DIFFERENT APPROACHES TO AGAROSE SULFATION WITH SULFAMIC ACID

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Sulfated polysaccharides are important promising biologically active substances with anticoagulant properties. Biological activity is affected by the method of preparation, as well as the type of the polysaccharide and its molecular structure. In this work, we have considered various methods for the synthesis of promising anticoagulants – polysaccharide sulfates using the example of obtaining agarose sulfate. We compared various sulfating agents: chlorosulfonic acid, sulfamic acid, with various activators, and a deep eutectic solvent mixture of sulfamic acid with urea (in the melt). It has been shown that when urea is used as an activator of the process of sulfation of agarose with sulfamic acid in 1,4-dioxane, agarose sulfate with a high sulfur content (up to 14.5 wt%) is formed, which is close to the use of chlorosulfonic acid as a sulfating agent (with the production of agarose sulfate with 15.0 wt% sulfur). The use of solid catalysts in the process of sulfation of agarose with sulfamic acid leads to the production of agarose sulfate with a sulfur content of up to 14.1 wt% (for a catalyst based on the oxidized carbonaceous material Sibunit-4®). Sulfation of agarose in a deep eutectic solvent – a mixture of sulfamic acid with urea – leads to the production of agarose sulfate with a sulfur content of up to 13.7 wt%. The resulting agarose sulfates were characterized by FTIR spectroscopy, X-ray diffraction, elemental analysis, atomic force microscopy and DFT.

Keywords: agarose, sulfation, sulfated agarose, catalysis, deep eutectic solvent, sulfamic acid

INTRODUCTION

Agar, a mixture of cell wall polysaccharides, including agarose and agaropectin, can be extracted from various species of marine red algae (Rhodophyta).[1] The main disaccharide repeat units of agarose consist of (1,3) linked β-d-galactose (G) and (1,4) linked α-1-3,6-anhydrogalactose (A).[2] Agarose has multifaceted properties, due to which it has found wide application in various fields, from medicine to bioengineering.[3] Agarose has found application for the production of hydrogels,[4] fibers,[5] 3D scaffolds[6] *etc*. Agarose, along with its derivatives and mixtures, is widely used in tissue engineering and regenerative medicine, for example, in cartilage formation,[7] bone regeneration,[8] wound healing,[9] *etc*.

Agarose derivatives also find application in various fields due to their wide spectrum of activity. Thus, agarose derivatives of poly(2-(dimethylamino)ethyl methacrylate) have an antimicrobial effect.[10] In previous research,[9] a bioplastic film based on agarose and glycerol was obtained, in which tetracycline was enclosed to release the drug, such as an antibiotic and antiseptic. Agarose derivatives are also known:

citrates,[11,12] poly(3-dimethyl (methacryloyloxyethyl) ammonium propanesulfonate) - agarose copolymer,[13] polycaprolactone derivative[14] and many others. Thus, agarose and its derivatives are important due to their unique characteristics that allow them to be used in medicine.

Among the derivatives of agarose, those that contain a sulfate group are noteworthy. Sulfated agarose, along with other sulfated polysaccharides, has anticoagulant, antioxidant, lipid-lowering properties and other bioactivities.[15,16]

Despite the obvious biological activity of sulfated agarose, methods for its sulfation are not sufficiently developed. Thus, B. Matsuhiro *et al*.[15] carried out agarose sulfation with the SO3-pyridine complex in formamide. In another study,[17] agar sulfation was carried out with sulfuric acid in DMSO in the presence of acetic anhydride. With this method of sulfation, a maximum degree of substitution of 1.02 was achieved. It should be noted that toxic and corrosive reagents are used in agarose sulfation. On the contrary, sulfamic acid is an environmentally safer sulfating agent.[18] It showed high efficiency in the reactions of sulfation of polysaccharides of various nature,[19,20] and lignin.[21,22]

The aim of this work was to develop methods for the preparation of agarose sulfates using sulfamic acid in the presence of activators and solid catalysts, as well as to study the products using a complex of physicochemical methods: FTIR, XRD, AFM, elemental analysis, DFT.

EXPERIMENTAL

Sulfation of agarose

Sulfation of agarose with sulfamic acid and urea derivatives

2.5 g of agarose, 6.2 g of sulfamic acid and an equivalent amount of urea (3.8 g) or its derivative (methylurea, ethylurea, hydroxyethylurea, thiourea, biuret) were placed in a 250 mL three-neck flask equipped with a stirrer and reflux condenser. 50 mL of 1,4-dioxane was added and heated to a temperature of 90 °C. The reaction was carried out for 2 hours. The reaction scheme is shown in Figure 1.

After 2 hours, the reaction mass was cooled to room temperature, the solvent was decanted, the remaining precipitate was dissolved in 50 mL of distilled water, and neutralized with a 5% ammonia solution.

