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Attachment of 2-Aminoethyl Sulfate Fragments to Polyaldehyde Starch: Structure-Charge-Anticoagulant Relationships

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Abstract

Starch is a natural polysaccharide containing reactive functional groups that enable chemical modification and targeted tuning of its physicochemical and biological properties. Incorporation of sulfate groups enhances the polyanionic character of the polymer and promotes interactions in biological environments. Although sulfated polysaccharides have been widely studied, anticoagulant systems based on conjugation of sulfate-containing fragments with polyaldehyde starch remain relatively underexplored. In this study, water-soluble polyanionic starch derivatives were synthesized via attachment of 2-aminoethyl sulfate fragments to polyaldehyde starch obtained by controlled acid hydrolysis followed by periodate oxidation. The degree of substitution (DS) was systematically varied to investigate structure-charge-activity relationships. Comprehensive physicochemical characterization (FTIR, ^{1}H NMR, XRD, SEM, GPC, ζ-potential, viscosity and pKa measurements) confirmed successful functionalization, reduced crystallinity, and formation of negatively charged polymer systems. In vitro coagulation assays (APTT, TT and PT) demonstrated significant prolongation of clotting times. Anticoagulant activity increased with DS and decreased molecular weight, reflecting the combined influence of charge density and macromolecular mobility. Highly substituted derivatives exhibited activity approaching that of heparin under the tested experimental conditions. In vivo studies using a thromboplastin-induced model confirmed the anticoagulant effect of Conjugate-65. Acute toxicity evaluation following intravenous administration indicated LD_{50} > 2,000 mg/kg, suggesting low toxicity. These results indicate that polyaldehyde starch derivatives bearing 2-aminoethyl sulfate fragments represent promising candidates for the development of plant-derived anticoagulant biomaterials.

Keywords: Starch derivatives, Polysaccharides, Chemical modification, Sulfate-containing starch conjugates, Polyanionic properties, Anticoagulant activity

Introduction

Polysaccharides are extensively applied in medical and chemical-pharmaceutical fields owing to their biocompatibility, low toxicity, renewability, and versatility for chemical modification. These characteristics make polysaccharide-based materials attractive platforms for the development of functional biomaterials. Consequently, the design and modification of polysaccharide derivatives remain an active area of research [1,2].

Among polysaccharides, starch is one of the most abundant, economically accessible, and non-toxic natural polymers. Despite these advantages, the physicochemical limitations of native starch - including poor solubility in cold water, limited solution stability, and weak biological activity - restrict its direct applications. Consequently, chemical modification has been widely employed to tailor the solubility, stability, and functional properties of starch. Introduction of appropriate functional groups enables regulation of macromolecular structure and the development of water-soluble and biologically active derivatives [3-5].

The introduction of ionogenic groups into the starch macromolecule enables targeted regulation of its physicochemical and biological properties. Although native starch primarily contains neutral hydroxyl groups, chemical modification allows incorporation of functional fragments with ionic character. In particular, attachment of negatively charged sulfate, carboxylate, or phosphate groups alters the overall charge state of the polymer, leading to the formation of polyanionic systems with modified functional behavior [6-10].

Sulfation reactions are widely employed to obtain polyanionic derivatives from various polysaccharides. Conventional approaches typically involve highly reactive sulfating agents, including sulfuric acid, chlorosulfonic acid, oleum, or sulfur trioxide complexes, followed by evaluation of the resulting biological activities [11-13]. Despite their effectiveness, such methods often require harsh reaction conditions and the use of toxic reagents, which may limit product stability and practical applicability. Consequently, the development of alternative modification strategies enabling controlled introduction of sulfate functionalities under milder conditions remains an important objective.

Periodate oxidation represents a selective and efficient route for generating polyaldehyde starch intermediates suitable for subsequent functionalization [14]. In this reaction, vicinal hydroxyl groups are oxidized to form reactive aldehyde moieties, producing polyaldehyde starch with enhanced chemical reactivity [15-17]. The electrophilic aldehyde groups serve as convenient sites for nucleophilic conjugation with primary amine-containing sulfate fragments, enabling the preparation of structurally tunable polyanionic derivatives. This strategy allows regulation of degree of substitution (DS) while preserving water solubility and facilitating targeted modulation of biological activity [18].

Polyanionic starch derivatives obtained through chemical modification exhibit enhanced hydration, reduced intermolecular hydrogen bonding, and increased macromolecular mobility, which collectively contribute to improved water solubility and solution stability over a broad pH range [19,20]. Owing to their pronounced negative charge, polyanionic polysaccharides are capable of interacting with biologically relevant macromolecules via electrostatic forces and hydrogen bonding. Such interactions are particularly important in processes involving blood coagulation, where charge density and molecular flexibility influence anticoagulant performance [21].

In our previous studies, methods for introducing 2-aminoethyl sulfate fragments into starch and their influence on physicochemical properties were investigated [25,26]. The effects of reaction conditions on nucleophilic conjugation were established, and a series of derivatives with varying degrees of substitution and molecular weights were obtained. Structural and morphological features of the synthesized materials were characterized using spectroscopic and microscopic techniques, and their anticoagulant activity was evaluated under in vitro conditions.

The objective of the present study is to develop water-soluble polyanionic starch derivatives with controllable structural parameters through systematic variation of degree of substitution (DS) and molecular weight. Particular emphasis is placed on elucidating structure-charge-anticoagulant relationships by combining comprehensive physicochemical characterization with evaluation of anticoagulant activity *in vitro* and *in vivo*.

Materials and methods

Materials

Starch (Mw = 3.715×10^5), sodium periodate, 2-aminoethyl hydrogen sulfate were obtained from Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using deionized water.

Synthesis of starch derivatives containing a 2-aminoethyl sulfate fragment

Hydrolysis of starch

Ten g of starch were placed into a 1,000 mL 3-necked flask, and 700 mL of isopropyl alcohol was added. Under continuous stirring, the flask was placed in a thermostated bath maintained at 75 - 90 °C. Subsequently, HCl was added at a starch:HCl molar ratio of 1.0:1.5, and the hydrolysis reaction was carried out for 30 - 120 min. After completion of the reaction, the resulting product was washed with distilled water and dried under vacuum in the presence of P_2O_5. As a result, starch hydrolysates with different molecular weights were obtained [19].

Periodate oxidation of starch

The periodate oxidation of starch was carried out in a 500 mL light-protected glass vessel. Dried starch (0.02 mol) was placed into the vessel, followed by the addition of 200 mL of acetate buffer (pH 4.3). Subsequently, an aqueous solution of sodium periodate (0.2 N $NaIO_4$) was added to the reaction mixture at a starch:periodate molar ratio of 1:1.5, calculated with respect to the molecular mass of the polysaccharide repeating unit. The reaction was conducted at room temperature for 9 h. The obtained polyaldehyde starch (PAS) samples were filtered using a Schott filter and subjected to a sequential purification procedure. Initially, the samples were washed with 0.5 - 1.0 L of aqueous solution adjusted to pH 1 with hydrochloric acid, followed by washing with 1.0 L of an aqueous acetone mixture. During the washing process, the removal of IO_4^- and IO_3^- ions was monitored using a silver nitrate test. In the final step, the products were washed with 0.2 - 0.25 L of acetone. The reaction products were dried under vacuum over P_2O_5 in the dark.

