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Influence of succinylation of a wide-pore albumin cryogels on their properties, structure, biodegradability, and release dynamics of dioxidine loaded in such spongy carriers

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ABSTRACT

The goal of this study was to reveal how the chemical modification, succinylation in this case, of the wide-pore serum-albumin-based cryogels affects on their osmotic characteristics (swelling extent), biodegradability and ability to be loaded with the bactericide substance – dioxidine, as well as on its release. The cryogels were prepared via the cryogenic processing (freezing – frozen storage – thawing) of aqueous solutions containing bovine serum albumin (50 g/L), denaturant (urea or guanidine hydrochloride, 1.0 mol/L) and reductant (cysteine, 0.01 mol/L). Freezing/frozen storage temperatures were either −15, or −20, or −25 °C. After defrosting, spongy cryogels were obtained that possessed the system of interconnected gross pores, whose shape and dimensions were dependent on the freezing temperature and on the type of denaturant introduced in the feed solution. Subsequent succinylation of the resultant cryogels caused the growth of the swelling degree of the pore walls of these spongy materials, resulted in strengthening of their resistance against of trypsinolysis and gave rise to an increase in their loading capacity with respect to dioxidine. With that, the microbiological tests showed a higher bactericidal activity of the dioxidine-loaded sponges based on the succinylated albumin cryogels as compared to that of the drug-carriers based on the non-modified protein sponges.

1. Introduction

Cryogels based on synthetic or/and natural macromolecular compounds are the macroporous polymeric gels formed in the non-deeply frozen systems that contain corresponding precursors [1–4]. Currently, many types of cryogels are widely used in various applied fields, commencing from biology and medicine [2,3,5–12], biotechnology [1–3,14–25], cell and tissue engineering [3,26–34], chemical catalysis and biocatalysis [35–40], sorption processes [41–48], carriers of immobilized nanoparticles [49–51], smart polymers [52–54], up to ecology [55–57], food technologies [58–60], construction of engineering structures in the permafrost regions [61,62], etc.

All polymeric cryogels are the heterophase macroporous gel materials, since during their formation the polycrystals of frozen solvent, ice crystals in case of cryotropic gelation in frozen aqueous media, play the role of porogens [1–4]. The properties of various cryogels and the spatial "architecture" of their pores are dependent on many factors. In particular, the factors that are of fundamental importance [1,63–65], include the chemical nature and concentration of precursors, the type of dispersion medium (solvent) and its cryoscopic properties, the presence or the absence of certain additives, both soluble and/or insoluble (dispersed fillers), their content, and also the temperature/time regimes of all stages of the cryogenic processing. The latter conditions are as follows: the temperature and cooling rate in the course of initial gelling system freezing, the regimes for keeping the samples frozen, the dynamics of their heating during defrosting, and the number of cryogenic treatment cycles [1,63–69]. By varying these parameters of cryotropic gelation, it is possible to modify widely the physicochemical characteristics of the resulting cryogels and their macroporous morphology,

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thereby adapting the totality of the polymeric material properties to the requirements of a particular area of its use for solving practical tasks.

In the last decade, the largest number of publications dealing with the applied potential of various polymeric cryogels has been related to the biomedical aspects of these materials use (see books and reviews [2,3,10–13,30,32–34,69–71]). This concerns both the non-biodegradable cryogels based mainly on synthetic polymers (e.g., physical cryogels of poly(vinyl alcohol) [8,11,12]) and the matrices that, when being placed into the patient's body, either decompose (for instance, via the enzymatic hydrolysis), or are disintegrated as a result of gradual dissolution. The cryogels prepared from various globular or fibrillar proteins are related exactly to the biodegradable materials [71]. The well-known examples of similar cryogenically-structured matrices that are hydrolyzed by the proteolytic enzymes of animals and humans are the cryogels based on gelatin [71–75] and serum albumin [71,76–79]. For instance, the efficiency of using the antibiotic-loaded albumin cryogels as the antibacterial sponges for the treatment of infected superficial and deep wounds has been demonstrated [79]. In this case, a high-dose antimicrobial agent attacks the pathogenic microorganisms directly in the infected wound, and the biopolymeric carrier of the drug is cleaved rather rapidly into harmless peptides and amino acids.

In certain cases, the lifetime of similar carriers can be of significance, therefore it would be of necessity to have a tool for controlling, e.g. elongation, the biodegradation dynamics. In this respect, the approach based on the chemical modification of proteins [80,81] in order to increase somewhat the resistance of the carrier matrix to the proteolysis was considered by us as such an useful tool. So, validation of this approach was the main aim of the present study. Its objects were the spongy proteinaceous cryogels based on serum albumin and their derivatives chemically modified by the succinylation. The in vitro enzymatic degradability of such biomaterials was examined, as well as the dioxidine loading in the albumin sponges and the release of this bactericide from such carriers was evaluated.

2. Experimental section

2.1. Materials

The following substances and reagents were used in the experiments without additional purification: bovine serum albumin (**BSA**) (≥98%); urea (**URE**) (ultra grade); succinic anhydride (≥99%); sodium hydrocarbonate (≥98%) (all from Sigma-Aldrich, USA); guanidine hydrochloride (**GHC**) (>99.5%; Helicon, Moscow, Russian Federation); L-cysteine (**CYS**) (ultra grade) (Fluka, Switzerland); 0.25% trypsin solution (Trypsin-EDTA 1×) (Gibco, Canada); sterile physiological solution (Nikopharm, Russian Federation); 2,3-bis-(hydroxymethyl) quinoxaline-N,N′-dioxide (dioxidine) (98,9%) (MIR-Pharma Co., Russian Federation).

All aqueous solutions were prepared using Milli-Q water; for rinsing albumin cryogels the boiled deionized water was employed.

2.2. Methods

2.2.1. Synthesis of BSA cryogels

Spongy BSA-based cryogels have been prepared essentially in accordance with the procedure described elsewhere [77]. In brief, BSA powder was dissolved in water to prepare the solution of the protein, followed by the addition and dissolution of required amounts of URE or GHC and CYS. The final solution was poured in the 2 mL-portions in the plastic Petri dishes with a 35 mm inner diameter. The dishes were sealed and placed onto the horizontal cooling platform connected with a MPC-K20 ultracryostat (Huber, Germany). The samples were frozen and incubated at a pre-set minus temperature for 20 h, and then immersed in water bath for 15 min to defrost the contents of the dishes. Further the resultant cryogel discs (~2 mm thickness) were removed from the dishes and rinsed at 6 °C for one day in a 100-fold excess of pure water, which was changed for a fresh portion every 8 h. The albumin cryogel discs thus treated were frozen at −20 °C and then lyophilized using an ALPHA 1–2 LD plus freeze-drier (Martin Christ, Germany). The final dry samples were stored in the hermetically-sealed vials at 6 °C.