The solution containing agarose sulfate and reaction products was dialyzed against distilled water on an MF-503-46 MFPI cellophane dialyzing sac (US) with a pore size of 3.5 kDa (-0.1 μm). The product was dialyzed for 10 h with replacing the water every 1-2 h.

After the dialysis process, the ammonium cellulose sulfate solution was transferred to a Petri dish and dried in an oven at a temperature of 50 °C to constant weight.

Sulfation of agarose with sulfamic acid in the presence of solid catalysts

2.5 g of agarose, 6.2 g of sulfamic acid and 50 mL of 1,4-dioxane were placed into a 250 mL three-necked flask equipped with a stirrer and reflux condenser and heated to a temperature of 90 °C, after which 0.5 g of solid catalyst (Amberlyst-15®, Aluminum oxide, Titanium dioxide, Sibunit-4® ox.). The reaction was carried out for 2 hours. Processing was carried out by the same procedure as described above.

Sulfation of agarose in a deep eutectic solvent – a sulfamic acid-urea mixture

In a three-necked flask with a capacity of 100 mL, 13 g of sulfamic acid and 16 g of urea were added, heated to 100 °C until a uniform liquid state was reached. After that, 2 g of agarose was added. The process was carried out for 2 hours. Processing was carried out by the same procedure as described above.

Sulfation of agarose with chlorosulfonic acid in pyridine

In a three-necked flask with a volume of 250 mL, equipped with a stirrer and a reflux condenser, 50 mL of pyridine was poured, chlorosulfonic acid (5 mL) was added dropwise while cooling, after which, the mixture was heated to 90 °C and 2.5 g of agarose was added. The reaction was carried out for 2 hours. Processing was carried out by the same procedure as described above.

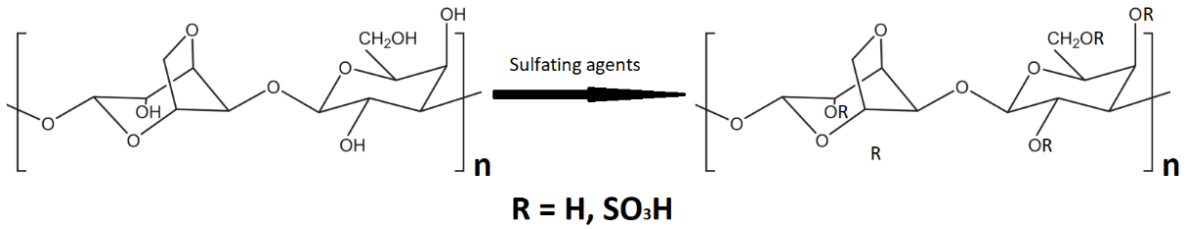


Figure 1: Agarose sulfation scheme

Preparation of the carbon catalyst

The oxidized carbon samples were prepared from a Sibunit-4 commercial meso-porous carbon material by oxidization in a mixture of 20 vol% of oxygen in N_2 in the presence of water vapors at temperatures of 400 °C for 2 h.[23]

Fourier transform infrared spectroscopy

The FTIR spectra of the initial agarose and sulfated agarose were recorded on a Shimadzu IRTracer-100 FTIR spectrometer (Japan) in the wavelength range from 400 to 4000 cm^{-1}. The spectral data were analyzed using the OPUS software (version 5.0). Solid specimens in the form of tablets in a KBr matrix (2 mg specimen/1000 mg of KBr) were prepared for the analysis.

X-ray diffraction

The XRD study was carried out on a DRON-3 X-ray diffractometer (Cu$\underline{K}\alpha$ monochromatized radiation with $\lambda = 0.154$ nm) at a voltage of 30 kV and a current of 25 mA. The scanning step was 0.02 deg and the intervals were 1 s per data point. The measurements were performed in the Bragg angle (2Θ) range from 5.00 to 70.00.

Atomic force microscopy

The preparation of sulfated agarose films was carried out as follows: sulfated agarose (1 grams) was dissolved in distilled water (30 mL) at the room temperature. The resulting solution of sulfated agarose was poured into a Petri dish and dried in the oven at the temperature of 45 °C to constant weight in an oven. The obtained films of sulfated agarose were separated from the Petri dish with tweezers, after which they were analyzed by atomic force microscopy. Study of the sulfated agarose films by AFM in the semicontact mode was carried out using a Solver P47 multimode scanning probe microscope (NT-MDT, Moscow). Scanning was performed at no less than 3–4 points on several sites. Scan speed was 1.5–2.0 Hz, the resolution of the resulting image was 256 × 256 pixels.

Computational details

The quantum-chemical computations were performed using the GAMESS US[24] program package on the cluster MVS-1000 M of the Institute of Computational Modeling SB RAS. Geometry optimization was performed by density functional theory (DFT) with PBE0[25] (under Grimme's empirical correction[26]) functional. The Def2-SVP[27] basis set functions were applied to C, H, O, N and S atoms.