Determination of aldehyde group content

A total of 0.01 mol of polyaldehyde polysaccharide and 10 mL of 0.2 N NaOH solution were placed into a 150 mL flask. The mixture was then heated in a water bath at 70 °C for 5 min. As soon as the solution turned dark orange, the flask was immediately removed from the water bath and rapidly cooled in cold water. After cooling, 10 mL of 0.2 N H_2SO_4 solution was added to the mixture and shaken until a pale yellow color was obtained. Phenolphthalein indicator was then added, and the solution was titrated with 0.2 N NaOH. The aldehyde group content was calculated using the following equation:

$$-CHO\ (\%) = \frac{(C_1V_1+C_2V_2-2\times C_3V_3)\times 161}{m\times 1000}$$

where −CHO (%) represents the number of formed polyaldehyde units per 100 repeating units (OD - degree of oxidation, mol%); C_1 is the initial concentration of NaOH (0.2 N); V_1 is the initial volume of NaOH (10 mL); C_2 is the concentration of NaOH used for titration (0.2 N); V_2 is the volume of NaOH aqueous solution consumed during titration; C_3 is the concentration of H_2SO_4 (0.2 N); V_3 is the volume of H_2SO_4 (15 mL); m is the mass of the sample (g); and 161 is the average relative molecular mass of the glucose unit of polyaldehyde starch [19-25].

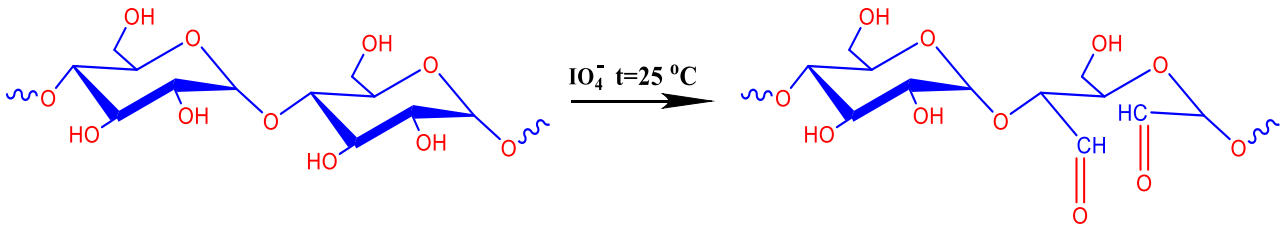


Figure 1 Schematic illustration of periodate-mediated oxidation of starch involving cleavage of vicinal diol groups and generation of dialdehyde starch.

Reaction of starch with a sulfate-containing fragment

The formation of new conjugates of polyaldehyde starch with a sulfate-containing primary amine was carried out as follows. First, 0.05 mol of polyaldehyde starch was taken and dissolved in water to obtain an aqueous solution. During the synthesis, the sulfate-containing fragment was added to the aldehyde groups at a molar ratio of 2.0:1.0 and dissolved in phosphate buffer (pH 8.2). The reaction was conducted at 20 ± 5 °C for 30 min under continuous stirring. After completion of the reaction, the obtained product was purified to remove unreacted components. For this purpose, the product was placed into a dialysis membrane (Mw = 3.5 - 7 kDa) and dialyzed for 24 h. Dialysis was performed against deionized water, which was replaced every 8 h. To accelerate the process, the water in the container holding the dialysis membrane was continuously stirred using a magnetic stirrer. After dialysis, the product was freeze-dried in the frozen state using a lyophilizer [25].

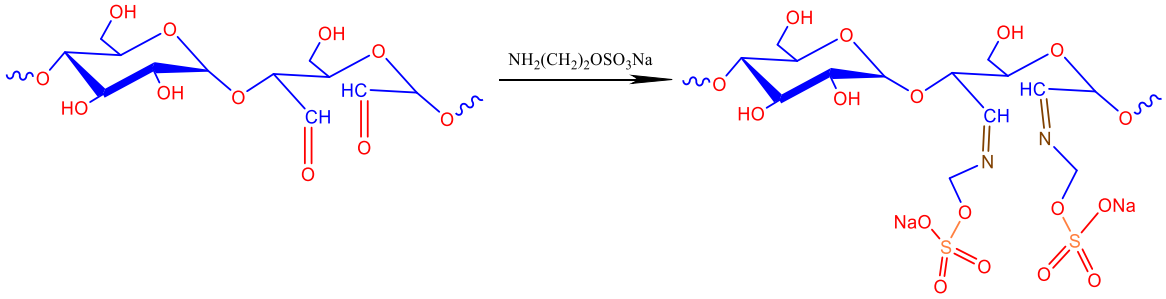


Figure 2 Schematic illustration of the nucleophilic conjugation reaction between polyaldehyde starch and 2-aminoethyl sulfate, involving the reaction of aldehyde groups with primary amine groups to form Schiff base (imine, −C=N−) linkages along the polymer backbone.

Characterization methods

FTIR spectra of starch and its derivatives were recorded using a Bruker Vector 22 IR spectrometer with KBr pellets (3 mg sample per 300 mg KBr) in the wavenumber range of 400 - 4,000 cm^{-1}. Spectra were collected at a resolution of 4 cm^{-1} with 32 scans (Bruker, Germany).

X-ray diffraction (XRD) and scanning electron microscopy (SEM) analysis. Structural changes in the samples were analyzed using an XRD-6100 powder X-ray diffractometer equipped with a Ni filter and CuKα radiation source (Shimadzu, Japan), operated at an accelerating voltage of 50 kV and a current of 40 mA. Diffraction patterns were recorded in the 2θ range from 3° to 80° at a scanning rate of 2° min^{-1}. The surface morphology of starch and its derivatives was examined using an EVO MA10 scanning electron microscope (SEM) (Zeiss, Germany) [26].

^{1}H NMR spectra of the synthesized conjugates were recorded on a high-resolution JEOL 600 MHz nuclear magnetic resonance spectrometer (JEOL Ltd., Japan). All samples were prepared under identical conditions using D_2O as the solvent. Chemical shifts (δ) were reported in ppm relative to tetramethylsilane (TMS). Spectral processing and analysis were performed using standard software provided by JEOL.

The ζ-potential values of the samples were measured using a Zetasizer Nano ZS instrument (Malvern, UK) equipped with a ZEN1002 immersion cell and plastic cuvettes. Each measurement was performed at least 3 times [27].