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

$$Y = (m_{dry} : m_{theor}) \cdot 100\%, \quad (1)$$

where m_{dry} is the weight of the dry sample, m_{theor} is the 'theoretical' weight of the sample calculated by assuming that all the initial protein amount was incorporated in the 3D network of the resultant cryogel.

2.2.2. Succinylation of BSA cryogels

Each freeze-dried disc was weighed and immersed in 5 mL of water for swelling, further 10 mg of sodium bicarbonate was introduced to adjust the pH value to about 8, and then succinic anhydride was added in such amount that its molar excess regarding the amount of amino-groups in the protein was equal to 4. The reaction mixture was gently shaken at 6 °C for one day followed by rinsing and drying of the succinylated sample analogously to the non-modified albumin sponges (see Section 2.2.1).

2.2.3. FTIR studies of non-modified and succinylated samples

The water-swollen albumin cryogels were freeze-dried and then additionally dried in vacuum desiccator over the burned $CaCl_2$ granules. FTIR spectra of the samples thus prepared were recorded on a Vertex 70 V Fourier spectrometer (Bruker, Germany) using a Pike ATR accessory with a diamond crystal (Nicolet, USA); the ATR spectra were averaged from 128 scans over a range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. All necessary corrections were done using an Omnic 8 program package (Nicolet, USA). The IR spectra of the dioxidine-loaded samples were recorded with a Bruker Tensor II spectrometer (Germany) with an ATR platinum attachment.

2.2.4. Characterization of cryogels samples

The swelling by weight ($S_{w/w}$) of the polymeric phase (the walls of macropores) of spongy non-modified and succinylated cryogels was measured gravimetrically as described earlier [77]. To this end, the free liquid was removed from a water-swollen albumin sponge on a glass filter under vacuum (water-jet pump). The sample "squeezed" in this way was weighed and dried in an SNOL 24/200 air thermostat (AB Utenos Elektrotechnika, Lithuania) at 105 °C to a constant weight. The $S_{w/w}$ values were calculated as follows:

$$S_{w/w} = (m_{wet} - m_{dry})/m_{dry} \text{ (g } H_2O \text{ per 1 g of dry polymer)}, \quad (2)$$

where m_{wet} is the weight of the wet squeezed sample and m_{dry} is the weight of the dried sample.

2.2.5. In vitro modeling of the biodegradation of albumin-based cryogels

The respective freeze-dried cryogel discs were cut to the segment-shaped fragments that were weighed and immersed individually into the 0.05% (w/v) aqueous solution of trypsin taken in an amount required to provide the enzyme-to-substrate ratio (w/w) equal to 1:500. The samples were incubated with gentle shaking at room temperature. After certain time intervals one partially degraded piece was removed from the reaction solution, rinsed with water and dried analogously to the case of the $S_{w/w}$ evaluation (Section 2.2.3). The biodegradation extent was expressed as the percent of the residual (R) dry matter amount at the current time point of trypsinolysis:

$$R = (m_{dry\_curr}/m_{dry\_init}) \cdot 100\%, \quad (3)$$

where m_{dry_curr} is the current dry weight of the sample and m_{dry_init} is the initial dry weight of the same sample.

2.2.6. Dioxidine loading in and release from the albumin-based spongy cryogels

The freeze-dried albumin discs were placed in an 1% (*w*/w) aqueous solution of dioxidine for swelling at room temperature for 30 min, then frozen at −30 °C and finally freeze-dried for 22 h at the pressure in the chamber $(8–6)\cdot10^{-2}$ Torr.

The kinetic curves for the release of the drug from the dioxidine-loaded sponges into the distilled water were recorded at λ = 375 nm with a Jasco V-770 spectrophotometer (Jasco, Japan).

2.2.7. DSC experiments

Differential scanning calorimetric studies of the freeze-dried dioxidine-loaded samples were carried out on a DSC 204 F1 Phoenix instrument (NETZSCH, Germany). Each sample of 5–10 mg weight was placed in an aluminum crucible. The temperature profile was as follows: heating from 25 to 400 °C with a rate of 10 K/min in the argon atmosphere.

2.2.8. H^1-NMR

The chemical shifts of the initial and cryogenically modified dioxidine were identified using a high resolution NMR-spectrometer VXR-400 (VARIAN, USA). The test samples were in the D_2O medium.

2.2.9. Microstructure of the wide-pore BSA-cryogels

The microstructure of water-swollen albumin cryogels was studied with an optical stereomicroscope SMZ1000 (Nikon, Japan) equipped with an MMC-50C-M system (MMCSoft, Russian Federation) for digital image recording. Prior to the experiments the disc gel samples were stained by treatment for 1 min with 0.125 mM aqueous solution of methylene blue dye followed by exhaustive rinsing the discs with water.

2.2.10. Antibacterial properties of the dioxidine-loaded albumin sponges

Determination of the antibacterial activity of the samples was carried out by the disc-diffusion method according to the earlier described procedure [49,50] using the albumin discs (4 mm in diameter and 2 mm in height) cut from the samples initially prepared in the 35-mm Petri dishes. Bacterial cells *E. coli* 52 and *S. aureus* 144 (Catalog of the collection of microorganisms of the Department of Microbiology, Biological Faculty, Moscow State University) were used as the test cultures. The experiments were carried out in Petri dishes containing 20 mL of agar nutrient medium dried during the day (thickness of the medium layer 4 mm). The bacterial cells of both test cultures in an amount of 10^8 have been seeded onto each 90-mm agar dish for further disc-diffusion experiments. Measurement of growth inhibition zones of the test cultures was carried out after 24 h of incubation. Statistically reliable results were obtained by a nine-fold repetition of the growth inhibition zones measurements for each series of samples.