The free energy of complexation ($\Delta\Delta G^{solv.}$) has been calculated taking into account three parts: gas phase energy (ΔG^{gas}), solvation free energy ($\Delta G^{aq.}$), and zero-point energy correction (ΔE^{ZPE}):[28]

$$\log K^{calc} = -\Delta\Delta G^{solv.}/(2.303RT) \quad (1)$$

$$\Delta\Delta G^{solv.} = \Delta G^{gas} + \Delta G^{aq.} + \Delta E^{zpe} + E^{corr} \quad (2)$$

$$E^{corr.} = \pm RT\ln([H_2O]) = 9.964 \text{ kJ/mol} \quad (3)$$

where $E^{corr.}$ is a term of free energy change associated with moving a solvent from a standard-state solution phase concentration of 1 M to a standard state of the pure liquid, 55.34 M.[29] Values of $G^{gas}(H^+)$ and $\Delta G^{solv.}(H^+)$ for proton (−26.28 and −1108.27 $kJ \cdot mol^{-1}$, respectively) were taken from previous research.[30] The solvent effects were evaluated using the SMD solvation model.[31] All theoretical calculations were performed with a temperature of 298 K.

RESULTS AND DISCUSSION

Polysaccharides have important biologically active characteristics, which leads to the search for various ways to obtain them. One of the directions is their extraction from seaweed.[32,33] An alternative for obtaining sulfated polysaccharides can be their chemical modification into the corresponding sulfates.[34] Chemical modification makes it possible to obtain a sulfated polysaccharide with a given degree of substitution and a certain molecular weight.[35]

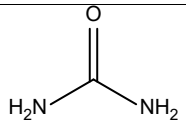
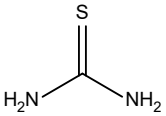
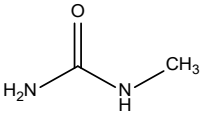
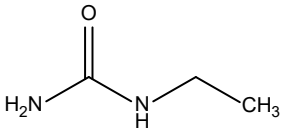
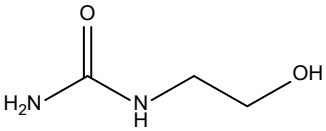
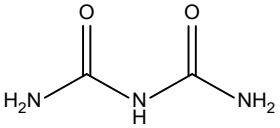
It is known[34,36] that for the sulfation of polysaccharides with sulfamic acid, it is necessary to use various activators of the process, which lead to the breaking of the S-N bond in its molecule, which leads to the production of NH_3 and SO_3. Various organic bases and urea derivatives have been studied as activators of the process of sulfation with sulfamic acid.[37] At the same time, during sulfation with ammonium sulfamate (by the example of arabinogalactan), it was shown that urea does not show high data on the sulfur content in the sulfated reaction product, and the best activation of the process is achieved using oxidizing agents ($KMnO_4$ and $K_2Cr_2O_7$).[38]

In this work, we conducted a comparative study of the processes of agarose sulfation with sulfamic acid and ammonium sulfamate in 1,4-dioxane, as well as the effect of process activators – urea derivatives.

As activators of the sulfation process were chosen: urea, thiourea, methylurea, ethylurea, hydroxyethylurea and biuret. Data on the sulfur content in agarose sulfates during sulfation with sulfamic acid and ammonium sulfamate are presented in Table 1.

Sulfation of agarose with sulfamic acid without an activator results in agarose sulfate with a low sulfur content (2.7 wt%) (Table 1). Various urea-based sulfation activators show different results with the sulfur content of agarose sulfate.

Table 1
Influence of sulfating reaction activator (or catalyst) with sulfamic acid on the sulfur content in agarose sulfate (conditions: temperature 90 °C, time 3 h)

№	Activator/Catalyst	Formula/Functional groups (for solid catalysts)	Sulfur content, wt%
1	Without activator	-	2.7
2	Urea		14.5
3	Thiourea		8.5
4	Methyl urea		8.8
5	Ethyl urea		8.2
6	Hydroxyethyl urea		7.5
7	Biuret		7.2
8	Chlorosulfonic acid*	HSO_3Cl	15.0
9	Amberlyst-15®	R- SO_3H	12.5
10	Aluminium oxide	Al_2O_3	8.1
11	Titanium dioxide	TiO_2	9.8
12	Sibunit-4® ox.	-	14.1
13**	Deep euthectic solvent (DES)	DES with urea	13.7

* chlorosulfonic acid was used instead of sulfamic acid without an activator/catalyst, instead of 1,4-dioxane, pyridine was used as a solvent; ** no organic solvent (1,4-dioxane) was used in this experiment

