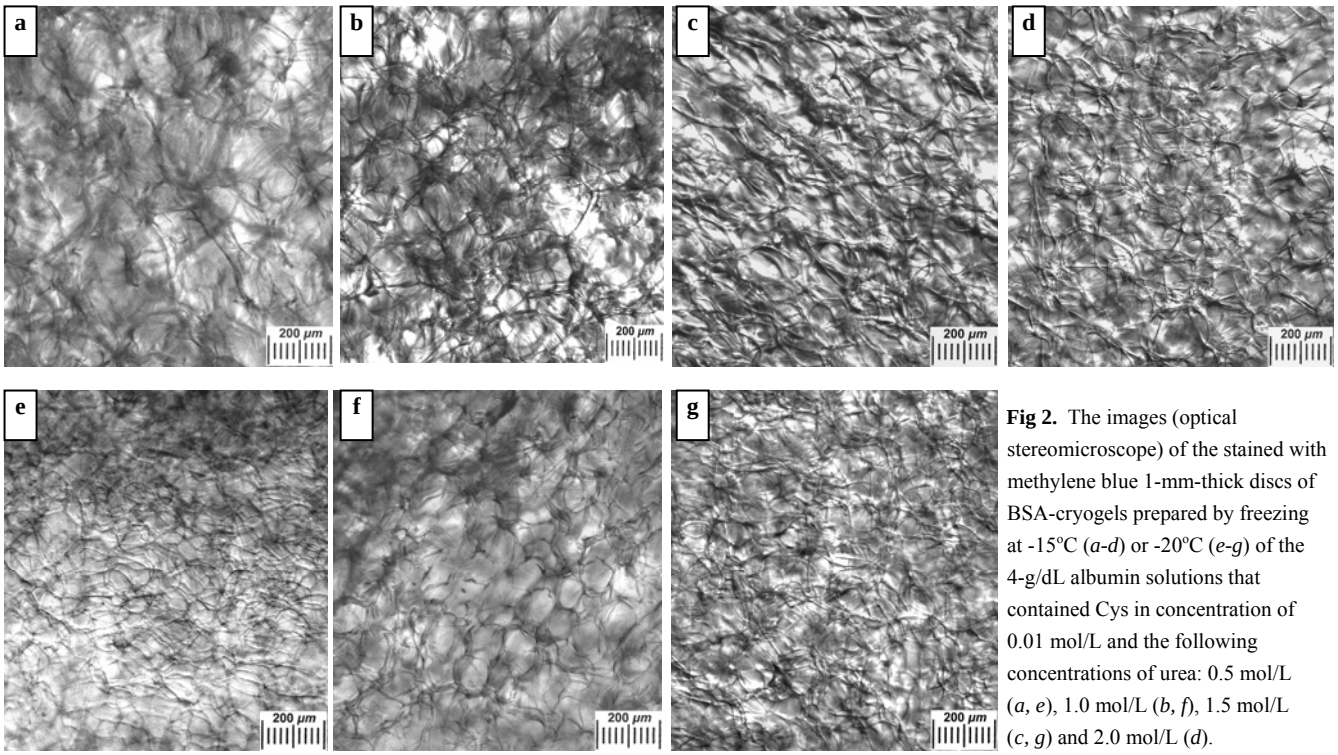

Fig 2. The images (optical stereomicroscope) of the stained with methylene blue 1-mm-thick discs of BSA-cryogels prepared by freezing at -15°C (*a*-*d*) or -20°C (*e*-*g*) of the 4-g/dL albumin solutions that contained Cys in concentration of 0.01 mol/L and the following concentrations of urea: 0.5 mol/L (*a*, *e*), 1.0 mol/L (*b*, *f*), 1.5 mol/L (*c*, *g*) and 2.0 mol/L (*d*).

Table III. Data of the quantitative analysis of microscopic images of BSA-cryogels and the respective columnar samples prepared by freezing [a] of protein solutions that contain different amount of denaturant additives.

