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Cryostructuring of polymer systems. 44. Freeze-dried and then chemically cross-linked wide porous cryostructurates based on serum albumin

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Abstract: Spongy cryostructurates based on bovine serum albumin (BSA) have been prepared *via* freezing the aqueous solutions of the protein followed by freeze-drying and subsequent cross-linking BSA macromolecules each together within the macropore walls using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) dissolved in ethanol. The gel-fraction yield values testifies high efficiency (>93%) of the protein building-up into the 3D polymeric network. Poor swelling of the pore walls of BSA-based sponges in water (1–2 g H_2O per 1 g of dry polymer) and even in the powerful protein-solubilizing media (8 M urea, 5 M guanidine hydrochloride, 1% SDS) indicates the multipoint character of albumin cross-linking *via* the pendant peptide bonds. As a result, strong cross-linking is able (as revealed by HS-DSC) to inhibit BSA thermal denaturation. The size of wide pores in the obtained cryostructures ranges from 40 to 250 μm and mainly depends on the freezing temperature.

Keywords: cross-linking; cryostructuring; freeze-drying; macroporosity; serum albumin.

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1 Introduction

Various gel-like matrices based on biopolymers and synthetic polymers are now well-known to be widely used as advanced materials of biomedical and biotechnological interests (1–7). Among those, hydrogels fabricated from proteins, e.g. from collagen, gelatine, elastin, silk fibroin, different blood proteins, etc., are of especial significance for biomedical applications because of biocompatibility, non-toxicity, biodegradability and easy availability of such gel precursors (6). Rather often the respective gels have to be macroporous in order to ensure the non-hindered penetration of biological particles (e.g. viruses, cell organelles, whole cells) into the bulk of such polymeric gel materials as bioaffinity sorbents, carriers of immobilized cells, scaffolds for cell culturing, tissue engineering constructs, covers on wounds and burns, and so forth (8–11). In this respect, many approaches have been elaborated for generating macroporosity in the corresponding gel matrices. The examples of the most frequently used methods being the phase-separation (12), foaming (13), incorporation of porogens that can be leached or extracted from the material after completion of gelation (14, 15), cryotropic gel-formation (16, 17), freeze-drying (18, 19), etc. In the latter two cases the function of porogens is fulfilled by the polycrystals of frozen solvent (ice crystals in case of frozen aqueous solutions or colloidal sols). If the gel-formation occurs exactly in the frozen system within the volume of unfrozen liquid microphase (20), the final "products" of such process after the system thawing are the so-called *cryogels* (21, 22), while if the frozen solvent is removed *via* sublimation, i.e. freeze-drying, without any gelation of the constituting solutes, such a procedure results in the solid freeze-structured macroporous polymeric matrices that can be called by the term *cryostructurates* (17).

There are several reports on the cryogels fabricated from serum albumins [the major by mass protein in blood plasma (23)], when the gel materials thus obtained were

employed as carriers of immobilized cells and plant cell organelles (24–28), as tissue engineering cell scaffolds (29), components of cryostructured food formulations (30). In our previous studies the formation and properties of two types of proteinaceous cryogels based on bovine serum albumin (BSA) have been explored: the gelation took place either through the intermolecular thiol-disulfide-exchange-driven cross-linking of the unfolded (under the action of added denaturants) protein chains (31), or the albumin cryogels were prepared in the moderately frozen reaction systems through the covalent cross-linking of BSA macromolecules *via* pendant amide bonds using water-soluble carbodiimide (32). In both these cases we sought to create the 3D network with junction knots that contain so-called cross-links of "zero length" (33, 34) in order to avoid the incorporation of potentially toxic fragments of the cross-linking reagents into the chemical structure of the resultant protein cryogels. With that, both types of such albumin cryogels were the wide-porous sponges, the size of their large pores was, depending of freezing conditions, within the range from ~ 50 to ~ 150 μm. The protein concentration in the proper polymer phase, i.e. in the thin gel walls of macropores, was of about 10 times higher than these values in the feed solutions before their freezing. The last fact evidently demonstrates the cryoconcentrating effect (16, 17, 35, 36), that is, how considerably, upon the large amount of solvent is frozen out, the concentration of gel precursors in the unfrozen liquid microphase (where the gel-formation occurs) increases as compared with initial solution.

In this respect it was of interest also to prepare, using the same freezing regimes, the BSA-based cryostructurates. However, although simple freeze-drying of serum albumin aqueous solutions gives rise to the formation of wide-porous solid matter, the latter one is water-soluble. Hence, additional cross-linking is required in order to obtain the insoluble BSA cryostructurates. In the present study this was attained by the treatment of lyophilized solid uncross-linked samples with carbodiimide dissolved in ethanol, which, in turn, is the non-solvent for BSA, but is able for wetting the protein materials, thus ensuring the penetration of cross-linker into the inner bulk of polymeric phase. So, the main differences of the wide-porous serum albumin *cryostructurates* are under consideration in the present study and the BSA-based *cryogels* previously reported are as follows:

(i) No gelation proceeded during the preparation of the cryostructurates, when the initial BSA solution was frozen, kept frozen at the pre-set minus temperature and then freeze-dried, whereas in the above-mentioned previous cases the formation of cross-linked 3D polymeric network of the respective albumin cryogels occurred indeed in the frozen systems.
(ii) Covalent cross-linking of BSA cryostructurates has been carried out after the polymeric matter acquired cryogenically-induced wide-porous texture, which only then has been fixed chemically with the coupling reagent used, whereas in the case of above-mentioned BSA cryogels their macroporous "architecture" was formed in parallel with cryotropic gelation taking place quite in the moderately-frozen samples.

In whole, the goals of present work were the search for the conditions of sponge-like BSA cryostructurates formation, as well as the study of their physico-chemical properties and wide pore morphology as dependent on the precursors' concentrations and preparation regimes.