Thus, the lowest value (7.2 wt%) of the sulfur content in agarose sulfate when using urea-based activators is observed in biuret. According to Table 1, urea derivatives, in an increasing order of their activating ability in the reactions of sulfation of agarose with sulfamic acid, can be arranged as follows: biuret > hydroxyethyl urea > ethyl urea > thiourea > methyl urea > urea. The low values for biuret can be explained by the fact that it does not form a donor-acceptor complex with sulfamic acid, or that this complex does not have the proper activity in sulfation reactions. The lower sulfur content when using hydroxyethylurea, compared to ethylurea, can explain that the hydroxyl group can enter into competitive reactions, itself undergoing sulfation. Among the described urea derivatives, urea exhibits the highest activity in agarose sulfation reactions. When urea is used as an activator of agarose sulfation with sulfamic acid in 1,4-dioxane, agarose sulfate with a high sulfur content (up to 14.5 wt%) is formed.

A comparison of this method with the traditional sulfation with chlorosulfonic acid yields similar values of sulfur content (14.5 and 15.0 wt%, respectively) in agarose sulfates. However, the sulfation method with sulfamic acid has many advantages, the main of which is lower toxicity and corrosiveness in comparison with chlorosulfonic acid.[19,20] In addition, the use of chlorosulfonic acid leads to a greater degree of polysaccharide hydrolysis,[39] which also explains the higher sulfur content in the sulfated product. The disadvantage of using the method of sulfation with sulfamic acid in the presence of urea is the difficulty of

regeneration and reuse of urea. Solid catalysts can eliminate this disadvantage.

When aluminum and titanium oxides are used as solid catalysts for the process of agarose sulfation with sulfamic acid, a sulfur content of up to 8.0 wt% is achieved. The use of Amberlyst-15® as a catalyst leads to higher values of sulfur content in agarose sulfates – up to 12.5 wt%. The use of an oxidized catalyst based on carbonaceous material Sibunit-4® as a catalyst for the sulfation process leads to the production of agarose sulfate with a sulfur content of up to 14.1 wt%.

Sulfation of agarose in a deep eutectic solvent – a mixture of sulfamic acid with urea – leads to the production of agarose sulfate with a sulfur content of 13.7 wt%, which is also a high indicator. It should be noted that this method has an advantage – the absence of organic solvents. Among the disadvantages are the high consumption of reagents, the complexity of processing and side reactions of carbamation.[40]

Thus, among the presented activators and catalysts for the process of agarose sulfation with sulfamic acid, the most promising is the use of oxidized carbonaceous material Sibunit-4® as a catalyst.

Sulfate group embedding into the agarose molecule was proven by FTIR spectroscopy (Fig. 2). In the FTIR spectrum of the original agarose (Fig. 2), there is an absorption band at 3435 cm^{-1}, corresponding to the vibrations of the hydroxyl group. The absorption band at 2897 cm^{-1} corresponds to the stretching vibrations of the -CH group, and at 1640 cm^{-1} to the vibrations of the -C=O group. Several absorption bands in the region of 920–1135 cm^{-1} are characteristic of vibrations of nonequivalent –C–O–C–1→4 glycosidic bonds.[41] The absorption band at 891 cm^{-1} corresponds to vibrations of the –C–O–C bond with 3,6-anhydrogalactose.[42] In the FTIR spectrum of the ammonium salt of agarose sulfate, new absorption bands appear in the region of 1248–1217 cm^{-1} and at 802 cm^{-1}, which correspond to the vibrations of the sulfate group.[19]

The initial and sulfated agarose was analyzed by X-ray diffraction (Fig. 3). According to Figure 3, the initial agarose is an X-ray amorphous material, which agrees with previous data.[43,44] In the process of sulfation, the agarose structure further amorphizes, which is also characteristic of other polysaccharides.[19,45] According to X-ray diffraction data, there are no peaks characteristic of sulfamic acid in the diffraction patterns of agarose sulfates.

The films of agarose sulfated by various methods were studied by atomic force microscopy (Fig. 4). According to the data presented in Figure 4, the surface of agarose films consists of particles of various shapes with an average size of 42.1 nm. The surface of the film of agarose sulfated with sulfamic acid consists of particles of a near-spherical shape with an average size of 79.6 nm.