3. Results and discussion

3.1. Spongy cryogels based on serum albumin

The procedure used in this study for the preparation of BSA-cryogels is grounded on the finding [76] that cryogenic processing (freezing – frozen storing – thawing) of the serum albumin aqueous solutions that also contain additives of denaturant and thiol reductant is able to result in the formation of spongy cryogels. It was shown that their 3D-polymeric network is supported by the de-novo-linked intermolecular disulfide bridges in the junction nodes [77]. In the present study, serum albumin cryogels were synthesized starting from the feed solutions that contained the gel precursors in the following concentrations: BSA – 50 g/L, URE or GHC – both 1.0 mol/L, CYS – 0.01 mol/L. These compositions were selected on the basis of preliminary experiments, where it was found that the resultant BSA cryogels possessed stable enough sponge-like texture and thus being the most convenient for further manipulations in comparison with the samples prepared at a lower (0.5 mol/L) and a higher (1.5–2.5 mol/L) denaturant concentrations.

The 2-mL-portions of the feed solutions were dosed in the plastic Petri dishes and frozen for 20 h either at −15, or −20, or −25 °C, then defrosted and rinsed with water from the sol-fraction. The attempts to prepare similar cryogels via the initial liquid systems freezing at −10 °C did not lead to the reproducible results mainly because of the supercooling effects, when very often the solutions did not freeze at all.

The gel-fraction yield for the samples formed with URE additives at −15 and −20 °C was a rather high, ~80–90%, while in the case of cryogels prepared at −25 °C a marked decrease in the cryotropic gel-formation efficiency ($Y \approx 40\%$) was observed (Fig. 1A, white columns). Such result testified that the processes responsible for the unfolding (denaturation) of albumin globules and thiol-disulfide-induced intermolecular cross-linking commenced to be significantly inhibited at the temperatures below −20 °C. One of such factors, as it was early shown for the water-ovalbumin-urea system [82], is a partial crystallization of urea at reduced temperatures, since the eutectic point of the water-urea system lies in the vicinity of −9 °C. Such solidification of urea decreases its concentration in the non-frozen liquid microphase, thus lowering the ability of the denaturant solution to unfold the protein globules. The same conclusion on a decrease in the efficiency of intermolecular cross-linking with lowering the cryotropic gelation temperature can also be drawn from the data on the swelling characteristics of BSA-based cryogels formed at analogous negative temperatures (Fig. 1B, white columns). The swelling ability of the cross-linked polymeric networks is essentially dependent on their cross-linking degree, i.e., the lower is the amount of interchain cross-links, to a higher level the respective spatial network will swell [83]. Therefore, an increase in the $S_{w\backslash w}$ values from ~1.8 to 6–8 g/g for the walls of macropores in these cryogels with lowering the cryotropic gelation temperature from −15 to −25 °C evidently points to a decrease in the amount of

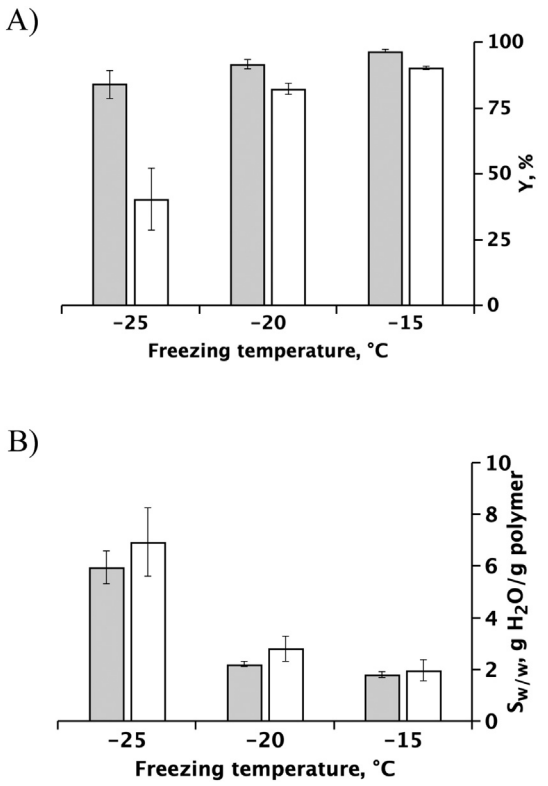

Fig. 1. Gel-fraction yield (A) and swelling degree (B) of the albumin-based cryogels prepared in the presence of 1-molar URE (white columns) or GHC (grey columns) in the aqueous BSA solutions (50 g/L) frozen at different negative temperatures.

the cross-links within the final 3D-network. These results also testify that the temperature interval, where the formation of such albumin cryogels occurs with high performance, is a rather narrow, namely, of about 5–7 °C.

In the case of albumin cryogels formed with GHC additives, the trend to a decrease in the gel-fraction yield with lowering the cryostructuring temperature from −15 to −25 °C was exhibited in a lesser extent: *Y* values declined from ~97 to ~84% (Fig. 1A, grey columns). In parallel, swelling capability of the polymeric phase in the respective spongy samples grew from about 2 to ~6 g of H_2O per 1 g of the protein core (Fig. 1B, grey columns). GHC is well-known to be a stronger than URE denaturant for proteins [84,85] because of the ability of GHC not only to cleave the hydrogen bonds but also to affect the ionic interactions. Therefore, a negative influence of temperature lowering on the BSA cryotropic gelation in the presence of GHC, which possesses a higher "denaturation power", is exhibited weaker compared to the URE-caused effects, since the efficient unfolding of albumin globules is the necessary condition for the formation of such cryogels [77].

The (denaturant + reductant)-induced cryotropic gel-formation of BSA gives rise to the proteinaceous sponges that have the interconnected capillary-size pores whose shape, dimensions and spatial "architecture" depend on both the conditions of cryogenic processing and the composition of the initial solution prior to its cryostructuring. Thus, microphotographs (optical stereomicroscope) in Fig. 2 show the porous morphology of the BSA-based cryogel discs formed in the presence of 1-molar additives of URE (A and B) or GHC (C and D) by freezing at −15 °C (A and C) and −20 °C (B and D). The samples contrasted by dyeing with methylene blue (see 'Experimental section') are in a water-swollen state; the dark areas in the black-and-white images of Fig. 2 are the polymeric walls of macropores, and the light areas are the macropores themselves filled with water.