The molecular weights of the polysaccharide derivatives were determined by gel permeation chromatography (GPC) using an Agilent 1260 Infinity II system. A PLgel Mixed-C column (5 µm, 300×7.5 mm) was used for analysis. Samples were prepared at a concentration of 3 mg/mL and dissolved in an aqueous solution containing 0.1 M $NaNO_3$ and 8.0 mmol/L NaN_3. Prior to analysis, the solutions were filtered through a 0.45 µm polyvinylidene fluoride (PVDF) filter and degassed. Calibration was performed using pullulan standards. The obtained data were processed using Agilent OpenLab CDS software to calculate the Mw and polydispersity index (PDI).

The pKa values of the synthesized conjugates were determined by potentiometric titration using a TitroLine 7000 automatic titrator (SI Analytics, Germany). In this procedure, hydrochloric acid present in the sample was titrated with 0.1 M NaOH solution [28,29].

The nitrogen content of the newly formed derivatives obtained from the reaction between the polysaccharide and 2-AES was determined using an EA 1108 elemental analyzer (Carlo Erba, Italy). The degree of substitution (DS) was calculated from the nitrogen content using the following equation.

$$\gamma_{DS} = \frac{N_{\%} \times 16200}{2800 - (N_{\%} \times 290)}$$

where γ_{DS} represents the number of substituted repeating units per 100 monosaccharide units (DS - degree of substitution, mol%); N% is the nitrogen content; 16200 is the mass of 100 monosaccharide units of the polysaccharide; 2800 corresponds to the mass of nitrogen when all 100 units are fully substituted; and 290 is the molecular mass of the 2-AES fragment introduced per repeating unit [29].

The intrinsic viscosity of the samples was determined using an Ubbelohde viscometer (d = 0.34) in

the presence of sodium chloride as a low-molecular-weight electrolyte at a concentration of 0.1 mol/L.

***In vitro* evaluation of the anticoagulant activity of the obtained starch derivatives**

The anticoagulant properties of the synthesized starch derivatives were evaluated under *in vitro* conditions. Citrated plasma obtained from animal blood was used for the experiments (3.8% sodium citrate, plasma:citrate ratio of 9:1). The citrated plasma was separated by centrifugation at 3,000 rpm for 15 min. The tested samples were added to the plasma at concentrations ranging from 10 to 100 μg/mL and incubated at 37 °C. Anticoagulant activity was assessed by determining the effect on the initial stages of coagulation using activated partial thromboplastin time (APTT) and prothrombin time (PT) assays. In addition, thrombin time (TT) was measured to evaluate the influence on the fibrin formation stage. Coagulation times were recorded using a coagulometer (CYANCoag, Belgium) with standard reagents. All standard tests were performed in the absence of samples (control) and in the presence of heparin as a reference anticoagulant. Each experiment was carried out in six replicates [30,31].

***In vivo* evaluation of the anticoagulant activity of the obtained starch derivatives**

The anticoagulant effect of the synthesized starch derivatives containing sulfate fragments was evaluated *in vivo* using a thromboplastin-induced procoagulant model. The thromboplastin-induced model was selected as a widely used approach for evaluating changes in coagulation parameters under experimental conditions. Male outbred laboratory rats (*Rattus norvegicus*, 200 - 250 g) were used in the study. The animals were housed under standard laboratory conditions and randomly assigned to experimental groups (n = 6). The conjugate solution was administered into the tail vein at a dose of 2 mg/kg once every 24 h for 7 consecutive days. Heparin (130 IU; 2 mg/kg) was used as a reference drug. Twenty-four hours after the last dose, thromboplastin was injected intravenously at a dose of 25 μg/kg. After 10 min, blood was collected and plasma was prepared. APTT, TT, and PT were measured using a CYANCoag coagulometer. Each experiment was performed in six replicates [32-34].

Determination of acute toxicity parameters of the obtained starch derivatives

The acute toxicity study was conducted in laboratory animals using a single fixed (limit) dose design. Male outbred white mice with a body weight of 20 ± 2 g, clinically healthy and sexually mature, were selected for the experiments. Each group consisted of five animals. Prior to the study, the animals were kept under quarantine conditions for at least 14 days. The mice were housed under standard vivarium conditions with controlled temperature, humidity, and light regime. The animals were provided with a standard diet and free access to clean drinking water. Husbandry conditions were identical for all groups.

The physiological condition of the animals was monitored throughout the experimental period. Humane endpoints were applied where necessary to minimize animal suffering. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee and conducted in accordance with the European Directive 2010/63/EU. Animals were randomly assigned to experimental groups. In addition, the samples were evaluated in a coded (blinded) manner to minimize potential bias during the assessment.

The experiment was performed in 2 stages. In Stage 1, 2 mice received a single intravenous administration of Conjugate-65 at a dose of 2,000 mg/kg and a volume of 0.2 mL, delivered by slow injection into the tail vein. No mortality was observed during the 2 - 3 day observation period. In Stage 2, the remaining 3 animals were administered Conjugate-65 at the same dose. Animals in the control group received an equivalent volume (0.2 mL) of distilled water intravenously. Following administration, the general clinical condition of the animals was monitored hourly during the first 24 h and daily for the subsequent 14 days [35,36].

Statistical analysis was performed using Student's t-test for pairwise comparisons. Where appropriate, differences among multiple groups were evaluated using one-way analysis of variance (ANOVA), followed by appropriate post hoc tests. A p-value < 0.05 was considered statistically significant.

Results and discussion

To reduce the molecular weight of starch, it was first subjected to heterogeneous acid hydrolysis in the

presence of hydrochloric acid. The reaction was carried out at 75 - 90 °C with a starch:HCl molar ratio of 1.0:1.5 for up to 120 min. As a result of HCl-assisted hydrolysis, starch samples with different and reduced molecular weights were obtained. This approach facilitated subsequent periodate oxidation and nucleophilic conjugation reactions, thereby increasing the possibility of synthesizing new conjugates with controlled molecular weights.

In the next step, starch samples with different molecular weights were subjected to periodate oxidation, resulting in polyaldehyde starch derivatives with various degrees of oxidation. The resulting polyaldehyde starch was reacted with 2-aminoethyl sulfate via nucleophilic conjugation, leading to the synthesis of new polyanionic starch derivatives containing sulfate-bearing fragments. Suitable reaction conditions for the conjugation process were established. As a result of chemical modification, the introduction of sulfate groups imparted pronounced polyanionic character to the polysaccharide derivatives. In addition, a decrease in molecular weight was accompanied by a reduction in polymer viscosity.

Table 1 summarizes the influence of the DS of the conjugates on the molecular weight, ζ-potential, viscosity, and pKa values of the modified polysaccharides.

Table 1 Effect of the DS of the conjugates on the molecular weight, ζ-potential, viscosity, and pKa values of the polysaccharides.