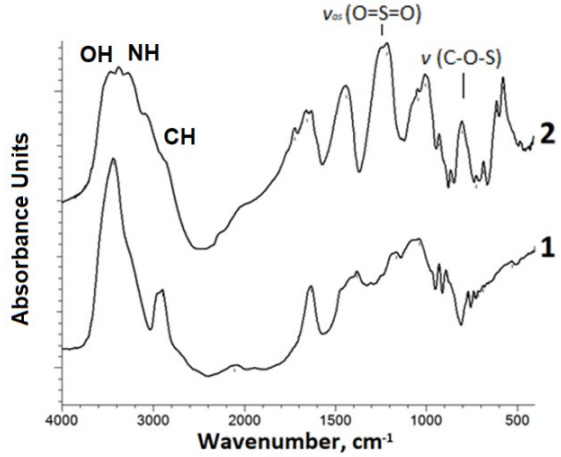


Figure 2: FTIR spectra of 1 – initial agarose, 2 – agarose sulfated with sulfamic acid

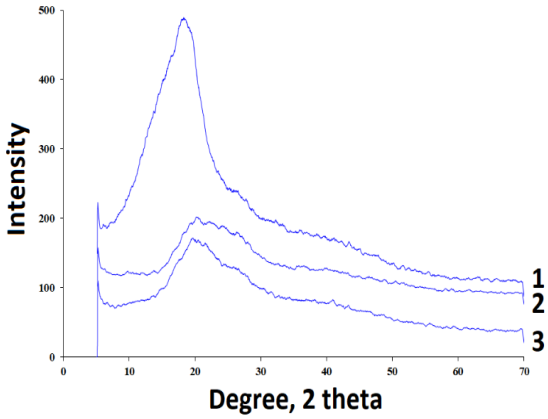


Figure 3: XRD diffraction patterns of 1 – initial agarose, 2 – agarose sulfated with sulfamic acid, 3 – agarose sulfated with chlorosulfonic acid

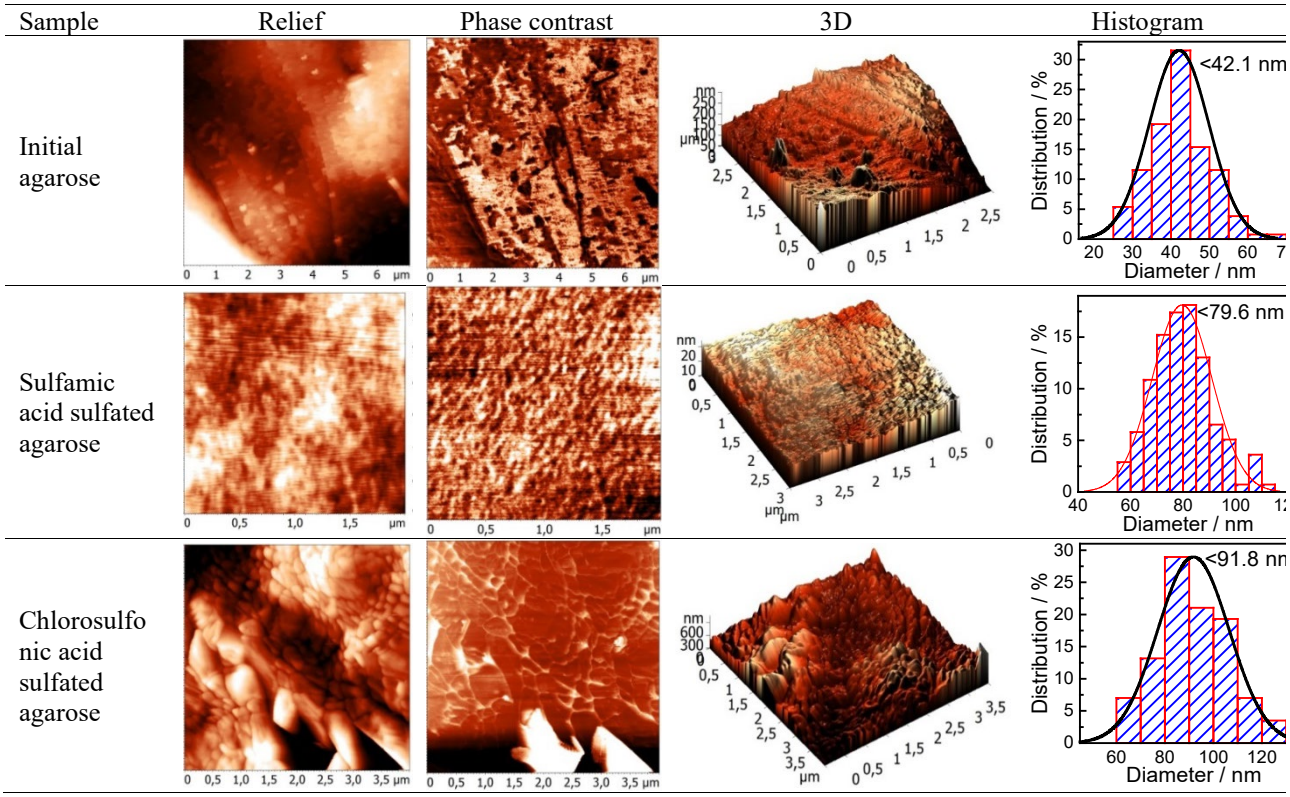


Figure 4: AFM data of original and sulfated agarose

In the case of chlorosulfonic acid sulfated agarose sulfate films, aggregated particles with an average size of 91.8 nm are observed. Thus, in the course of agarose sulfation, an increase in the particle size on the film surface is observed. This is due to aggregation caused by the functionalization of the polysaccharide with groups that enhance its inter- and intramolecular interactions or polyelectrolyte effects.[46,47]