These micrographs visually testify that such albumin cryogels are the heterogeneous wide-pore spongy matrices whose macroporous morphology is dependent on the cryotropic gelation temperature and on the nature of the denaturant used, i.e. URE or GHC. Since the succinylation did not cause any noticeable changes in the structural features of the respective cryogels, their micrographs are not shown. Lowering the cryotropic gelation temperature from −15 to −20 °C (that is, only by 5 Centigrade) in the case of cryogels formed with URE additives resulted in a decrease in average cross-section of macropores from 50 to 110 μm (Fig. 2A) to 20–70 μm (Fig. 2B). At a qualitative level, such an effect is typical of the processes giving rise to the formation of various polymeric cryogels [1–3,16,17,63–67], since the lower is the freezing temperature, the smaller are the solvent polycrystals thus formed that act as porogens (see 'Introduction').

However, in the case of BSA-based cryogels prepared with additives GHC a quite different trend was observed. If the roundish shape of macropores (average cross-section of 60–140 μm) in the samples formed at −15 °C (Fig. 2C) was similar to the cryogels prepared with URE additives at the same temperature (Fig. 2A), the macroporous morphology of spongy gel samples formed at −20 °C in the presence of 1-molar GHC turned out to undergo a significant transformation: such pores acquired the shape of oblong ellipsoid with the minor axis average size of 15–50 μm and the major axis up to 100–400 μm and even longer (Fig. 2D). This fact evidently points to the unequal influence of GHC on the ice crystallization at different freezing temperatures. Since the higher amount of the solvent is frozen-out upon decrease of the temperature, the solutes concentration in the so-called unfrozen

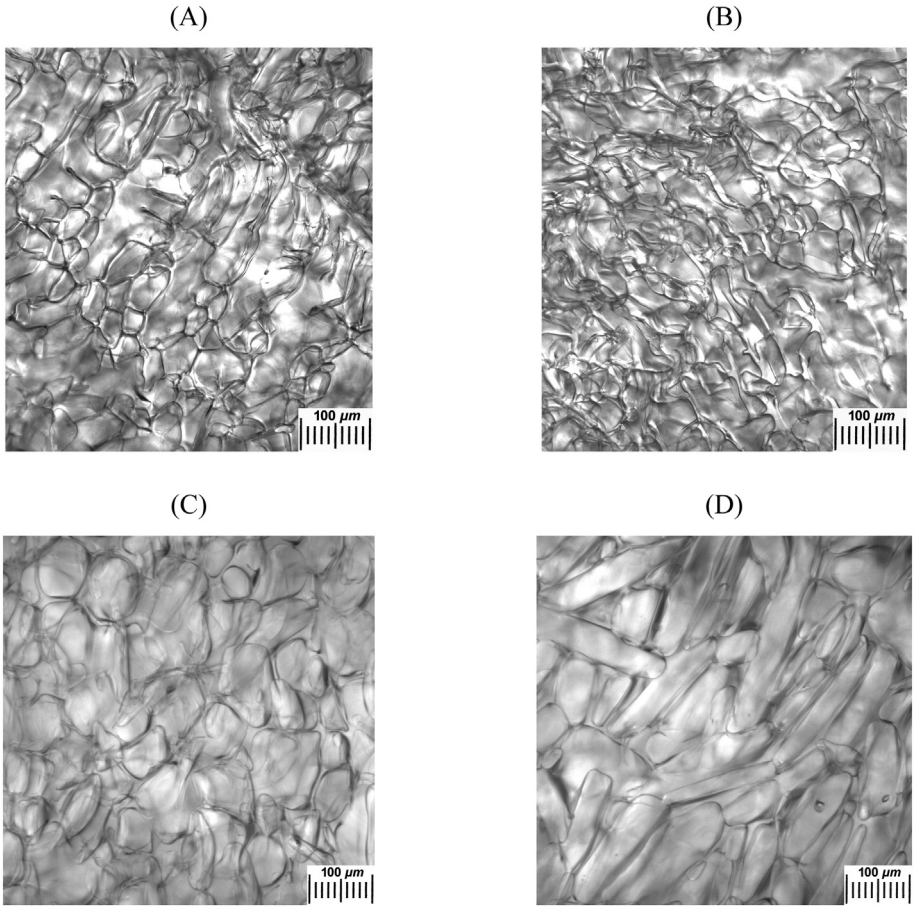

Fig. 2. Micrographs (optical stereomicroscope) of the albumin-based cryogels prepared in the presence of 1-molar URE (A and B) or GHC (C and D) in the aqueous BSA solutions (50 g/L) frozen at −15 (A and C) or −20 °C (B and D).

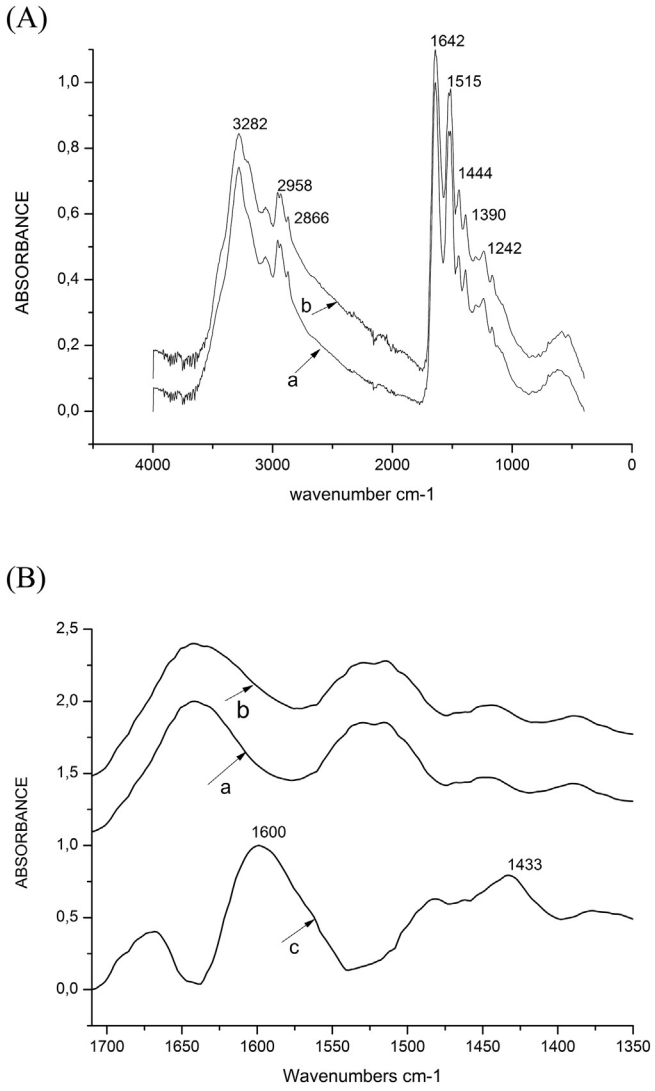

Fig. 3. FTIR spectra in the wavenumber ranges of 4000–400 cm^{-1} (A) and 1710–1350 cm^{-1} (B) of the non-modified (**a**) and succinylated (**b**) dried albumin-based cryogel samples, as well as the result (**c**) of the spectra subtraction, i.e. (**b**) − (**a**).

microphase [1,92] increases and it can start, commencing from a certain GHC concentration level, to affect markedly the growth dynamics of different facets of ice crystals. No doubts, this phenomenon requires additional separate exploration, which was out of the frameworks of the present study.