Number	DS, Molar%	N content, %	S content, %	pKa	ζ-potential, mV	Mw×10^3	η, g/dl
1	12.1 ± 2.6	1.72 ± 0.2	3.93 ± 0.2	5.7 ± 0.4	−8.2 ± 6.3	7.97 ± 0.1	3.14 ± 0.2
2	25.2 ± 2.3	3.01 ± 0.6	6.86 ± 0.6	5.1 ± 0.3	−22.0 ± 6.1	8.06 ± 0.3	3.11 ± 0.3
3	45.3 ± 3.4	4.32 ± 0.7	9.88 ± 0.7	4.5 ± 0.3	−36.4 ± 5.8	8.11 ± 0.2	3.05 ± 0.1
4	58.2 ± 3.1	4.93 ± 0.9	11.3 ± 0.9	4.2 ± 0.2	−48.8 ± 5.6	8.17 ± 0.1	2.89 ± 0.1
5	**65.0 ± 3.7**	**5.19 ± 0.4**	**11.9 ± 0.4**	**4.0 ± 0.1**	**−51.9 ± 5.1**	**8.23 ± 0.2**	**2.70 ± 0.3**

DS - degree of substitution, defined as the number of substituted repeating units per 100 polysaccharide units.

As shown in **Table 1**, an increase in the content of sulfate-containing fragments in the starch structure led to a shift of the overall macromolecular charge toward more negative values, accompanied by a decrease in the pKa value. The introduction of sulfate groups into the macromolecule imparted negative charge to the polymer, resulting in the formation of pronounced polyanionic character in the starch derivatives. With increasing DS, a partial increase in Mw was observed, while a slight decrease in solution viscosity was detected. This behavior can be attributed to the nucleophilic conjugation of 2-aminoethyl sulfate fragments to the polysaccharide backbone and to partial macromolecular degradation occurring under the mildly alkaline conditions employed during the nucleophilic conjugation reaction.

A clear correlation was observed between the degree of substitution and the electrokinetic properties of the derivatives. Increasing DS resulted in progressively more negative ζ-potential values, confirming the growth of surface charge density due to the incorporation of sulfate groups. This increase in negative charge is expected to enhance electrostatic interactions with positively charged domains of coagulation-related proteins, thereby contributing to the observed anticoagulant activity.

IR spectroscopy was employed to analyze the structural features of the native and chemically modified starch samples and to identify changes in functional groups resulting from the modification process. This technique enables the detection of bond formation, the appearance of new functional groups, and variations in their relative intensities. The corresponding spectra are presented in **Figure 3**.

The characteristic absorption bands observed in the IR spectra provide information on the structure of the investigated materials. In **Figure 3**, spectrum 1 exhibits a broad absorption band in the range of 3,310 - 3,460 cm^{-1}, which is attributed to O−H stretching vibrations typical of polysaccharides. The absorption band at 2,930

cm^{-1} corresponds to asymmetric stretching vibrations of C−H groups.

In the fingerprint region, bands observed in the range of 1,000 - 1,200 cm^{-1} are associated with C−O and C−O−C stretching vibrations of the polysaccharide backbone [25]. The band at 1,620 cm^{-1} is attributed to bound water in starch, confirming that this spectrum is characteristic of native starch.

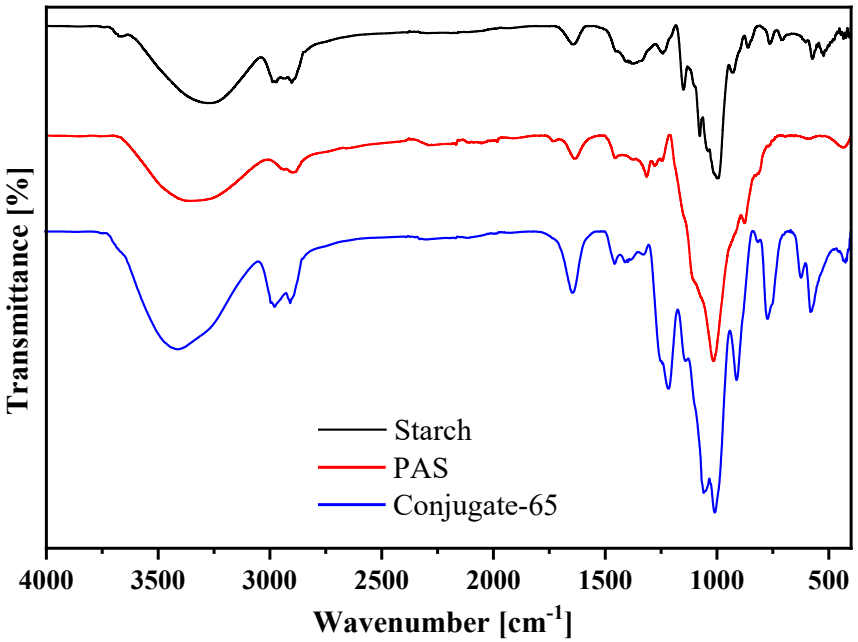


Figure 3 FTIR spectra of native starch, polyaldehyde starch (PAS), and its conjugate (Conjugate-65), illustrating structural modifications and the appearance of characteristic functional groups after periodate oxidation and nucleophilic conjugation.

In addition to the characteristic peaks described above, spectrum 2 shows an absorption band at approximately 1,720 cm^{-1}, which corresponds to C=O stretching vibrations. This observation confirms the formation of the polyaldehyde form of starch as a result of oxidation. At the same time, a decrease in the intensity of the O–H band is observed, indicating a reduction in hydroxyl group content due to their conversion into aldehyde groups.

In the IR spectra of starch derivatives chemically modified with a sulfate-containing primary amine, characteristic absorption bands appear in the range of 1,655 - 1,675 cm^{-1}, confirming the presence of –C =N– bonds. In addition, vibrations characteristic of sulfate groups are significantly enhanced in the range of 800 - 1,000 cm^{-1}, providing further evidence for the incorporation of sulfate groups into the polymer structure. The disappearance of the C=O absorption band at 1,720 cm^{-1} indicates that the aldehyde groups participated in reactions with amino groups, leading to the formation of Schiff base structures.

^{1}H NMR spectroscopy was employed to examine the structural features of the synthesized conjugates (**Figure 4**). Compared with native starch, the spectrum of Conjugate-65 exhibits an additional signal at δ 8.31 ppm, attributed to protons of the –CH=N– group. This signal confirms the formation of azomethine bonds between aldehyde groups of polyaldehyde starch and the 2-AES fragment.