DFT optimization

Agar-agar, which includes agarose, is used in the electrochemical, biotechnological and pharmaceutical fields as a stabilizer and gelling material. The physicochemical properties of agarose and its derivatives are closely related to its optimized structure.[48,49]

The first task of theoretical calculations was to optimize the structures of agarose and its derivatives with one, two, and three sulfate groups. The structure of agarose was approximated by one functional unit. All structures were optimized as ammonia salts of the corresponding sulfates. The data are shown in Figure 5. It can be seen that the introduction of sulfated groups significantly changes the structure of the agarose skeleton.

Spectroscopic analysis makes it possible to obtain data on the types of bonds, interactions, and other characteristics of the system.[50] In this direction, theoretical methods are being actively developed, which allow us to assume the spectroscopic characteristics of substances based on the structure.[51,52] Figure 6 shows the calculated IR spectra of agarose and its sulfated derivatives with different contents of sulfate groups.

OH-group vibrations

The absorption bands for the hydroxyl group are observed in the theoretical spectra in the region of 3200-3600 cm^{-1} (νOH). The introduction of the sulfate group has little effect on the spectroscopic characteristics of the vibrations of the hydroxyl group by changing its intensity.

CH-group vibrations

In the theoretical FTIR spectrum, the absorption bands corresponding to vibrations of the CH group are observed in the region of 2900–3000 cm^{-1} (νCH). The introduction of sulfate

groups into the agarose molecule does not significantly affect the vibrations of the CH group.

SO-group vibrations

The vibration of the sulfate group for mono-, di- and trisulfate agarose is observed in the areas of 1242-1268 cm^{-1} (vSO), 803-819 cm^{-1} (vSO). The results obtained are consistent with the data given in previous works.[37,52]

The place of introduction of sulfate groups into the agarose molecule was chosen according to the most negative charges of the oxygen atoms of the hydroxyl groups. For a thermodynamic evaluation of each structure, their theoretical spectral data were compared. Sulfation of agarose was carried out by various methods with different yields of the final product. For the thermodynamic evaluation of the sulfation reaction, the Gibbs free energies of formation of the corresponding reactions were calculated. The thermodynamic cycle diagram for evaluating these parameters is shown in Scheme 1 and the FTIR spectra in Figure 6. Table 2 contains the theoretical parameters for sulfation reactions for various sulfation agents. The choice of these models is based on the fact that sulfation with chlorosulfonic acid is a traditional method for sulfation of polysaccharides, sulfation with sulfamic acid is an alternative method that has begun to develop actively in recent years. As a comparison, a new method has been added to the calculation – sulfation with ammonium sulfamate.[38]