In any case, the micrographs in Fig. 2 show that albumin-based cryogels that were formed in the non-deeply-frozen protein solutions containing also the denaturant (URE or GHC) and the thiol reductant (CYS), possess a sponge-like morphology with the system of interconnected wide pores. Such 3D structure of these heterophase matrices allows loading the sponges with the solutions of desired substances by a very simple immersion of the material in the respective liquid. Quite similar procedure has been used in this work for loading the cryogenically-structured albumin sponges with the bactericide agent, dioxidine. The results of these experiments are discussed later in the Section 3.4.

3.2. Chemical modification of BSA cryogels by their succinylation

The succinylation (mainly, be means of the N-acylation of lysyl amino-groups) is popular, experimentally simple, non-dangerous toxicologically and rather widely implemented method for the modification of physicochemical and functional properties, as well as the enzymatic digestibility, of various proteins including food and medicamentous proteins [80,86]. For instance, succinylation of the plant protein glycinin has been applied for increasing its foaming ability [87], and aqueous solution of succinylated gelatin (trade mark 'Gelofusin®') is a therapeutic agent for the replacement of blood plasma [88]. Therefore, the use of this type of chemical modification of the albumin-based cryogenically-structured spongy cryogels that are of interest for the biomedical application should not cause any undesirable toxic effects.

In this study both types of BSA cryogels, i.e. protein sponges formed in the presence of URE or GHC, have been subjected to chemical modification of this type. With that, the 4-fold molar excess of succinic anhydride relatively to the amount of NH_2-groups in the protein was introduced into the reaction. The fact of succinylation has been confirmed by the FTIR spectroscopy; the osmotic properties of the resultant succinylated specimens have been evaluated through the measurement of their swelling characteristics.

Thus, Fig. 3A shows the FTIR spectra of the dried non-modified albumin cryogel (**a**) and the respective succinylated sample (**b**). Both spectra are very similar and typical of proteinaceous substances. Broad absorption with the maximum at 3282 cm^{-1} extending to 2500 cm^{-1} belongs to NH modes of protonated amino groups. The Amide I and Amide II bands at 1642 and 1515 cm^{-1}, respectively, show that the succinylation accomplished under the conditions used in this study does not affect significantly the chemical composition of the modified albumin sponges as compared to that of the non-modified ones.

It is known that the Amide I and Amide II bands are a result of coupling of C═O stretches with NH deformational modes; besides, the bands of carboxylate groups are observed in the same range and can overlap with the amide bands. Thorough examination of the bands in Fig. 3B shows that the Amide I band at 1642 cm^{-1} of the succinylated sample (**b**) is somewhat broadened as compared to that of the non-modified sample (**a**). The differential spectrum (**c**) representing the subtraction result (b)-(a) shows that the Amide I and Amide II bands the of the modified sample contain, as compared with the corresponding spectrum of the non-modified sample, two new bands at 1600 and 1433 cm^{-1} that could be assigned to the asymmetric and symmetric modes of carboxyls in the succinate fragments. Thus, the FTIR data could be considered as one of the evidences for the succinylation consequence. In addition, the same conclusion can be drawn from the comparison of swelling characteristics peculiar for the non-modified and succinylated samples.

Since the succinylation shifts the isoelectric point of a protein towards the acidic zone because of the replacement of the basis amino groups for the carboxylic ones, the osmotic characteristics (swelling

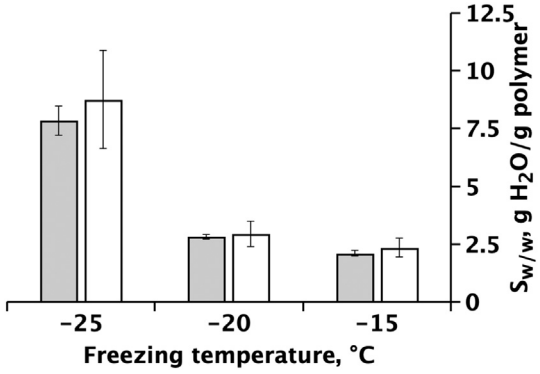

Fig. 4. Swelling degree of the succinylated albumin-based cryogels prepared in the presence of 1-molar URE (white columns) or GHC (grey columns) in the aqueous BSA solutions (50 g/L) frozen at different negative temperatures.

ability) of thus modified BSA cryogels are changed. The graphs in Fig. 4 presents the $S_{w\backslash w}$ values for all tested samples that prior to the succinylation were formed at −15, −20 and −25 °C.

As expected, the polymeric phase of succinylated spongy cryogels swelled somewhat higher (by 15–30%) than in case of the respective non-modified samples. Therewith, it turned out that in contrast to the non-modified cryogels (Fig. 1b) the differences in the $S_{w/w}$ values for the samples gelled cryogenically at equal negative temperature, but under the action of URE/CYS or GHC/CYS, virtually disappeared after the succinylation of these samples (Fig. 4). Most probably, it was stipulated by a higher hydration ability [89] of carboxylic groups (coordination number of five) grafted to the albumin chains instead of the initially less hydrated amino-functions (coordination number of three), thus smoothing not very strong differences in swelling degrees. In any case, both types of the albumin cryogels retained their integrity after succinylation and did not lose a sponge-like texture.

3.3. Modeling of the biodegradation of non-modified and succinylated BSA cryogels

The studies of the enzymatic hydrolysis of BSA-based cryogels had the goal to compare the dynamics of the trypsin-induced decomposition of non-modified and succinylated protein sponges. In the preliminary experiments it was found that the most convenient for the manipulation with the tested samples was the enzyme-to-substrate ratio equal to 1:500 (*w*/w); in this case it was possible to track the biodegradation process up to almost 80% completion. The results of such measurements are given in Fig. 5 as the respective diagrams for the non-modified and succinylated albumin cryogels formed either in the presence of URE (Fig. 5A), or GHC (Fig. 5B).