Example number	Concentration of feed components [b]			Freezing temperature [b]	Porosity characteristics of the resultant BSA-cryogels			Flow rate of water through the 3 mL column of BSA-cryogel
	[BSA] (g/dL)	[Urea] (mol/L)	[Cys] (mol/L)	(°C)	D_n (μm)	D_w (μm)	$k = D_n/D_w$	(mL/h)
1 ***a***	4.0	**0.5**	0.01	**-15**	105.4 ± 31.7	148.0 ± 59.3	1.40	562 ± 55
b		**1.0**			102.0 ± 25.8	149.9 ± 61.1	1.47	539 ± 78
c		**1.5**			98.9 ± 26.1	127.3 ± 42.9	1.29	528 ± 38
d		**2.0**			92.2 ± 24.6	120.1 ± 40.9	1.30	[c]
2 ***a***	4.0	**0.5**	0.01	**-20**	68.2 ± 14.9	86.0 ± 25.4	1.26	580 ± 15
b		**1.0**			73.9 ± 17.3	95.5 ± 31.5	1.29	607 ± 40
c		**1.5**			57.7 ± 16.6	75.8 ± 29.0	1.31	587 ± 24
d		**2.0**			[c]	[c]	[c]	[c]

[a] Freezing/frozen storage duration in all the cases was 20 hrs.
[b] The main parameters varying within the series are shown in bold type.
[c] The mechanically weak samples whose textural morphology was unsuitable for the measurements.

since the lower the cryostructuring temperature, the smaller porogen particles, i.e. solvent crystals, are commonly formed, if the supercooling phenomenon does not interfere with freezing process.[13,23]

Besides the influence of the cryogenic treatment temperature, a certain effect of the urea initial concentration on the morphometric parameters of BSA-cryogels was also registered. Thus, with the increase in the feed urea content the size of macropores of samples formed at -15°C (nos. 1*a*-1*d*, Table III) somewhat diminished or passed through a slightly exhibited maximum in the case of cryogels formed at the lower temperature of -20°C (nos. 2*a*-2*c*, Table III). The same tendency was found for the rate of water flow through the albumin-cryogel-filled syringes (the rightmost column in Table III). These results testify that urea additives affect not only the efficiency of BSA cryotropic gel-formation (Tables I-II and footnote [c] in Table III) but the geometry of ice crystals, as well. A very similar impact of urea influence was earlier observed for the denaturant-caused gelation of ovalbumin

in moderately frozen systems,[19], so that this effect obviously appears to be a common feature.

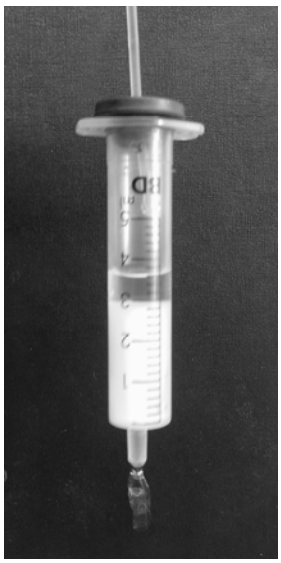

Fig 3. The sample of plastic syringe filled with BSA-cryogel (the liquid is passed through the syringe at hydrostatic pressure of 900 mm water-column).

3.4. The (urea+Cys)-induced changes of BSA conformation in the course of gel-formation in moderately frozen media.

As it was already indicated in the 'Introduction', the ovalbumin-based cryogels are formed *via* freeze-thaw treatment of the urea-containing aqueous solutions of the protein without any additives of the SS-reductant additives.[19] In this earlier study it was found that ovalbumin globular macromolecules in the UFLMP medium undergo a certain partial unfolding (partial denaturation) thus "opening the vacancies" for the interchain hydrophobic interactions capable of forming the junction knots of spatial network in the resulting cryogels. Therefore, it was of significance to elucidate also the conformational changes of the BSA globular structure in the cryogels produced from this particular protein in the presence of urea and Cys. For this purpose a high-sensitivity differential scanning calorimetry (**HS-DSC**) was applied.

Figure 4a shows the thermogram of the native BSA dissolved in buffer solution (trace 1) and the thermograms of the BSA-cryogel suspensions in the same buffer medium (traces 2-4). The thermogram of the native BSA reveals a distinct biphasic denaturation profile with two heat capacity maxima at 67 and 80°C typical for an incompletely defatted serum albumin.[31] Additionally, the thermogram shows a well-known feature of the BSA denaturation behaviour – a post-denaturation aggregation appearing as a decrease in the heat capacity at temperatures above 90°C. The thermograms of the BSA cryogels differ notably from those of the native protein regardless of the protein concentration in the reaction mixture: both denaturation peaks of BSA are degenerated. This proves a crucial perturbation of the BSA tertiary structure in the cryogel – the protein is strongly denatured. However, the aggregation behaviour of BSA in the post-denaturation area in the cryogels is similar to that of the native BSA. It means that conformation of BSA in the cryogel does not correspond to a completely unfolded coil; it retains some functionality and is able to form supermolecular structures.