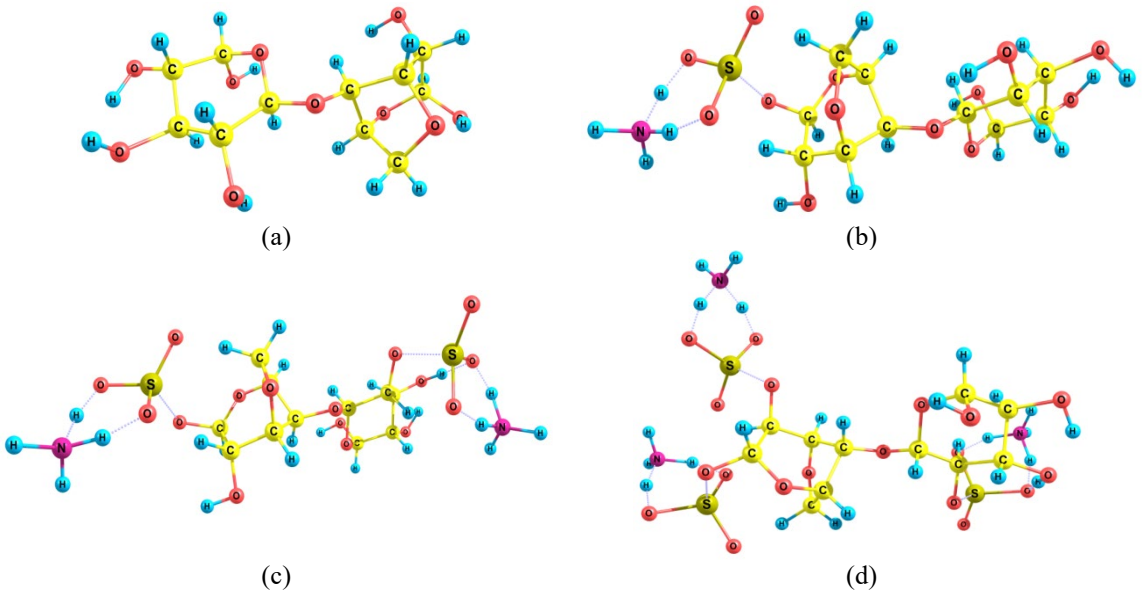
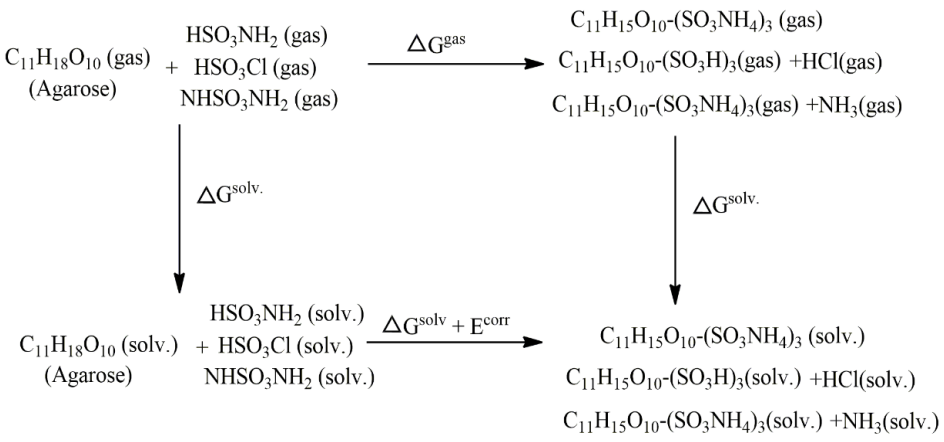


Figure 5: Optimized structures of (a) pure agarose, (b) agarose monosulphate, (c) agarose bisulphate, (d) agarose trisulphate



Scheme 1: Thermodynamic cycle for calculation $logK^{calc}$

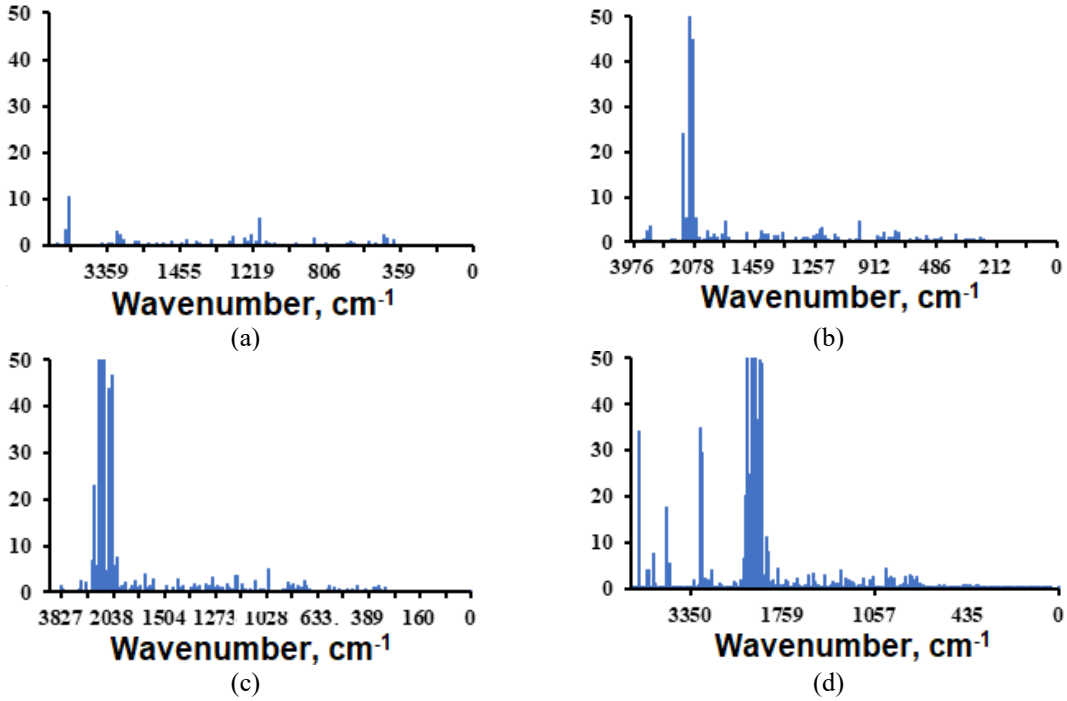


Figure 6: Theoretical FTIR spectra of (a) pure agarose, (b) agarose monosulphate, (c) agarose bisulphate, (d) agarose trisulphate