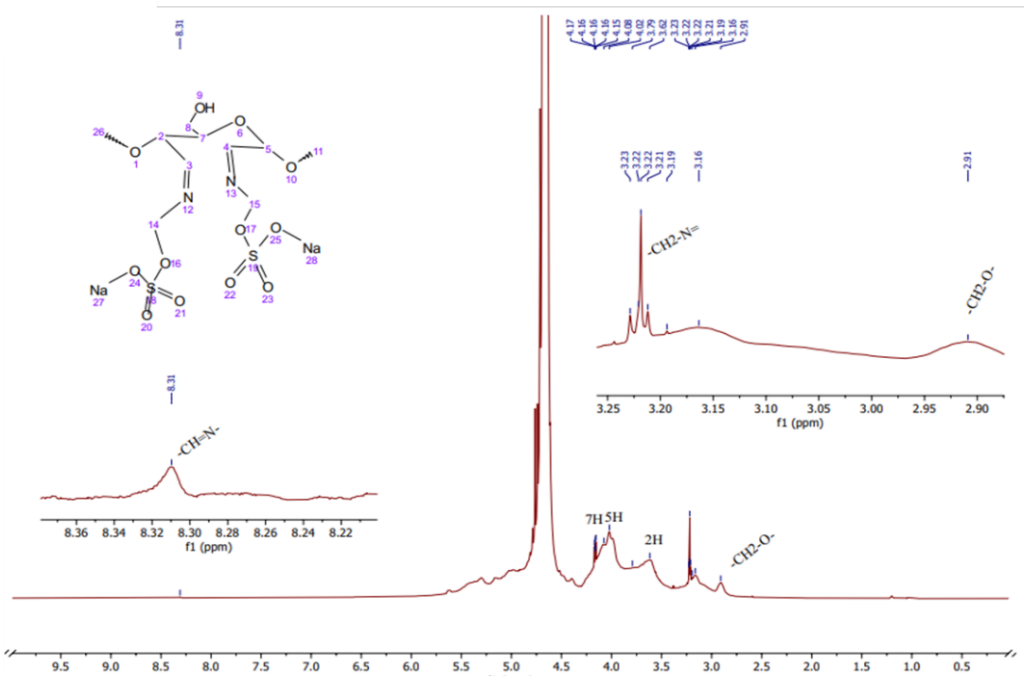


Figure 4 ^{1}H NMR spectrum of Conjugate-65, illustrating the characteristic proton signals of the polysaccharide backbone in the region of 3.0 - 5.5 ppm, along with the distinct imine proton (–CH=N–) signal at ~8.3 ppm, confirming the formation of Schiff base linkages. Additional signals corresponding to the introduced 2-aminoethyl sulfate moieties further support successful conjugation and structural modification of the polymer.

Several weak signals observed in the δ 3.21-3.25 ppm region correspond to =N–CH_2– protons associated with the azomethine linkage. The signal at δ 2.91 ppm is assigned to –CH_2–O– protons, indicating the presence of the ethyl chain connected through the sulfate group. Signals detected within the δ 3.0 - 4.5 ppm range are attributed to protons of the starch backbone, suggesting preservation of the main polysaccharide structure after modification.

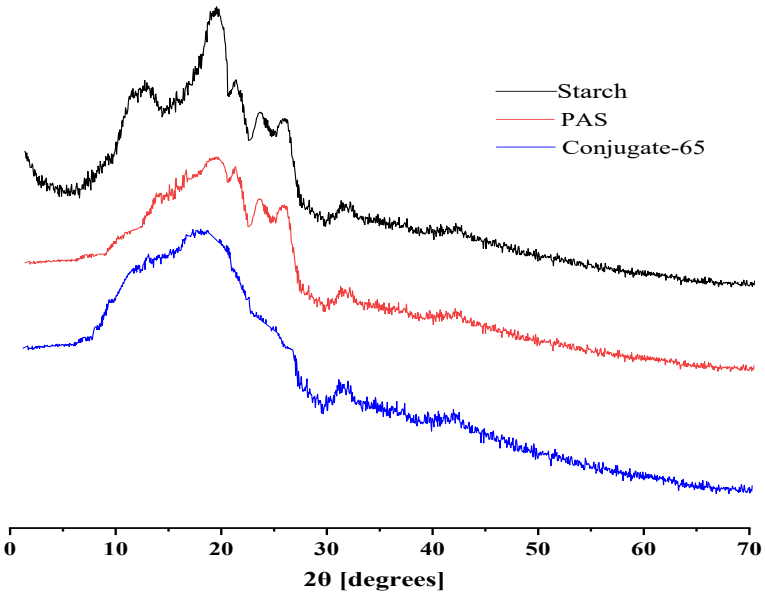


Figure 5 XRD patterns of native starch, polyaldehyde starch (PAS), and its conjugate (Conjugate-65), showing the reduction of crystalline peaks and increased amorphous character following periodate oxidation and nucleophilic conjugation.

Figure 5 presents the comparative X-ray diffraction (XRD) patterns of native starch, polyaldehyde starch, and starch derivatives containing sulfate-bearing fragments. In diffractogram 1, a distinct and intense diffraction peak is observed at 2θ ≈ 20°, indicating the semicrystalline structure of native starch and a relatively high fraction of the crystalline phase. In diffractogram 2, a pronounced decrease in diffraction peak intensity is observed as a result of periodate oxidation. This behavior is attributed to the reduction of hydroxyl groups, cleavage of C2 - C3 bonds, and disruption of intermolecular chain ordering within the macromolecule. Consequently, the proportion of crystalline regions decreases, while the contribution of the amorphous phase increases.

In the diffractogram of the final reaction product, namely the starch derivative conjugated with a 2-aminoethyl sulfate fragment, the diffraction peaks around 2θ ≈ 20° are almost completely flattened. This observation indicates that the crystalline characteristics of the polysaccharide macromolecule are nearly entirely lost. At the same time, weak diffraction peaks detected at 2θ ≈ 14° and 26° suggest the presence of partially preserved ordered regions within the polymer structure.

Numerous anticoagulant compounds have been synthesized through the sulfation of polysaccharides. Sulfate groups are capable of interacting with amino acid residues of antithrombin and thrombin in blood, thereby influencing their biological activity. Among anticoagulants, heparin is the most widely used compound; due to its sulfate groups, it binds to antithrombin III and enhances its activity by several orders of magnitude. The synthesized conjugates obtained in this study also contain sulfate groups that exhibit pronounced polyanionic character [31-33]. Therefore, it can be hypothesized that the sulfated starch derivatives are capable of interacting with antithrombin in blood and activating antithrombin-mediated mechanisms. To verify this hypothesis, the anticoagulant properties of conjugates with different DS were investigated. In addition, the content of sulfate groups and their distribution along the polysaccharide backbone may significantly affect the anticoagulant activity of the conjugates. An increase in polyanionic character is expected to enhance electrostatic interactions between the macromolecule and coagulation-related factors. Therefore, a comparative analysis of conjugates with different DS values is important for elucidating the relationship between chemical structure and anticoagulant performance, as well as for identifying possibilities to control biological activity (**Figure 6**).