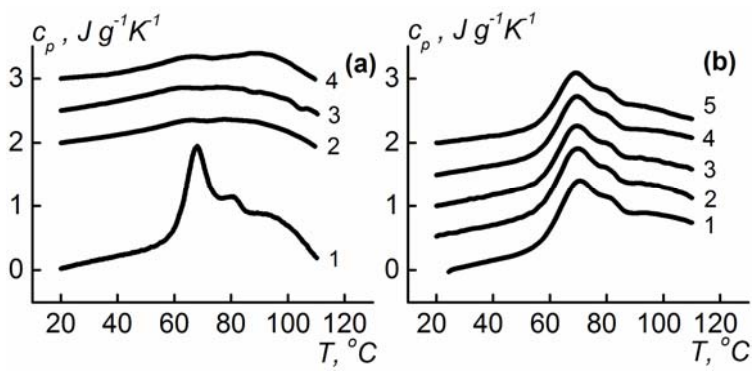

Fig 4. (a) Thermograms of native BSA (1) and of BSA-cryogels prepared by freezing (-20 °C for 20 h) of the reaction mixtures with protein concentration of 3.0 (2), 4.0 (3) and 5.0 (4) g/dL in the presence of urea and cysteine (measured in 20 mM Na-phosphate buffer, 0.15 M NaCl, pH 7.4). **(b)** Thermograms of native BSA (1) and of the BSA solutions with the protein concentration of 0.5 (2), 3.0 (3), 4.0 (4) and 5.0 (5) g/dL subjected to freezing at -20 °C for 20 h (measured in water, pH 6.5-6.6). All curves are arbitrary shifted along the ordinate axis for clear presentation.

Therefore, a question arises: whether the protein loses its tertiary structure through the so-called irreversible cold denaturation during freezing or because of cryogel network formation induced by the denaturant and the reductant? In order to clarify this point we have carried out the calorimetric measurements for the native BSA dissolved in water without additives of urea and/or Cys (trace 1, Fig. 4*b*) and for the BSA solutions subjected to freezing-thawing (traces 2-5, Fig. 4*b*). The thermograms of all protein solutions after freezing-thawing regardless of the protein concentration showed denaturation profile similar to that of native BSA. It implies that in the absence of urea and Cys the freeze-thaw treatment of BSA in solution does not lead to any substantial change in its tertiary structure. In other words, the cold denaturation of BSA in water is reversible. Thus, the observed conformational changes of BSA in the cryogels are caused by network formation in the presence of denaturant/reductant mixtures.

3.5. On the chemical nature of intermolecular crosslinks in BSA-cryogels

Analyzing the data discussed in the sections 3.1-3.4 leads to the question on the possible mechanisms of the formation of

intermolecular disulfide links in the nodes of the 3D network of the gel phase in spongy BSA-cryogels produced within the space of UFLMP under the combined action of urea and Cys on albumin macromolecules. Indeed, at first glance, it is unclear how such SS-bonds can appear in the presence of the added free thiol, i.e. cysteine, especially when this reductant is in an excess (nos. 3*c* and 3*d* in Table I) with respect to the amount of disulfide bridges in the 'initial' globules of BSA?

For Cys feed concentration of 0.001 and 0.01 mol/L (nos. 3*a* and 3*b*, Table I) the reductant, as pointed in Section 3.2, was, respectively, in deficiency and close to molar equivalence with respect to the protein's intramolecular SS-bridges. Therefore, in these cases the formation of intermolecular disulfide links could occur *via* the well-known mechanism of thiol-disulfide exchange[27] as shown in the following scheme:

(i) ***(Prot)-SS-(Prot′) + (Cys)-SH → (Prot)-SS-(Cys) + (Prot′)-SH***

(ii) ***(Prot′)-SH + (Prot′′)-SS-(Prot′′′) → (Prot′)-SS-(Prot′′) + (Prot′′′)-SH → …,***

and so forth according to, say, relay-race sequence. In this scheme, ***Prot*** and ***Prot′*** are the segments of one protein chain, whereas ***Prot′′*** and ***Prot′′′*** are the segments of another chain. In parallel with such chemical reactions, the denaturing agent, i.e. urea, facilitates unfolding of BSA macromolecules thus making easier the diffusion of Cys towards the intramolecular SS-bridges inside the protein globules.

On the other hand, when the reductant amount exceeds manifold the amount of disulfides in the systems, i.e. at [Cys] = 0.1 and 0.7 mol/L in our case, some other and rather specific mechanism should act in order to "protect" the *de novo* formed interchain SS-links against the reduction by the excessive thiol.