2 Materials and methods

2.1 Materials

The following substances were used as recieved from Sigma-Aldrich Inc. (St. Louis, USA): BSA (≥ 98%; catalog # A7906), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; catalog # E7750) and sodium dodecylsulphate (SDS; catalog # L3771). Urea (ultra pure grade) was from Reakhim (Moscow, Russian Federation); guanidine hydrochloride (> 99%) was from Helicon (Moscow, Russian Federation); 1,4-dithiotreitol (DDT) was purchased from Panreac Quimica S.A.U. (Barcelona, Spain); methylene blue dye was from Merck GmbH (Darmstadt, Germany). All aqueous solutions were prepared using water of Milli-Q quality.

2.2 Preparation of BSA cryostructurates

The 1-ml portions of BSA aqueous solution, which contained the protein in the concentration of either 30, or 40, or 40 mg/ml, were poured in the flat-bottomed glass vials (inner diameter of 18 mm) and frozen for 3 h in the chamber of a programmable precision cryostat F-32 (Julabo GmbH, Seelbach, Germany) at a pre-set minus temperature. Then the 3.9-mm-height frozen samples were lyophylized in a ALPHA 1–2 LD plus freeze-drier (Martin Christ, Osterode am Harz, Germany) for 18 h. After that, 1.5 ml of EDC ethanolic solution of necessary concentration was added to each vial, which was sealed and further incubated in a refrigerator at 5°C for 24 h with periodical shaking. Finally,

thus treated samples were rinsed with a 100-fold excess of water, incubated at room temperature for 24 h in the 0.1 M aqueous solution of glycine for blocking possible reactive residues of EDC-derived carboxyl groups of the protein and again rinsed with an excess of water.

2.3 Characterization of BSA cryostructurates

2.3.1 The yield of gel-fraction

The gel-fraction yield (Y) values were found according to the procedure described earlier (31). In brief, free capillary liquid was, at first, removed for 5 min under vacuum (water pump) from each equilibrium-water-swollen spongy sample placed onto a glass filter, a 145-g load being put on the top of the sample. Thus squeezed matter was weighed, frozen at – 20°C and freeze-dried followed by final drying to a constant weight at 105°C in a SNOL 24/200 oven (AB Utenos Elektrotechnika, Utena, Lithuania). Y values were calculated with a formula:

$$Y = (m_d : m_{th}) \cdot 100\%, \quad [1]$$

where m_d is the weight of the dried sample, m_{th} is the "theoretical" weight of the sample assuming that all the protein containing in the feed solution was incorporated into the 3D network of the resultant BSA cryostructurate. Some weight addition owing to possible grafting of glycine was not taken in account.

2.3.2 Swelling degree of the polymeric phase in spongy BSA cryostructurates

The values of water-swelling degree by weight ($S_{w/w}$) of the gel phase (the walls of macropores) for the samples of interest were calculated with a formula:

$$S_{w/w} = (m_{ws} - m_d) : m_d (\text{g } H_2O \text{ per 1 g of dry polymer}), \quad [2]$$

where m_{ws} is the weight of the squeezed BSA sponge after removal of free water, m_d is the weight of the dried sample.

2.3.3 Swelling behavior of BSA cryostructurates in denaturing media

Per one piece of spongy BSA cryostructurate was placed in each glass Petri dishes (dia. 90 mm) filled with 40 ml of aqueous solution of either urea (8 mol/l), or guanidine hydrochloride (5 mol/l), or SDS (1 wt.%), where the samples were incubated at room temperature for 24 h with periodical photographing of the sample's appearance. Then aqueous solution of DTT was added to each dish in the amount necessary to obtain the reductant concentration equal to 0.1 mol/l; the systems were kept 24 h and photographed again.

2.3.4 Macroporous morphology of BSA cryostructurates

The preparations for the microscopy studies were formed as the 1-mm-thick discs using plastic Petri dishes (38 mm inner diameter) as moulds; the cryoctructuring conditions were the same as indicated in Section 2.2. The BSA discs, prior to exploration, were water-swollen and stained by treatment for 1 min with 0.125 mM aqueous solution of methylene blue dye followed by rinsing with water. Porous morphology of BSA cryostructurates thus prepared was examined with the aid of optical stereomicroscope SMZ1000 (Nikon, Tokyo, Japan) equipped with a MMC-50C-M system (MMCSoft, St. Petersburgh, Russian Federation) for digital image recording. The linear size of macropores was measured by treatment of the images using Image J software (http://imagej.net).

The values of the numerical root-mean-cube diameter (D_n) and the weighted root-mean-cube diameter (D_w) of wide pores in the BSA cryostructurates were calculated as described elsewhere (31, 32, 37) using the ratios (38):

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

where $\sum N_i \geq 100$ is the sum amount of pores measured. The ratio D_w/D_n is the polydispersity index k.

2.3.5 DSC studies of BSA cryostructurates

A study of conformational state of BSA macromolecules built-in the cross-linked network of cryostructurates was performed by high-sensitivity differential scanning calorimetry (HS-DSC) using the procedure applied earlier for BSA cryogels (31, 32). In brief, the protein sponge samples were milled in water with a manual homogenizer of the Potter type followed by determining of protein concentration in the suspension after its drying at 105°C to a constant weight of solid residue. The suspension for calorimetric measurements with concentration of 5 mg/ml was prepared in the buffer solution (20 mM imidazole, 0.15 M NaCl, pH 7.4). Prior to HS-DSC experiments the suspension was incubated for 20 h at 5°C under continuous stirring. 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 10–90°C and at an excess pressure of 0.25 MPa. The data acquisition and the conversion of thermograms to the apparent partial heat capacity functions were performed using COMPORT and NAIRTA software, respectively (Institute of Organoelement Compounds, Moscow, Russian Federation).