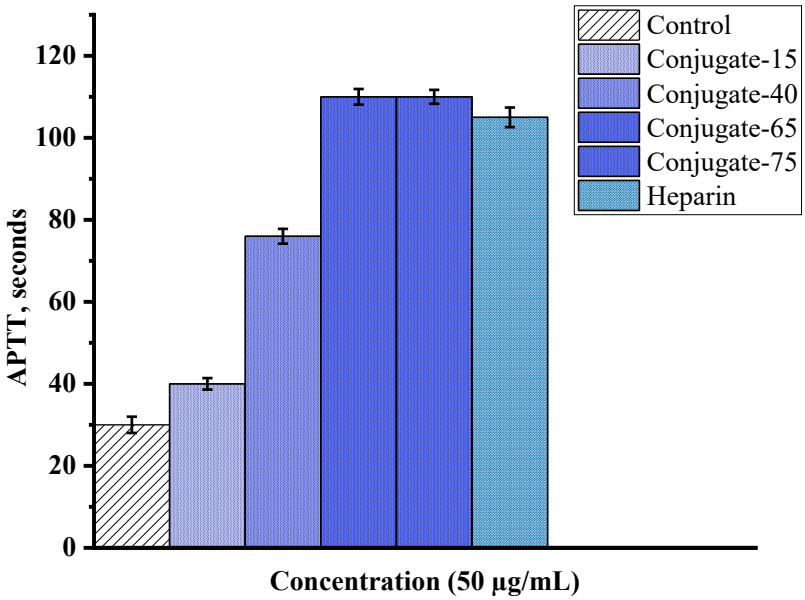


Figure 6 APTT values of starch-based conjugates with varying degrees of substitution (DS = 15, 40, 65, and 75), in comparison with control and heparin, demonstrating a DS-dependent prolongation of clotting time, with higher substituted conjugates exhibiting anticoagulant activity approaching that of heparin under the tested conditions.

To evaluate the anticoagulant activity of the starch-based conjugates, preliminary in vitro screening was performed using the activated partial thromboplastin time (APTT) assay, which is widely applied in clinical practice. First, the effect of variations in the content of sulfate-containing fragments in the conjugates on APTT values was investigated. An increase in anticoagulant activity was observed with increasing sulfate group content, expressed as the DS. Specifically, the control APTT value was 30 s, while the conjugate with a DS of 15 mol% prolonged APTT to 39 s, and the conjugate with a DS of 65 mol% extended APTT up to 110 s. Notably, the conjugate with a DS of 65 mol% exhibited activity comparable to, or slightly greater than, heparin under the tested conditions. The results indicate that a DS of 65 mol% represents an optimal value for starch derivatives, as no further increase in activity was observed at higher substitution degrees. Therefore, the conjugate with a DS of 65 mol% was selected for subsequent studies. In this set of experiments, molecular weight was kept at its lowest value to isolate the effect of DS.

In further experiments, the influence of molecular weight on the anticoagulant activity of the selected conjugate was evaluated.

Table 2 Anticoagulant activity of Conjugate-65 at different molecular weights (concentration: 50 μg/mL).

Name	Mw, KDa	TT (s)	APTT (s)
Conjugate-65	50 ± 2.1	*91 ± 0.7	*94 ± 0.3
	20 ± 1.4	*107 ± 0.9	*101 ± 0.4
	8.1 ± 0.2	**115 ± 0.7	**110 ± 0.9
Heparin	16.0 ± 1.2	*109 ± 0.7	*106 ± 0.8
Control	-	**20 ± 0.2**	**30 ± 0.1**

Note: (*- $p < 0.05$; **- $p < 0.01$, n = 6).

As shown in **Table 2**, a decrease in molecular weight resulted in an enhancement of anticoagulant activity. In particular, for the conjugate with a DS of 65 mol%, when the molecular weight was 50 kDa, TT and APTT values were 91 and 94 s, respectively. When the molecular weight was reduced to 8 kDa, TT and APTT increased to 115 and 110 s, respectively. Overall, a clear enhancement of anticoagulant activity was observed with decreasing molecular weight. For comparison, the reference anticoagulant heparin with a molecular weight of 16 kDa showed TT and APTT values of 109 and 106 s, respectively. The observed enhancement of anticoagulant activity with decreasing molecular weight may be associated with improved molecular mobility and accessibility of functional sulfate groups. Lower molecular weight derivatives likely possess higher diffusion capability and reduced steric hindrance, facilitating more efficient interactions with coagulation-related proteins.

This behavior is consistent with previously reported effects in sulfated polysaccharide systems, where molecular weight plays a critical role in determining biological performance. In this analysis, the degree of substitution (DS) was fixed at 65 mol% to evaluate the effect of molecular weight.

In the next stage of the study, the effects of different concentrations of the newly synthesized starch derivative on blood coagulation factors were investigated in animal plasma under in vitro conditions using these 2 assays. **Table 3** presents the concentration-dependent effects of the conjugate on standard coagulation tests (APTT, TT and PT) in comparison with heparin. The obtained results demonstrate that with increasing concentration, the effect of the modified polysaccharide on the intrinsic coagulation pathway-reflected by APTT and TT values-consistently increased.

In the control group, the activated partial thromboplastin time (APTT), which reflects the intrinsic

coagulation pathway, was 28.2 ± 0.2 s. In the presence of the starch derivative at a concentration of 50 μg/mL, APTT increased to 109.1 ± 0.4 s, while heparin prolonged APTT to 104.6 ± 0.5 s. These results indicate that both substances reduce the activity of coagulation factors involved in the intrinsic pathway, such as factors VIIIa, IXa, and Xa.

A significant increase in thrombin time (TT) was also observed. TT increased from 21.5 ± 0.7 s in the control to 115.2 ± 0.5 s for Conjugate-65 at 50 μg/mL and to 110.4 ± 0.7 s in the presence of heparin. This finding indicates inhibition of thrombin activity and a slowdown in the conversion of fibrinogen to fibrin. In contrast, prothrombin time (PT) values remained almost unchanged, suggesting that the tested compounds predominantly affect the intrinsic coagulation pathway. Moreover, increasing the conjugate concentration above 50 μg/mL did not result in a further significant prolongation of coagulation times.

Table 3 Dose-dependent effects of the conjugate and heparin on blood coagulation times in APTT, TT, and PT assays.

Examples	Concentration, μg/mL	APTT	TT	PT
Control	-	28.2 ± 0.2	21.5 ± 0.7	9.7 ± 0.6
Conjugate-65	10	41.5 ± 0.8	43.5 ± 0.8	11.0 ± 0.3
	20	76.4 ± 0.7	77.7 ± 0.1	11.9 ± 0.7
	30	90.3 ± 0.9	**93.4 ± 0.7	**12.2 ± 0.7
	50	**109.1 ± 0.4	**115.2 ± 0.5	**12.9 ± 0.9
	70	*110.7 ± 0.7	*116.3 ± 0.6	*13.7 ± 0.4
	100	**111.5 ± 0.9	*117.7 ± 0.8	*13.9 ± 0.8
Heparin	20	*75.1 ± 0.9	79.9 ± 0.8	11.4 ± 0.5
	50	**104.6 ± 0.5	**110.4 ± 0.7	**12.4 ± 0.6
	100	*171.3 ± 0.7	*176.9 ± 0.4	*52.7 ± 0.6

Note: (*- $p < 0.05$; **- $p < 0.01$, n = 6).

At a concentration of 100 μg/mL, heparin caused a sharp increase in both APTT and TT, reaching 171.3 ± 0.7 and 176.9 ± 0.4 s, respectively. While this confirms the strong anticoagulant effect of heparin, such high concentrations may excessively thin the blood and increase the risk of internal bleeding. In contrast, the presented results indicate that this risk can be controlled for starch-based conjugates by adjusting their concentration.