Since the spatial polymeric network of the previously studied ovalbumin cryogels prepared in frozen urea-containing solutions is stabilized by the intermolecular hydrophobic crosslinks formed during such cryotropic gelation,[19] we supposed that a similar process occurs, along with the thiol-disulfide exchange, in the cryotropic gel-formation of BSA, as well. If it is so, the simultaneously taking place unfolding of BSA globules, liberating the "new" vacancies for hydrophobic interactions, and, as a result, the formation of interchain hydrophobic crosslinks can, all together, create the regions (domains) enriched with hydrophobic amino acid sequences that surround the above-mentioned *de novo* formed SS-bridges. We hypothesize that even in the case of excess of added Cys, such areas are able, sterically or because of increased hydrophobicity, to hinder somewhat the diffusion of water-dissolved reductant to these regions of the cross-linked covalent nodes in BSA cryogels, thus protecting them against the chemical cleavage in the highly viscous medium of unfrozen liquid microphase of the macroscopically-frozen reaction system. In turn, during defrosting of the system, the concentration of soluble components (both denaturant and reductant) sharply declines owing to dilution by the thawed solvent thus also sharply decreasing the probability of the reductive dissolution of such cross-linked nodes. Therefore, it was possible to solubilise the resultant BSA-cryogels immersed in high-concentrated urea, Gu·HCl or SDS solutions for swelling only when the strong SS-reducing reagent DTT was added to cleave the interchain disulfide crosslinks (Fig. 1).

Certainly, this explanation of the experimentally observed dissolution behaviour of the BSA-cryogels prepared in the presence of Cys and urea is still an assumption, which requires additional experimental confirmation. However, this working hypothesis is grounded, first, on the known data on the severe conditions required to denature BSA globules in solutions almost completely, when the concentration of urea should be higher than 5 mol/L, at least, and the reductant should be in a considerable excess.[20,28,32] This is so because such globules have very "conservative" tertiary structure with the majority of SS-bridges buried in the core areas of protein macromolecule,[15] except for the only bridge which is more spatially available.[33] Therefore, at low concentration of thiol-bearing reductant only the thiol-disulfide exchange reactions (which can lead to the oligomerization of BSA macromolecules in solutions) can occur.[28,33] The second argument in favour of the suggested mechanisms is the above-mentioned hydrophobic nature of the junction knots in the 3D network of ovalbumin cryogels.[19] That is why we suppose that in case of the formation of the (urea+Cys)-induced BSA-cryogels, the combination of both chemical (covalent) cross-linking of unfolded protein chains *via* the thiol-disulfide exchange reactions and non-covalent cross-linking *via* the hydrophobic interactions takes place. It results in the generation of the polymeric network nodes that include the intermolecular SS-bridges "immersed" in a microenvironment with hydrophobicity higher than that of the other segments of polypeptide chains, which, in turn, are well solvated in aqueous medium.

4. Conclusions

The wide-pore serum-albumin-based cryogels are of interest, similar to other reported proteinaceous cryogels,[1] as biocompatible gel matrices for biomedical and biotechnological purposes. Therefore, the recognition of fine mechanisms responsible for the formation of such cryogels, as well as revealing the factors influencing the properties and the structure of these biopolymeric materials, are important from both fundamental and applied viewpoints. In the present study it was shown that concerted action of denaturant (urea) and thiol-bearing reductant (cysteine) on BSA macromolecules in moderately-frozen aqueous systems leads to the formation of covalent cross-links between the denatured polypeptide chains, while the proper crosslinks are the *de novo* interchain disulfide bridges formed during such kind of gelation. Significant role of hydrophobic interactions in the stabilization of the 3D network of these cryogels is also assumed. The resultant BSA-cryogels possess a wide-pore sponge-like morphology with the size of gross pores ranging, depending on the cryostructuring regimes, from ~50 to ~150 μm. The optimum conditions for preparing such BSA-based cryogels were found to be as follows: [BSA] = 3-5 g/dL, [urea] = 0.5-2.0 mol/L, [Cys] = 0.01 mol/L, freezing temperature – within the range from -15 to -20°C. To the best of our knowledge, before these studies there were no data in the

scientific literature either on such mechanisms for the denaturant/reductant-induced formation of BSA-cryogels, or on the influence of various factors of the cryogel preparation process on their properties and microstructure of the resultant cryogels. This information was first obtained in the course of present study.

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Legend for graphical abstract:

Freeze-thaw cycle of serum albumin (BSA) – urea – cysteine solutions yields spongy cryogels. Combined denaturant and reductant action in frozen system causes unfolding of albumin globules and is accompanied by the formation of interchain disulfide bridges being the covalent nodes of the resulting polymeric network. Intermolecular hydrophobic interactions additionally facilitate the nodes' stability. Gross pores in such BSA-cryogels are of 50-150 μm in cross-section.

Graphical Abstract