It turned out that under the above-indicated conditions both types of the non-modified albumin cryogels were decomposed enzymatically rather quickly – for about 3 days (Fig. 5, grey columns). At the same time, succinylated samples were markedly more resistant with respect to the trypsin-catalyzed hydrolysis: for the same 3 days the sponges lost no >10–12% of the initial dry matter content, and to the lapse of one week around 40–50% of these albumin sponges remained undigested (Fig. 5, white columns). Calculated values of the half-life for the proteinaceous cryogels of these types upon their trypsinolysis were as follows:

(i) non-modified freeze-thaw gel formed in the presence of URE – 38 h;
(ii) succinylated cryogel derived from the sample (i) – 192 h;
(iii) non-modified freeze-thaw gel formed in the presence of GHC – 42 h;
(iv) succinylated cryogels derived from the sample (iii) – 151 h.

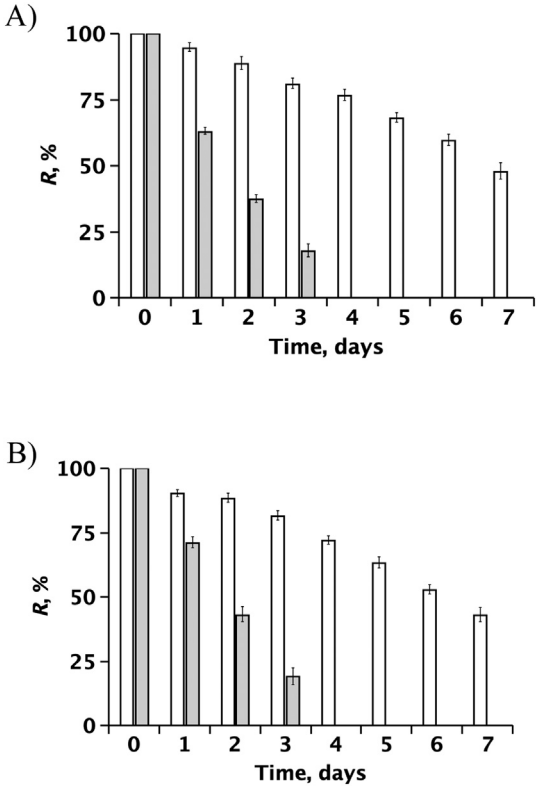

Fig. 5. Trypsin-induced degradation of the non-modified (grey columns) and succinylated (white columns) albumin-based cryogels prepared in the presence of 1-molar URE (A) or GHC (B) in the aqueous BSA solutions (50 g/L) frozen at −20 °C.

These results evidently testify that the suitability of the initial idea of this study was confirmed, thus showing the opportunities to vary the biodegradation rate of the serum-albumin-based spongy cryogels by applying the chemical modification (the succinylation in this case) approach.

The reasons for the observed deceleration of the trypsin-catalyzed hydrolysis of the succinylated BSA cryogels are sufficiently clear and can be explained from the knowledge on the substrate specificity of trypsin. Since this enzyme splits amide bonds in proteins and peptides after at the basic lysyl or arginyl monomeric units [90], the succinylation of the pendant lysyl ε-NH_2-groups lowers the amount of the recognition sites for the enzymatic attack, i.e. “deteriorates” the substrate for this enzyme. As a result, the proteolysis efficiency decreases. The primary structure of BSA macromolecules is known to include 583 amino acid units, among them are 60 lysyl units [91], thus, there is a wide range for the variation of modification degree. It is also evident that the extent of similar modification can be adapted to the desirable level by a simple selection of the modifying reagent concentration and/or the reaction duration.

3.4. Dioxidine loading into the non-modified and succinylated BSA cryogels and release of the bactericide from such spongy carriers

In order to impart the antimicrobial properties to the cryogenically-structured albumin-based sponges, the respective non-modified and succinylated biopolymeric matrices have been loaded with the bactericide agent, dioxidine (see Experimental Section 2.2.6). The main goal of this part of our study was to demonstrate the potential of similar sponges as the drug carriers and to show their possibilities to the efficient drug release. These experiments were carried out using the albumin cryogels prepared from the feed solutions that contained BSA (50 g/L), URE (1 mol/L) and CYS (0.01 mol/L). The dioxidine amount in the loaded sponges was found gravimetrically as the difference in the weight of the freeze-dried drug-loaded sponge and the weight of the respective freeze-dried non-loaded sample. The values thus determined were as follows (in milligrams of dioxidine per 1 g of dry sponge): 79 ± 1 mg/g in the case of non-modified albumin sponges and 114 ± 2 mg/g for the succinylated samples.

The IR spectra of both above-indicated types of loaded carriers turned out to be virtually akin (Fig. 6A). They contain bands that inherent in the BSA matrices themselves, e.g. the Amide I and II peaks (Fig. 3A), and, in addition, the spectra of dioxidine-loaded samples also have the bands related to the drug substance (Fig. 6B). Namely, these are the set of peaks at 1050–1020 cm^{-1} refers to plane deformation vibrations of the CH groups of aromatic circle, vibrations at 974 and 825 cm^{-1} relating to the off-plane deformation vibrations of these groups; the band at 1110 cm^{-1} associating with the C-O-H group, and the peak at 1320 cm^{-1} relating to the NO group. With that, the signal at 1515 cm^{-1} is related to the Amide II band and quinoline ring vibration in the cryomodified form of dioxidine. The latter one is formed as