Table 2
Theoretical calculated parameters for sulfation reactions

Type of reaction	$\Delta G^{gas} + \Delta G^{aq}$, kJ/mol	ZPE, kJ/mol	$\Delta\Delta G$, kJ/mol
Agarose + sulfamic acid	-634.8	14.3	-616.41
Agarose + chlorsulfonic	128.3	-43.7	88.66
Agarose + $NH_4SO_3NH_2$	-167.3	-8.9	-172.06

As can be seen from Table 2, the most thermodynamically advantageous is the sulfation reaction using pure sulfamic acid. The reaction with chlorosulfonic acid shows the least thermodynamic direction among the three reactions. It is probably due to kinetic rather than thermodynamic factors, since the Gibbs energy for it is significantly positive. The reaction of agarose with ammonium sulfamate is three times less favorable than a similar reaction with pure sulfamic acid, which also explains the use of stronger activators of the sulfation process (for example, potassium permanganate compared to urea for sulfamic acid).[38] It can be seen that the greatest contribution for all types of reactions is made by the difference in the reaction energies in the solid and liquid states, while the zero-point energies play a significant role only for the reaction of sulfamic acid (Table 2).

Molecular orbitals are used in boundary electron density to predict the most reactive position in π electron systems. In molecular interactions, the LUMO accepts electrons and its energy corresponds to the electron affinity (EA), while the HOMO is an electron donor and its energy is related to the ionization potential (IP).[53,54] The HOMO-LUMO energy gap explains the final intermolecular charge transfer interaction and is useful for determining the properties of molecular electrical transport. A molecule with a large boundary orbital gap (HOMO-LUMO energy gap) has low chemical activity and high kinetic stability.[53-57]

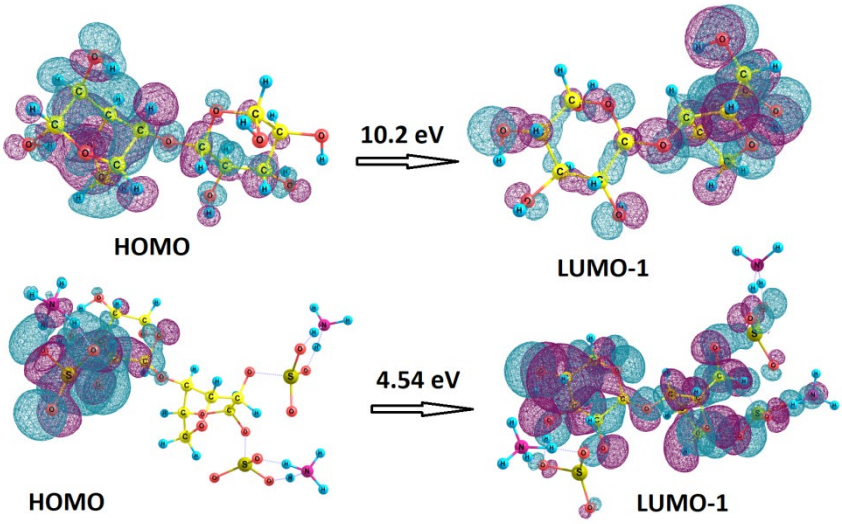


Figure 7: 3D representation of HOMO-LUMO orbitals

According to Figure 7, the largest value of the energy gap (10.2 eV) is observed in agarose, while agarose sulfate has a smaller value (4.54 eV). Thus, it can be assumed that agarose is a more chemically stable substance compared to agarose sulfate. The data are partly consistent with our previous work.[58] In addition, a decrease in the energy gap value is also observed when a sulfate group is introduced into the structure of the substance.

CONCLUSION

Different methods of agarose sulfation with both chlorosulfonic and sulfamic acids are compared. It has been shown that the maximum sulfur content (up to 15.0 wt%) is achieved by sulfation with chlorosulfonic acid. Sulfation of agarose with sulfamic acid in 1,4-dioxane in the presence of urea leads to the production of agarose sulfate with a sulfur content of up to 14.5 wt%. The use of the oxidized carbon material Sibunit-4® as a catalyst for sulfation with sulfamic acid leads to the production of agarose sulfate with a sulfur content of up to 14.1 wt%. It should be noted that the use of solid catalysts based on aluminum and titanium oxides for sulfation with sulfamic acid led to the production of agarose sulfates with a sulfur content of up to 8.0 wt%. At the same time, sulfation of agarose in a deep eutectic mixture – sulfamic acid-urea[59] – led to the formation of the corresponding sulfates with a sulfur content of up to 13.7 wt%. The introduction of a sulfate group into the agarose molecule is proved by the appearance of absorption bands in the FTIR spectra in the region of 1248-1217 cm^{-1}. According to X-ray diffraction and atomic force microscopy, agarose sulfate does not contain impurities in the form of sulfamic acid.

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