3 Results and discussion

3.1 Preparation of EDC-cross-linked spongy BSA cryostructurates

The procedure used in this study for producing wide pore BSA cryostructurates included following sequential stages:

(i) preparation of feed aqueous solutions of BSA and their pouring in the moulds;
(ii) freezing the samples at a pre-set minus temperature;
(iii) freeze-drying such frozen systems;
(iv) incubation of the dried solid wide-porous cryostructurates in the ethanol solution of EDC for chemical tunning of proteinaceous material;
(v) rinsing the cross-linked spongy cryostructurate out of the soluble admixtures;
(vi) incubation of the BSA-based sponge in glycine solution for the deactivation of unreacted grafted EDC-residues;
(vii) final rinsing of the target cross-linked BSA cryostructurates.

The resultant samples represented soft white sponges that could be easily squeezed from free liquid upon moderate compression (Figure 1A) and quickly swelled with complete restoring their size and shape being again placed in water (Figure 1B). Such swelling behavior is typical for various wide-porous cryogels usually possessing the open interconnected pores. The interpore connections are formed since in the course of freezing each porogen crystal (ice in case of aqueous systems) grows till a tight contact with a facet of neighbouring another crystal, and upon defrosting the contact space is filled with thawed liquid (16, 17, 35, 39). With the respect of wide-pore morphology, the BSA cryostructurates under consideration turned out to be very similar to the earlier described BSA cryogels (31, 32).

All the stages (i)–(vii) were operationally simple, the main influence on the physicochemical properties of the resultant cryostructurates being exert the initial BSA concentration in the feed solutions at the stage (i) and the ratio BSA/EDC at the stage (iv) of protein cross-linking, while the freezing temperature during stage (ii) is the main factor influencing the macroporous morphology of BSA sponges. Exactly the effects of these three parameters on the characteristics of final biopolymeric matrices were explored in our study. Preliminary searching experiments allowed elucidating the working ranges of both the biopolymer concentration in feed solutions and freezing temperature that gave rise to BSA cryostructurates with a set of physical properties and texture capable of ensuring for the samples to withstand (without destruction) mechanical stresses upon squeezing-swelling cycles in the course of rinsing protein sponges at the stages (v) and (vii).

It was found that the most operationally stable BSA sponges were formed originating from the initial solutions with the protein concentrations from 30 to 50 mg/ml. At lower than the inferior value of this range the resulting cryostructurates had low strength and poorly maintained their shape after taken from the water excess. On the other hand, at initial BSA concentration higher than 50 mg/ml the resulting samples turned out to be non-uniform in their texture and rather fragile in a dry state. Analogous

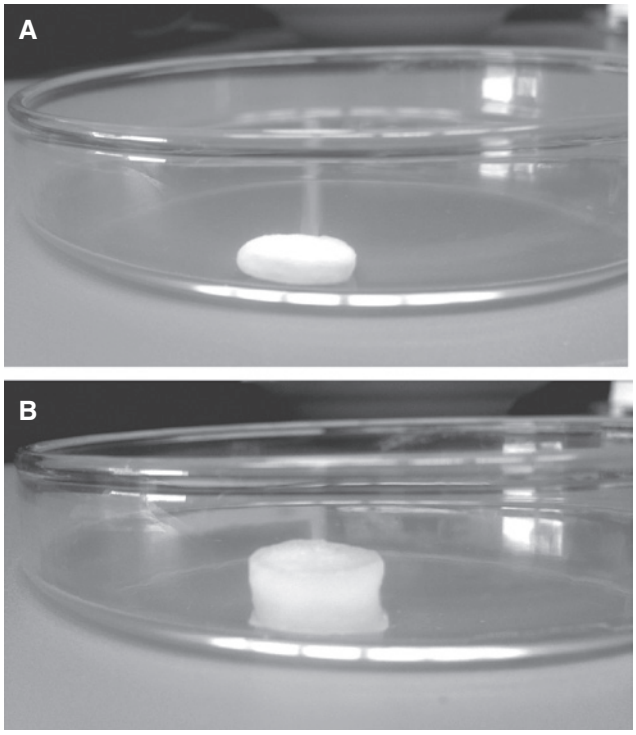

Figure 1: Appearance of BSA cryostructurate prepared by freezing of 40 mg/ml aqueous protein solution at – 15°C followed by freeze-drying, cross-linking with EDC in ethanol medium at the 1 : 3 n_{COOH}/n_{EDC} ratio and then rinsing with water: (A) the sample after water squeezing; (B) the sample re-swollen in water.

preliminary trials regarding freezing conditions showed that when the minus temperature of cryogenic processing was higher than – 15°C the initial BSA solutions, especially of 50 mg/ml concentration, did not, rather often, freeze because of the supercooling effects, whilst if the freezing temperature was as low as – 25°C, the pore size in the obtained cryostructurates fell down drastically. Therefore, in the subsequent experiments we froze feed BSA solutions at one of the temperatures over the range from – 15 to – 25°C (see Section 2.2).

The indicator used for the evaluation of the efficiency of BSA embedding into the 3D network of final cryostructurates was the parameter *Y* (the gel-fraction yield accordingly eq.1), and it was found that these values depend to the certain extent, but not very markedly, on such factors as the polymer concentration in initial solutions at the stage (i), freezing temperature at the stage (ii) and BSA/EDC ratio upon cross-linking of the cryogenically structured protein at the stage (iv). In all the cases, within the examined ranges of the indicated variables, the efficiency of BSA cross-linking was high and exceeded 93% (Table 1). The results obtained are presented here as a cumulative Table (and further in Table 2, as well) rather than some set of plots since the amount of data is large, and the intervals of experimental error (as we observed) overlap each together in such plots thus interfering to differ their relation to the respective points on many curves.

The *Y* values in Table 1 testify that the majority of the protein macromolecules presented in the initials solutions was, in fact, incorporated in the cross-linked network of polymeric phase of final spongy material, and the amount of the sol-fraction, which did not built-in the walls of macropores, was rather small. Also, a slight growth of *Y* took, as a rule, place with increase in the amount of coupling reagent, i.e. EDC, added to the freeze-dried BSA-based matter. In some cases the gel-fraction yield was found even to exceed 100% (the examples in Table 1 indicated by the index [b]), especially when large excess of EDC was applied.