Based on the *in vitro* studies, the polysaccharide derivatives synthesized from starch exhibit anticoagulant activity under the tested experimental conditions. These samples significantly affect the blood coagulation process and are characterized by their ability to prolong coagulation times. During the study, an optimal concentration of 50 μg/mL was identified for starch derivatives containing 2-aminoethyl sulfate fragments, exhibiting pronounced anticoagulant activity. Importantly, no excessive prolongation of coagulation times was observed at higher concentrations within the studied range, indicating a low risk of excessive anticoagulation. Under the tested experimental conditions, the anticoagulant activity approached that of heparin in clotting-time assays under the tested conditions. It should be noted that direct quantitative comparison with heparin requires further dedicated mechanistic and pharmacodynamic studies.

In subsequent studies, the anticoagulant activity of the obtained starch derivative was evaluated *in vivo* using an experimental thrombosis model. Anticoagulant

properties were assessed by standard coagulation assays, namely APTT and TT. The results are presented in **Figure 7**.

The anticoagulant effect of Conjugate-65 was evaluated in vivo using a thromboplastin-induced procoagulant model. As shown in **Figure 7**, administration of thromboplastin in the control group resulted in activation of the coagulation system, which was characterized by relatively reduced APTT and TT values. In animals treated with heparin (130 IU, 2 mg/kg) and Conjugate-65 (2 mg/kg), a pronounced prolongation of blood clotting time was observed. A significant increase in APTT indicates suppression of the intrinsic coagulation pathway. The elevation of TT reflects a deceleration of thrombin-mediated fibrin formation. The anticoagulant effect observed in the Conjugate-65 group can be attributed to the high density of negative charges generated by the introduced sulfate groups. These negatively charged fragments likely interact electrostatically with coagulation-related proteins, particularly antithrombin III, thereby attenuating key stages of the coagulation cascade. Overall, the obtained results demonstrate that Conjugate-65 exhibits strong anticoagulant activity *in vivo*, showing an effect comparable to that of heparin under the studied conditions.

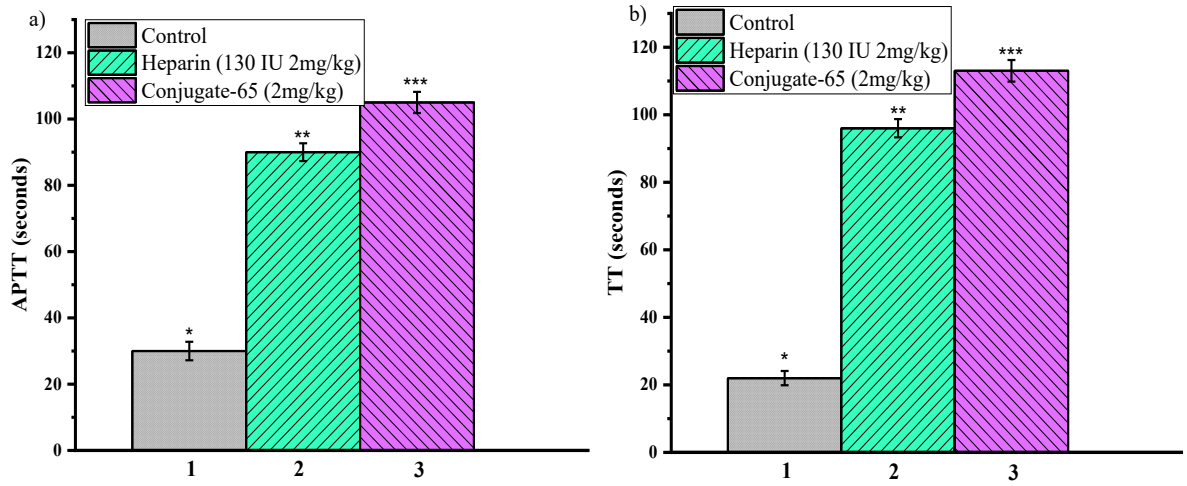


Figure 7 Effect of Conjugate-65 (2 mg/kg) on blood coagulation parameters in a thromboplastin-induced procoagulant model: (a) APTT and (b) TT, compared with control and heparin (130 IU, 2 mg/kg), showing statistically significant prolongation of clotting times (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n = 6).

To assess the changes in the lung tissues of animals subjected to the induced model, a macroscopic examination was performed following necropsy (**Figure 8**).

In this study, animals in the thromboplastin-induced procoagulant control group died. In contrast, no mortality was observed in the groups treated with Conjugate-65 or heparin. These findings suggest that the tested compounds prevented blood coagulation disturbances and inhibited thrombus formation in animal organs. In the thromboplastin-induced procoagulant model, numerous thrombi were detected in the lung tissues of rats, accompanied by a dark red discoloration of the lungs. In animals treated with Conjugate-65 and heparin, thrombus formation was minimal, and the lung color remained similar to that of healthy tissue, exhibiting a pale pink appearance. These results confirm the anticoagulant effect of the investigated compounds.

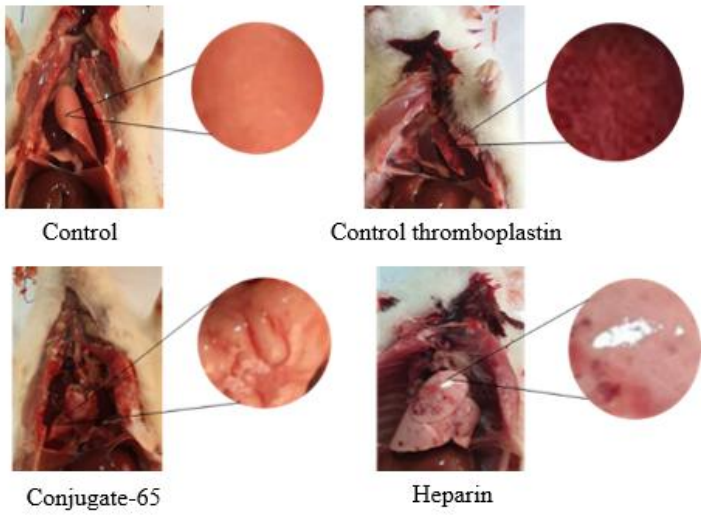


Figure 8 Macroscopic appearance of lung tissues of animals subjected to the induced model following treatment with Conjugate-65 and heparin, illustrating visible morphological alterations and possible inflammatory responses.

Furthermore, in subsequent experiments, the duration of action of the synthesized Conjugate-65 was studied in comparison with heparin. For this purpose, blood samples were collected from the venous vessels of outbred white rats following administration of Conjugate-65 (2 mg/kg) and heparin (130 IU, 2 mg/kg), and the persistence of their effects on blood coagulation was evaluated (**Figure 9**).