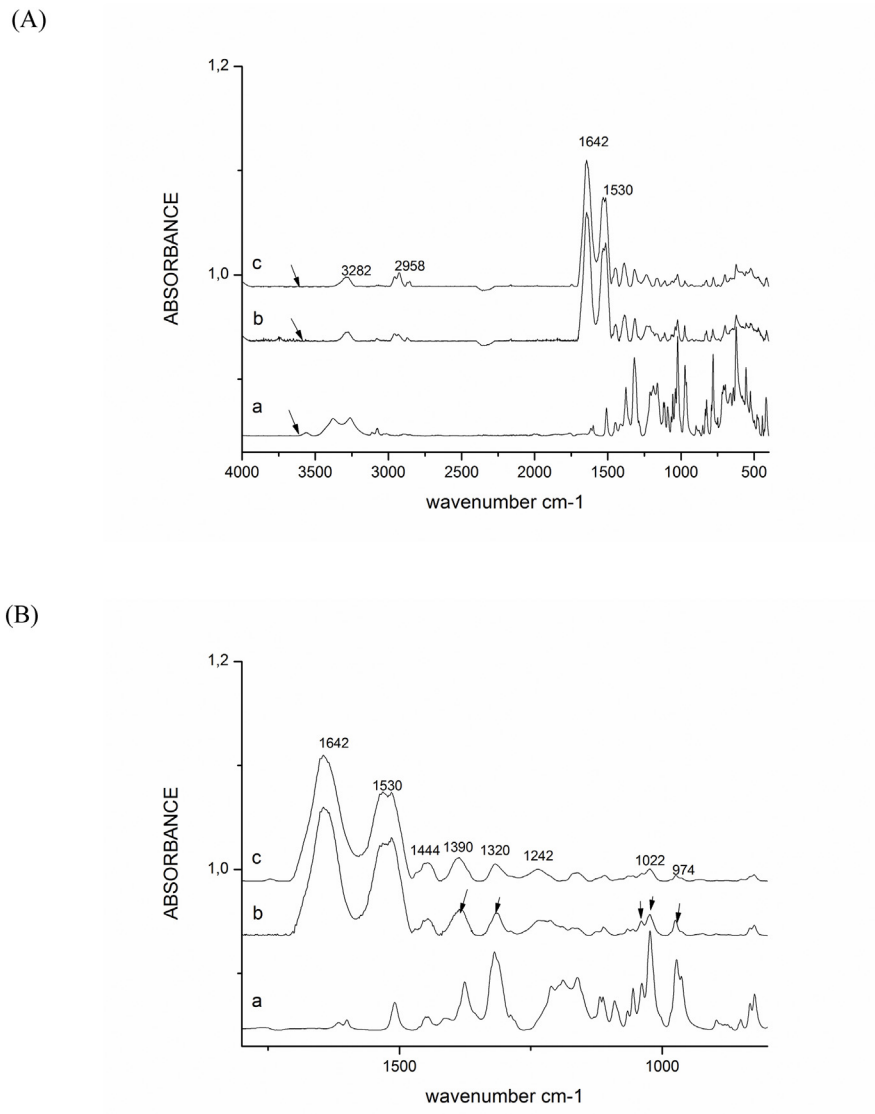

Fig. 6. IR spectra of the systems of dioxidine with non-modified (**a**) and succinylated (**b**) albumin-based cryogenically-structured sponges: 4000–400 cm^{-1} wavenumber range (A) and 1600–800 cm^{-1} wavenumber range (B).

a result of freezing and freeze-drying of dioxidine aqueous solutions; this form mainly contains the amorphous phase, some amount of hydrated crystalline phase and trace amount of anhydrous monoclinic crystal polymorph phase [92,93]. Broad absorption with the maximum at 3280 cm^{-1} is concerned both with the NH modes of protonated amino groups in the protein and with the OH groups of dioxidine, which are linked into poly-associates, which is also characteristic for the dioxidine cryomodified form.

The presence of dioxidine in the drug-loaded sponges was also confirmed in the DSC experiments (Fig. 7). The thermograms obtained for the non-modified and succinylated albumin-based samples, both the dioxidine-bearing, contained two broad endothermic peaks. The first one is associated with the loss of water from the matrix at 355 K, and the second one at 503 K is concerned with thermal decomposition of the protein matrix. In turn, sharp exothermic peaks in both thermograms at 446 K relate to the thermal decomposition of dioxidine, and such peak is typical for this substance [92,93].

As it was indicated in the 'Introduction', the cryogenically-structured serum-albumin-based sponges are of biomedical interest as the biodegradable carriers for various medicaments, when such drug-loaded sponges are implemented for the treatment and healing of infected wounds [79]. In this regard, it was necessary to compare the character of the dioxidine release from the bactericide-bearing non-modified and succinylated cryogels in order to reveal possible differences induced

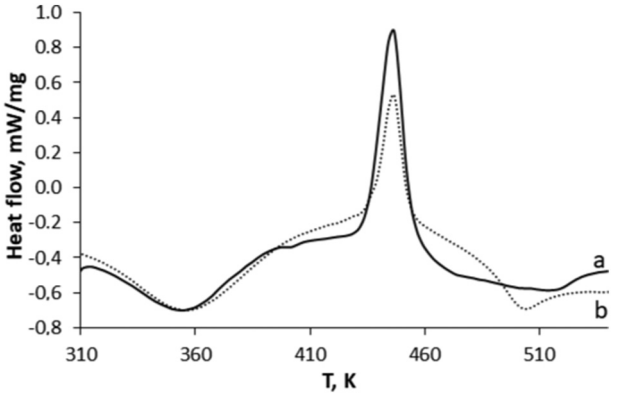

Fig. 7. DSC thermograms for the non-modified (**a**) and succinylated (**b**) albumin-based cryogenically-structured sponges both loaded with dioxidine.

by the succinylation. The respective experiments have been carried out using two independent procedures: (i) with the spectrophotometric analysis of dioxidine release into a pure water from the loaded sponges followed by the dioxidine identification by UV and NMR spectroscopy (see Sections 2.2.6 and 2.2.8), as well as (ii) the efficiency of the bactericide properties of the same samples has been evaluated in the microbiological tests (see Section 2.2.10).

The kinetic curves of dioxidine release from the drug-loaded sponges are shown in Fig. 8. The dioxidine release from the non-modified cryogels reached the maximum level for about 2 h (**a**, Fig. 8), while in the case of dioxidine-loaded succinylated sponge the release turned out to be considerably longer. The drug release in the outer solution continued for the >3 h (**b**, Fig. 8), and the resultant dioxidine concentration was higher. This result indicates that succinylated cryogenically-structured albumin-based sponge holds dioxidine molecules somewhat stronger compared to the non-modified sponge. With that, when the samples of these two kinds of loaded freeze-dried sponges had been immersed into the medium of D_2O, and solutions of the released dioxidine have been analyzed with H^1 NMR, no differences between the samples, as well as the reference solution of pure dioxidine, were observed. All the spectra had the multiplet signals at the following δ values: 4.93–5.21 ppm, CH_2 (4); 7.85–8.05 ppm, H Ar (2) and 8.38–8.52 ppm, H Ar (2). Since such spectral parameters are characteristic of the intact dioxidine, these data demonstrate the absence of changes in the structure of this substance being absorbed inside the wide-pore polymeric matrices of both the non-modified and succinylated albumin cryogels.