We believe that possible reason for the latter results could be some weight addition at the stage (vi) due to grafting of glycine moieties (Figure 2D) to those O-acylisourea residues (Figure 2C) that were unable to participate in the formation of intermolecular peptide crosslinks because of the lower amount of lysine ω-NH_2-groups comparing to the number of pendant aspartate and glutamate

Table 1: Influence of the initial BSA concentration, the temperature of freezing and the ratio protein/cross-linker on the gel-fraction yield of the resultant BSA cryostructurates.

BSA concentration in initial solution (mg/ml)	$n_{COOH} : n_{EDC}$ (mol/mol)[a]	*Y* values (%) for BSA cryostructurates formed at freezing temperature		
		−15°C	−20°C	−25°C
30	1 : 0.5	96.1 ± 0.6	94.5 ± 0.5	93.3 ± 0.2
	1 : 1	98.0 ± 0.7	95.3 ± 0.1	94.7 ± 0.5
	1 : 2	99.2 ± 0.3	95.4 ± 0.2	95.7 ± 0.6
	1 : 3	100.0 ± 0.2[b]	95.9 ± 0.3	96.0 ± 0.8
	1 : 4	99.9 ± 1.3[b]	96.9 ± 1.1	95.7 ± 0.5
	1 : 6	101.0 ± 1.0[b]	98.4 ± 0.2	96.8 ± 1.5
40	1 : 0.5	95.3 ± 0.3	95.0 ± 2.0	94.9 ± 0.7
	1 : 1	96.3 ± 1.6	95.5 ± 0.7	95.3 ± 0.9
	1 : 2	97.1 ± 0.1	97.0 ± 0.7	97.9 ± 0.1
	1 : 3	99.1 ± 1.1	98.3 ± 0.3	96.8 ± 2.6[b]
	1 : 4	100.2 ± 0.2[b]	98.8 ± 0.6	100.5 ± 0.3[b]
	1 : 6	101.0 ± 0.2[b]	99.3 ± 0.9	103.9 ± 5.3[b]
50	1 : 0.5	93.9 ± 0.1	95.2 ± 0.6	95.7 ± 0.5
	1 : 1	94.8 ± 0.4	94.9 ± 1.9	96.8 ± 0.8
	1 : 2	95.6 ± 0.4	95.4 ± 0.8	98.3 ± 1.1
	1 : 3	96.5 ± 0.6	93.5 ± 3.9	97.9 ± 0.1
	1 : 4	98.7 ± 0.5	96.7 ± 1.7	97.7 ± 0.7
	1 : 6	99.4 ± 0.9	96.6 ± 2.9	98.0 ± 0.2

[a] $n_{COOH} : n_{EDC}$ is the molar ratio of COOH-groups in BSA to the amount of EDC at the process stage (iv) upon cross-linking of the protein.
[b] It is believed that when *Y* values exceeded 100%, it could be the consequence of some weight "additive" owing to grafted glycine moieties (see Experimental, Section 2.2).

Table 2: Influence of the initial BSA concentration, the temperature of freezing and the ratio protein/cross-linker on the swelling extent of the polymeric phase in the resultant wide-porous BSA cryostructurates.

BSA concentration in initial solution (mg/ml)	$n_{COOH} : n_{EDC}$ (mol/mol)[a]	$S_{w/w}$ values (%) for BSA cryostructurates formed at freezing temperature		
		−15°C	−20°C	−25°C
30	1 : 0.5	1.23 ± 0.05	1.43 ± 0.17	1.20 ± 0.20
	1 : 1	1.24 ± 0.06	1.38 ± 0.13	1.37 ± 0.16
	1 : 2	1.94 ± 0.20	1.48 ± 0.03	1.39 ± 0.26
	1 : 3	1.48 ± 0.18	1.66 ± 0.09	1.67 ± 0.28
	1 : 4	1.41 ± 0.12	1.54 ± 0.17	1.85 ± 0.14
	1 : 6	1.36 ± 0.08	1.39 ± 0.10	1.46 ± 0.18
40	1 : 0.5	1.34 ± 0.08	1.34 ± 0.11	1.45 ± 0.03
	1 : 1	1.58 ± 0.06	1.43 ± 0.06	1.30 ± 0.08
	1 : 2	1.53 ± 0.08	1.56 ± 0.19	1.19 ± 0.06
	1 : 3	1.42 ± 0.02	1.65 ± 0.26	1.07 ± 0.14
	1 : 4	1.24 ± 0.16	1.96 ± 0.36	1.01 ± 0.03
	1 : 6	1.36 ± 0.05	1.33 ± 0.25	0.92 ± 0.13
50	1 : 0.5	1.20 ± 0.04	1.32 ± 0.06	1.09 ± 0.31
	1 : 1	1.27 ± 0.13	1.30 ± 0.22	1.43 ± 0.11
	1 : 2	1.45 ± 0.14	1.61 ± 0.16	1.24 ± 0.15
	1 : 3	1.45 ± 0.03	1.47 ± 0.21	1.11 ± 0.10
	1 : 4	1.42 ± 0.13	1.47 ± 0.10	1.03 ± 0.18
	1 : 6	1.41 ± 0.05	1.39 ± 0.08	0.94 ± 0.06

[a] $n_{COOH} : n_{EDC}$ is the molar ratio of COOH-groups in BSA to the amount of EDC at the process stage (iv) upon cross-linking of the protein.

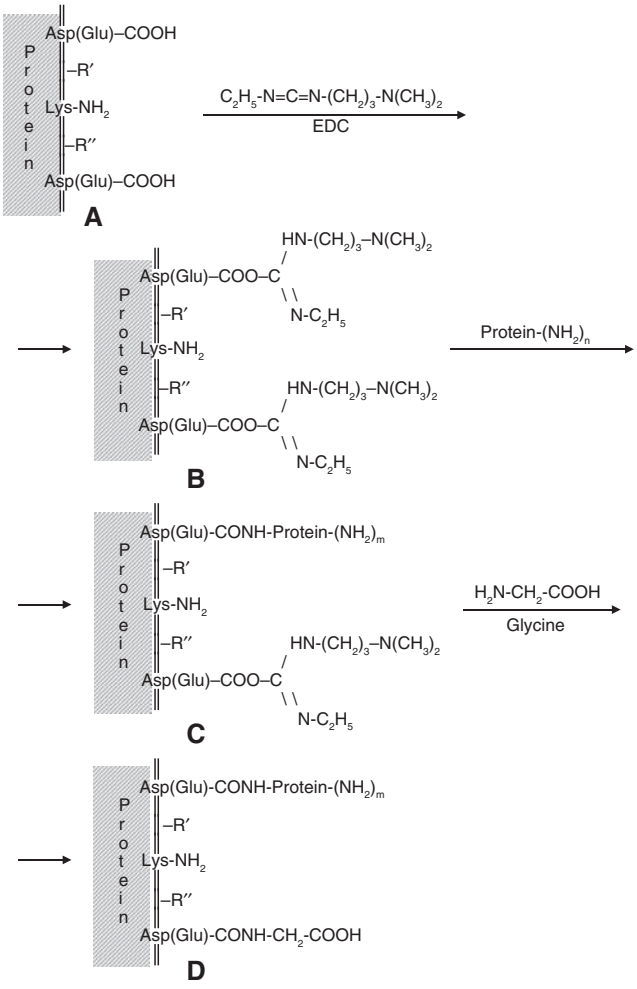

Figure 2: The scheme of reactions occurring upon the BSA macromolecules cross-linking and chemical modification with an excess of EDC at the process stage (iv) followed by the inactivation of residual O-acyl-isourea functions using glycine at the process stage (vi) [the scheme is based on the known mechanisms of peptide coupling induced by EDC (33, 40)]: (A) – BSA macromolecules in the composition of freeze-dried cryostructurate; (B) – O-acyl-isourea derivative of the protein formed upon reaction of EDC with pendant COOH-groups of aspartate and glutamate, and C-end carboxyl group; (C) – cross-linked BSA network containing grafted "excessive" O-acyl-isourea groupings; (D) – cross-linked BSA network containing grafted glycyl residues ($m << n$).

COOH-groups, and also because of sterical unavailability of some of the reactive residues inside the bulk of freeze-dried cryostructurate.

3.2 Swelling behavior of EDC-cross-linked BSA cryostructurates

As it is commonly known, the swelling extent of covalently cross-linked spatial polymeric networks is a rather sensitive indicator of their cross-linking density, i.e. the higher the number of cross-links in the network, the lower its swelling capability (41). Therefore, swelling behavior of BSA cryostructurates in several aqueous media was searched in this study, both qualitatively and quantitatively (in pure water).

It is also worthy to point out that, based on the data of the samples swelling in the liquid milieus capable of selective splitting definite types of intra- and intermolecular bonds, it is possible to judge about the involvement of one or another sort of non-covalent or covalent interactions in the stabilization of spatial polymeric network. As owing to the chemical hetero-functionality of pendant groups in proteins, different types of interactions exist in the proteinaceous gels, certainly including the BSA-based ones, we examined the influence of the following media on the swelling behavior of cross-linked BSA cryostructurates. The tests were carried out in: (I) 8 M aqueous solution of urea which is well-known to disrupt hydrogen bonds; (II) 5 M aqueous solution of guanidine hydrochloride which heavily interferes hydrogen and ionic bonding in proteins; (III) 1wt.% aqueous solution of sodium dodecylsulfate (SDS) affecting the hydrophobic interactions; and (IV) in the same three solutions additionally containing 0.01 M of dithiotreitol (DTT) – the reductant of the disulfide bonds. The results of these experiments (for the procedure used see Section 2.3.3) are shown in the pictures of Figure 3.

Analogous testing was performed previously for two kinds of BSA cryogels cross-linked, respectively, *via* the intermolecular thiol-disulfide exchange (31) and through the intermolecular peptide bonds (32). As in different media such samples swelled differently, often very strongly; or were dissolved in the case of the former cryogels after the addition of DTT, this allowed drawing conclusions on the nature of the links in the nodes of 3D networks of the polymeric phase in these gel matrices. However, in the case of BSA cryostructurates under consideration in the present study, only not very significant upswelling was observed in the denaturant solutions (I), (II) and (III). After the 24 h-incubation in these liquid media the samples' volume somewhat increased, in a larger extent in the urea solution (I), and then for the subsequent 24 h did not virtually changed (not shown in Figure 3). With that, the size of the samples was independent on the type of immersion medium. Even in the presence of DTT which had to cleave the intramolecular SS-bridges in BSA globules thus opening vacancies for chain unfolding and additional swelling of the samples in the media (IV), no marked changes in the sponge volume were registered, maybe, except for the SDS + DTT case where BSA cryostructurate swelled at some larger extent. Such results obviously indicated that