A higher capacity of succinylated cryogel with respect to dioxidine and a longer drug release in comparison with the drug-loaded non-modified cryogel is, most probably, stipulated by the succinylation-caused larger amount of carboxylic groups in the chemical structure of the former polymeric matrix. Since the dioxidine molecule itself has two OH groups (Fig. 9) capable of H-bonding with the carboxylic ones, their higher amount in the sorbent could bind a higher amount of the OH-bearing sorbate and, probably, could hold such substance somewhat stronger than the sorbent with a lower content of COOH-functions.

The data on the dioxidine release was shown to be in a good agreement with the results on the evaluation of antibacterial activity of the systems studied. These experiments were carried out using two test bacterial cultures: *Escherichia coli* and *Staphylococcus aureus* (see Section 2.2.10). The disc-diffusion procedure was employed, when the diameter of the so-called growth inhibition zones was measured. Such zones are formed owing to the bactericide release from its carrier in the microbial mat onto the solid nutritional medium. It was found that around the non-modified dioxidine-loaded cryogel discs these zones were 15.7 ± 1.0 mm for the *E. coli* cells and 20.1 ± 0.8 mm for the *S. aureus* bacteria (1; Fig. 10). In turn, the growth inhibition zones around the

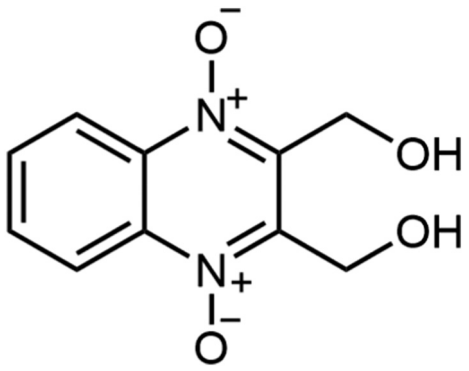

Fig. 9. Chemical structure of dioxidine molecule according to the data of ref. [94].

succinylated dioxidine-bearing cryogel discs were wider, namely, 17.9 ± 1.2 mm in the case of *E. coli* cells and 22.9 ± 1.2 mm for the *S. aureus* bacteria (2; Fig. 10). Thus, these experiments showed that the bactericide activity of the dioxidine-loaded sponges based on the succinylated albumin was higher in comparison with the activity of dioxidine-loaded sponges based on the non-modified albumin. Primarily, it was because of the higher dioxidine content in the former sponges.

4. Conclusions

Serum-albumin-based spongy cryogels, thanks to their biocompatibility and biodegradability, have certain applied potential as the materials for biomedical implementation. In this study we examined the possibilities that the approaches for the chemical modification of proteins, their succinylation in this case, could provide for slowing the biodegradation of such wide-porous gel matrices. The latter ones have been prepared via the cryogenic processing (freezing – frozen storage – thawing) of aqueous solutions containing bovine serum albumin, denaturant (urea or guanidine hydrochloride) and reductant (cysteine). It was shown that the gel-fraction yield values were higher for the systems when guanidine hydrochloride was used as the denaturant in comparison with the cases when urea additives were introduced in the feed solutions. The treatment of the resultant cryogels with succinic anhydride yielded the N-acylation of free lysyl amino groups of the protein and some increase in the water-absorbing capability of the polymeric phase (walls of macropores) of these heterophase materials. The juxtaposition of the digestibility by trypsin of the non-modified and succinylated albumin cryogels revealed an increase in the resistance of the latter ones against the proteolysis. Such result clearly shows the opportunities to vary the biodegradation rate of the serum-albumin-based spongy cryogels by applying the chemical modification

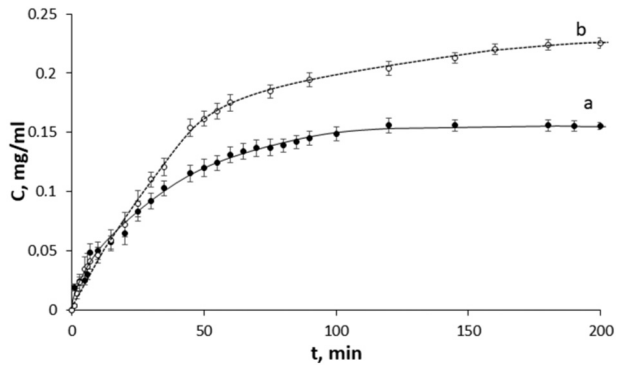

Fig. 8. Kinetic curves of dioxidine release from the drug-loaded non-modified (**a**) and succinylated (**b**) albumin-based cryogel samples.

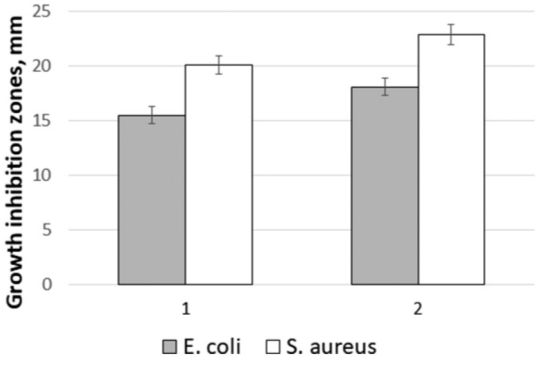

Fig. 10. The growth inhibition zones of *E. coli* and *S. aureus* cells around the dioxidine-loaded non-modified cryogel discs (1) and the dioxidine-loaded succinylated cryogel discs (2).

(the succinylation in this case) approach. In addition, both the non-modified and succinylated sponges have been loaded with dioxidine, the bactericide substance, and we found that increased amount of carboxylic groups in the succinylated matrices allowed absorbing a higher dioxidine specific amount. Subsequent microbiological tests also showed a higher bactericide activity of dioxidine-loaded succinylated albumin cryogels compared to the dioxidine-loaded non-modified sponges. As a whole, the results of this study testify on certain promising prospects (e.g., for the biomedical aims) of both the non-modified and modified cryogenically-structured serum-albumin-based sponges capable of serving as the biodegradable drug-carriers.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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