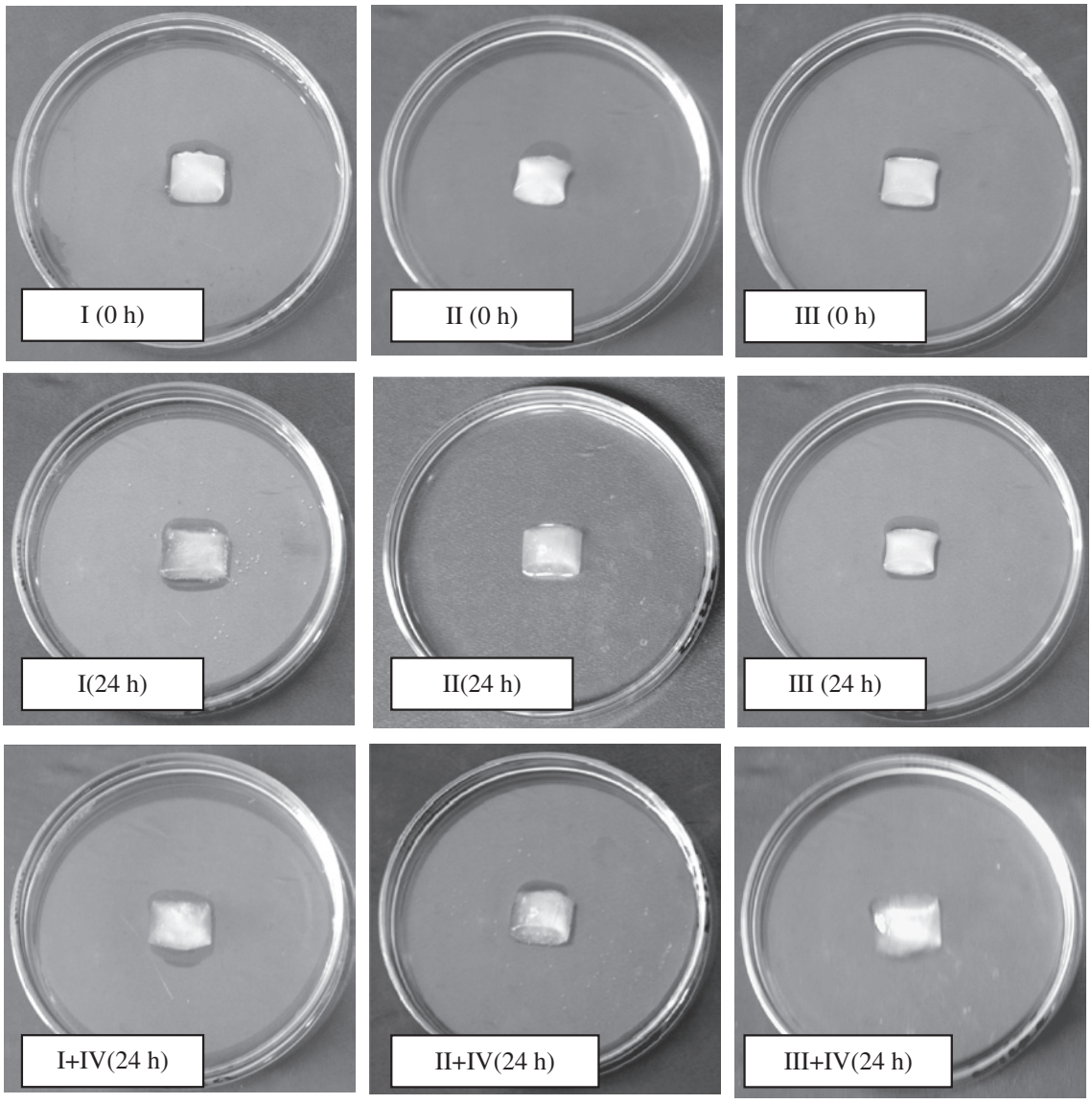

Figure 3: Photographs showing the swelling behaviour of the samples of BSA cryostructurate prepared by freezing of 40 mg/ml aqueous protein solution at – 15°C followed by freeze-drying, cross-linking with EDC in ethanol medium at the 1 : 3 n_{COOH}/n_{EDC} ratio and then rinsing with water (denotations of swelling media are given in the text).

albumin macromolecules in the composition of the examined cryostructurates were cross-linked each together and, obviously, intramolecularly, as well, extremely thick by the EDC-induced multiple peptide bonds in side chains, and thus formed multipoint "nodes" did not allow the globules to be unfolded to a significant extent.

The data on the $S_{w/w}$ values of the water-swollen polymeric phase in such spongy BSA cryostructurates also confirm the latter conclusion on their dense cross-linking. In general, these values (Table 2) were rather low, of the order from ~ 0.9 to ~ 2 g H_2O per 1 g of dry polymer, that is, for the cross-linked protein in the composition of gel phase within the walls of macropores was accounted from ~ 52 to ~ 33% by weight, i.e. a very high. In other words, when BSA macromolecules within the concentrated polymeric phase of freeze-dried matter have been cross-linked with EDC in ethanol medium, the resulting covalent network of the walls of macropores appeared to possess very high cross-linking density and, as a consequence, poor ability to swell even in such thermodynamically good solvent for BSA as water.

As for the dependence of cross-linked BSA cryostructurates' swelling characteristics on the preparation conditions, it was found that the lowest $S_{w/w}$ values (0.9–1.1 g/g) were peculiar to the samples formed by freezing of the 40–50 mg/ml BSA solutions at the lowest used temperature

– 25°C. The character of the influence of EDC concentration in the cross-linking solution (expressed in Table 2 as a molar ratio of protein-belonging COOH-groups to the carbodiimide) on swelling degree of the resultant BSA cryostructurates turned out to be variable. For all the samples, whose freezing was carried out at – 15 and – 20°C, such dependence passed through a maximum, and its position was the function of BSA/EDC ratio. For the samples prepared by freezing of 30- and 50-mg/ml BSA solutions at – 25°C the tendency was the analogous, but when the 40-mg/ml albumin solution was frozen at – 25°C the $S_{w/w}$ values systematically decreased with increasing the coupling agent concentration at the cross-linking stage (vi). The reason for this latter result is not yet clear. Also, we were unable to reveal evident trend with respect of the freezing temperature influence on the swelling characteristics of the prepared BSA cryostructurates, when the samples with equal concentration of the precursors have been compared. In some cases the dependences in the series: – 15, – 20 and – 25°C turned out to be descending, in other cases – ascending, and sometimes the dependences were bell-like in their shape (Table 2) in spite of the fact that all equi-concentrated samples after their freezing at different temperatures were then lyophilized under identical conditions. From the one hand, such results testified on the impact of freezing temperature *per se* on the swelling capability of polymeric phase of the resultant protein sponges. But, on the other hand, the observed ambiguous trends indicated on the sophisticated, multifactor influences of the process parameters on the properties of final BSA cryostructurates. Thus, initial BSA concentration controls the amount of protein in the pore walls of the freeze-dried matter and, obviously, the thickness of the walls; freezing conditions affect the amount of frost-solidified ice and macroporous morphology of such albumin sponges (see Section 3.3), while the EDC concentration at the processing stage (vi) governs the cross-linking density, as well as the amount of O-acyl-isourea residues (Figure 2C) and then grafted glycyl substituents (Figure 2D) in the final BSA cryostructurates. Apparently, because such factors exerted influence on the properties of cryostructured albumin sponges in different extent and in a competitive fashion, this could be the reason for the above-mentioned ambiguous trends.

3.3 Features of the wide-pore morphology of BSA cryostructurates

As it was already noted above, the characteristic feature of various polymeric cryogels and cryostructurates is their macroporous morphology generated by the porogens, i.e. by the polycrystals of frozen solvent (16–19, 39). Different factors are known to affect the porosity characteristics of such polymer matrices. These factors include type of the solvent, nature and concentration of initial solutes, and certainly, regimes of cryogenic processing (42, 43). The pore size is mainly governed by the freezing temperature, since the lower the temperature, the smaller ice crystals are formed and their amount is higher (44) providing that the supercooling effects do not interpose in the crystallization dynamics thus influencing the "pore size-freezing temperature" dependence (37).

Recorded with optical stereomicroscope micrographs in Figure 4 exemplify the spongy structure of

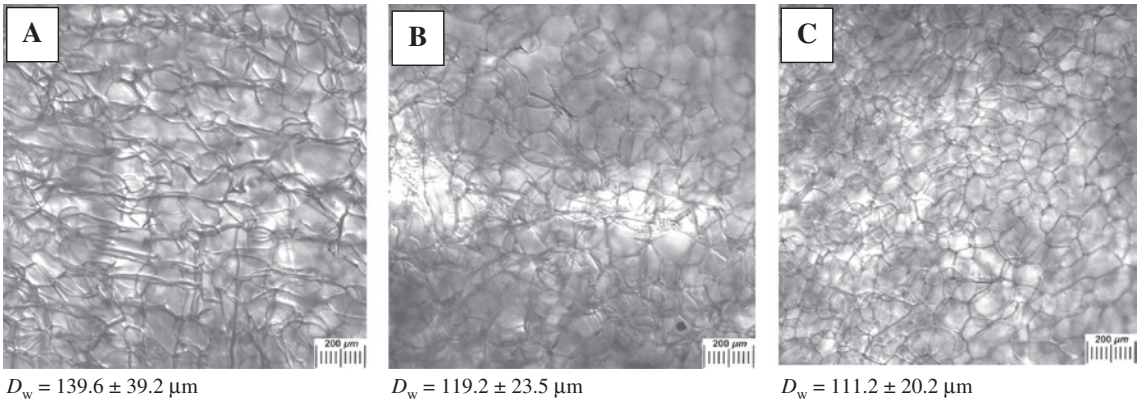

Figure 4: Optical stereomicroscopy images of the 1-mm-thick discs of BSA cryostructurates prepared by freezing of 40 mg/ml aqueous protein solution at – 15°C (A), – 20°C (B) and – 25°C (C) followed by freeze-drying, cross-linking with EDC in ethanol medium at the 1 : 3 n_{COOH}/n_{EDC} ratio and then rinsing with water.

water-swollen 1-mm-thick discs of such albumin cryostructurates. These are the samples prepared by freezing of initial 40-mg/ml-BSA solution at – 15, – 20 and – 25°C followed by lyophilization and treatment with coupling reagent at the 1 : 3 n_{COOH}/n_{EDC} ratio. The pictures show rather heterogeneous wide pore web-like texture of the samples, where the dark structural components are the walls of large pores (the swollen polymeric phase), and the lighter areas are such pores themselves. The thickness of the pore walls does not exceed 5–15 μm, and the cross-section of the capillary-size pores ranges from about 40 to 250 μm; particular values of morphometric indexes D_n, D_w and k are given under the corresponding micrographs. These data show certain decrease in the average pore size with lowering the freezing temperature, the common trend described many times for the cryogenically structured matrices based on synthetic or natural polymers, prepared in frozen aqueous, as well as in crystallizable organic media, cross-linked by covalent bonds or by the non-covalent interactions (16, 17, 21, 22, 39, 42, 43).

In general, similar wide-porous texture as seen in the pictures of Figure 4 is typical for the freeze-dried polymeric matter (18, 19), and the individual structural peculiarities are dependent on the specific combination of the parameters of preparation conditions. The overall morphology and pore size of the freeze-dried and then EDC-cross-linked BSA cryostructurates prepared in the present study appeared to be also very similar to analogous properties earlier observed for the equi-concentrated with respect of BSA cryogels that were fabricated over the same range of minus temperatures, when the coupling reagent was introduced in the feed protein solution prior to its freezing (32). This similarity evidently testifies that the major factor defining macroporous characteristics of both the cryogels and the cryostructurates is the freezing/frozen storage regime. At the same time, values of the root-mean-cube diameters D_n and D_w for the BSA cryostructurates turned out to be larger by the factors of about 1.5–2 in comparison with the pore size of the respective, i.e. formed originated for the equi-concentrated feeds and at the same freezing temperatures, BSA cryogels. Meanwhile, polydispersity indexes k were very close for both cases. These facts testify, if to compare with the cryotropic gel-formation occurring upon BSA cross-linking in the moderately frozen reaction system, that ice sublimation results in the additional stronger compaction of the polymeric phase thus widening the pores in the freeze-dried matter, which was then tightly cross-linked chemically.

3.4 Conformation status of BSA macromolecules within the cross-linked network of polymeric phase in the wide pore cryostructurates

Taking into account the above discussed data on a rather low swelling of the polymeric phase in walls of macropores of the BSA cryostructurates both in water (Table 2) and even in the strong solubilizing media (Figure 3), high polymer concentration and multipoint character of cross-linking of the protein within the 3D network (Section 3.2), it was of interest to evaluate the conformational state of BSA macromolecules in such albumin sponges. These studies have been accomplished using HS-DSC (for the procedure see Experimental, Section 2.3.5).

Thermograms in Figure 5 show temperature dependences of the apparent partial heat capacity for the following samples: (1) commercial serum albumin used in this work upon the preparation of BSA cryostructurates; (2) an albumin specimen prepared by freezing of the 40 mg/ml aqueous BSA solution at – 15°C followed by freeze-drying and subsequent treatment of the wide-pore dry matter with the EDC-free ethanol under the same conditions that were employed for cross-linking of the protein; (3) BSA cryostructurate prepared under the freezing – freeze-drying conditions analogously to the case (2) and then cross-linked with EDC at the 1 : 3 n_{COOH}/n_{EDC} ratio.

Traces (1) and (2) contain the denaturation maxima that testify unfolding of BSA globules. The peak

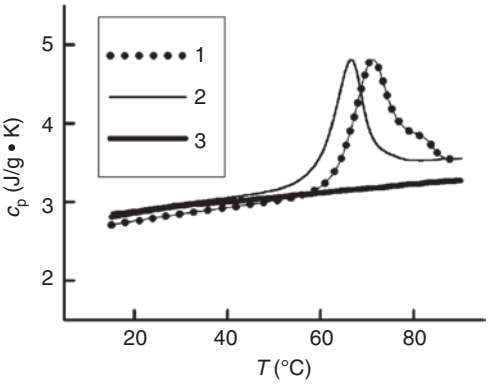

Figure 5: Apparent partial heat capacity functions of BSA solutions and cryostructurate in the medium of 20 мм imidazole, pH 7.4, 0.15 м NaCl: 1 – solution of BSA commercial sample; 2 – solution of BSA subjected to freeze-drying followed by the treatment with pure ethanol; 3 – BSA cryostructurate prepared by freezing of 40 mg/ml aqueous protein solution at – 15°C followed by freeze-drying and cross-linking with EDC in ethanol medium at the 1 : 3 n_{COOH}/n_{EDC} ratio.

temperatures for these samples are, respectively, 71.0°C and 66.4°C, and their denaturation enthalpies are 15.8 J/g and 11.8 J/g. In addition, the denaturation curves of the samples (1) and (2) differ in their shape: the thermogram for the solution of commercial BSA is of bimodal, while the thermogram of BSA, which was treated in a dry state with ethanol, is of monomodal. Such differences most probably relate to different contents of higher fatty acids in the samples (1) and (2). The fatty acids commonly present in the reagent-grade albumin preparations owing to a rather strong their binding to hydrophobic pockets of the protein. Such binding induces a considerable increase in the conformational stability of BSA macromolecules, which is pronounced in the relatively higher values of denaturation temperatures and enthalpy as compared with fatty-acids-free albumin specimens (45). The removal of the fatty acids usually leads to lowering the denaturation temperature and decreasing the denaturation enthalpy. As a rule, similar removal is not so easy, it can be reached using special procedures, e.g. by the prolonged treatment of albumin solutions with active charcoal (46). In our case, decreasing of the denaturation temperature and enthalpy of BSA after its treatment with ethanol in a dry state, i.e. transformation of the sample (1) to the sample (2), seemed to result in a marked removal of the fatty acids thus exhibiting in the observed character of thermogram of the sample (2). It is also necessary to point out that introducing ethanol into the solutions of various albumins is able to induce almost complete unfolding of protein globules even at room temperature (47). Therefore, the fact of preservation of tertiary structure of BSA after the treatment with ethanol of the freeze-dried albumin emphasizes again a very significant role of water in the solvent denaturation of proteins (48).

As for the thermal behavior of the EDC-cross-linked wide-porous BSA cryostructurate, the absence of the denaturation heat absorption peak in the thermogram [trace (3) in Figure 5] could point to the preservation of the major elements of tertiary structure of the protein within the temperature range used in the calorimetric experiments. We believe that this fact is a consequence of "sealing" the structure of BSA macromolecules by multiple covalent cross-links formed upon the action of EDC in high concentrated protein phase of pore walls of the spongy freeze-dried matter. Just such multipoint cross-linking, along with rather probable intramolecular that, increases apparently the conformational stability of BSA globules being tightly inserted in the spatial polymeric network of these pore walls.

4 Conclusions

There are many approaches for creating polymeric gel matrices (in general) and hydrogels (in particular) that possess macroporous morphology. Characteristic feature of the cryostructuring method, where the polycrystals of frozen solvent act as porogens, is the ability to result in the formation of interconnected pore system. It is because the growth of a particular facet of each crystal stops when it comes into a tight contact with some facet of the neighbor growing crystal, thus giving rise to the connections between the "future" macropores. In the present study this approach was employed to produce the sponge-like albumin-based cryostructurates by freezing aqueous 30–50-mg/ml-solutions of the protein at either −15, or −20, or −25°C followed by freeze-drying the samples and subsequent cross-linking of BSA macromolecules each together within the matter of macropore walls using EDC dissolved in ethanol. The evaluation of the gel-fraction yield values showed high efficiency (>93%) of the protein macromolecules building-up into the 3D polymeric network thus formed. In addition, poor swelling of the pore walls of these heterophase BSA-based sponges in water (1–2 g H_2O per 1 g of dry polymer) and even in the powerful protein-solubilizing liquids (8 M urea, 5 M guanidine hydrochloride, 1% SDS) indicates to the multipoint character of albumin cross-linking *via* the EDC-induced pendant peptide bonds. As a result, such strong cross-linking is able, how it was revealed in the HS-DSC experiments, to inhibit BSA thermal denaturation. The size of wide pores in the BSA cryostructures thus obtained ranges from about 40 to 250 μm and mainly depends on the freezing temperature. As such albumin cryostructurates consist of the proteinaceous matter only, i.e. do not contain any non-protein inclusions, these BSA-based sponges could be of biomedical interest. The respective studies are in progress now in the authors' team.

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