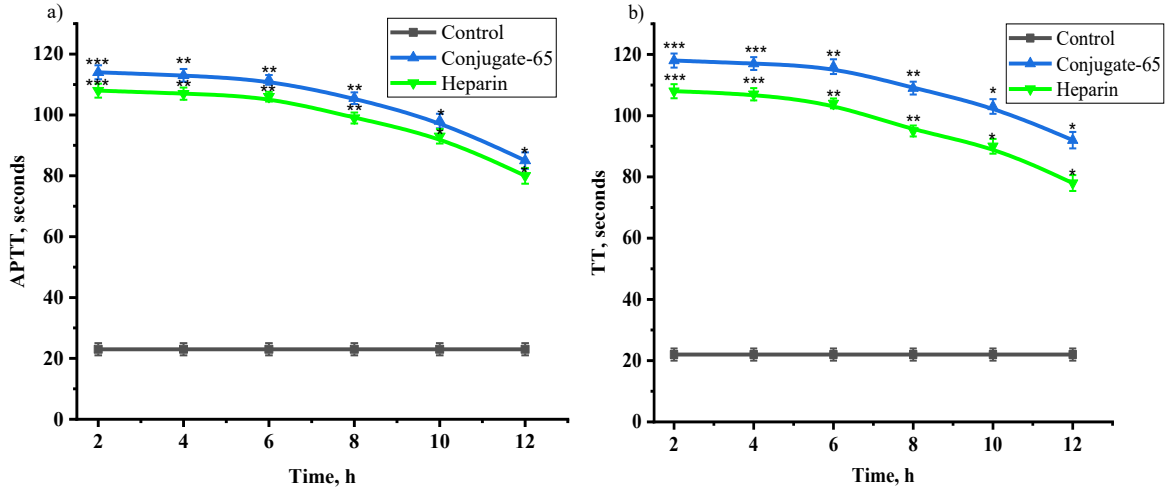


Figure 9 *In vivo* duration of anticoagulant activity of Conjugate-65 over time: (a) APTT and (b) TT, illustrating time-dependent changes in clotting parameters after administration (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 6).

The synthesized polysaccharide samples affected coagulation parameters for approximately 2 - 8 h. After 8 h, a significant decrease in activity was observed. This reduction is attributed to enzymatic degradation of the polysaccharide system and systemic clearance *in vivo*.

Furthermore, as reported in the literature, Schiff base (–C=N–) linkages are relatively stable under physiological conditions and predominantly undergo hydrolysis under acidic environments [37]. Therefore, the observed decrease in anticoagulant activity is more reasonably attributed to degradation of the polysaccharide backbone rather than instability of the imine linkage. However, direct time-dependent spectroscopic evaluation of imine bond stability was not performed and represents a limitation of the present study.

The anticoagulant mechanism of heparin is primarily based on its highly sulfated structure, which

enables binding to antithrombin III and may subsequently influence thrombin-related processes. Numerous negatively charged sulfate groups within the heparin macromolecule interact electrostatically with coagulation-related proteins, thereby slowing the coagulation process. In a similar manner, starch modified with 2-aminoethyl sulfate becomes a negatively charged polymer due to the incorporation of sulfate groups along the polymer backbone. Under physiological conditions, these groups are ionized, generating a high negative surface charge. Such a structure can interact with positively charged regions of coagulation factors, leading to a reduction in their enzymatic activity, particularly through interactions with thrombin and factor Xa, which slows key stages of the coagulation cascade.

In addition, sulfated starch may form complexes with antithrombin III, thereby enhancing its natural inhibitory effect and promoting faster inactivation of coagulation enzymes. The hydrophilic and negatively charged polymer surface can also reduce platelet adhesion and limit platelet activation, resulting in a slowdown of primary thrombus formation. Furthermore, sulfate groups may interact with fibrin monomers, hindering proper fibrin fiber formation and leading to a looser and less stable thrombus structure. The degree of anticoagulant activity depends on the sulfate group content within the molecule, with increasing DS leading to higher charge density and enhanced biological activity. Therefore, starch conjugates containing 2-aminoethyl sulfate fragments can be considered promising anticoagulant biomaterials that may exert their effects via a mechanism analogous to that of heparin. However, it should be noted that the exact anticoagulant mechanism has not been directly confirmed in this study and requires further investigation using specific assays such as anti-factor Xa and anti-thrombin activity tests.

Randomization and blinded evaluation were applied in the experimental design, ensuring the reliability of the obtained results. However, additional parameters such as bleeding time assessment and histological analysis were not included in the present study.

In the safety evaluation of new anticoagulant drugs, determination of their acute toxicity represents one of the essential stages. This assessment is particularly important for agents administered by injection, as it allows the preliminary evaluation of their suitability for clinical application. In this study, the newly synthesized anticoagulant Conjugate-65 sample was tested by intravenous administration in mice. This approach enabled the evaluation of the compound's direct effect on the circulatory system and, consequently, its acute toxic effects. Based on the obtained results, the acute toxicity dose (LD_{50}) of the synthesized anticoagulants was determined (**Table 4**).

Table 4 Acute toxicity parameters of the Conjugate-65 polysaccharide derivative (M ± m; n = 5).

Sample name	Dose (mg/kg, mL)	Total number of animals / number of dead animals	Mean body weight (g) (Day 1)	Mean body weight (g) (Day 7)	Mean body weight (g) (Day 14)	LD_{50} (mg/kg)
Control	Distilled water 0.2 mL	5/0	21.0 ± 0.22	22.2 ± 0.25	23.4 ± 0.3	-
Conjugate-65	2,000	5/0	21.1 ± 0.23	22.3 ± 0.26	23.6 ± 0.31	> 2,000

Following intravenous administration of the test sample at a dose of 2,000 mg/kg, mice exhibited an increased respiratory rate, reduced locomotor activity, and pupil constriction within the first 5 min. These changes were transient, and the animals returned to a normal physiological state after 15 - 20 min. No mortality was recorded during the 14-day observation period (0/5). Body weight in the experimental group did not show a significant decrease compared with the control group at Days 7 and 14 ($p > 0.05$).

Based on the obtained results, the median lethal dose was estimated as LD_{50} > 2,000 mg/kg. According to Sidorov's toxicity classification, the test sample belongs to toxicity class VI (relatively non-toxic substances).

However, it should be noted that the limit test design and the small sample size do not allow precise determination of LD_{50} and represent a limitation of the present study.

The obtained results are consistent with previously reported studies on sulfated polysaccharides, where anticoagulant activity is closely related to charge density and molecular structure. Compared to other polysaccharide-based systems, the present starch-derived conjugates offer advantages in terms of synthetic accessibility and tunable physicochemical properties. In addition, the synthesis approach employed in this study is based on an original method for which a patent has been granted (Uzbekistan Patent No. IAP 8059). These features highlight the potential of the developed materials as promising candidates for anticoagulant applications.

Conclusions

The incorporation of sulfate-containing fragments into the starch backbone significantly modifies its physicochemical behavior under aqueous and physiological conditions, particularly by enhancing solubility and dispersion stability. The increase in negative ζ-potential and decrease in pKa values indicate a higher density of negative charges within the polymer structure. Physicochemical analyses confirmed successful functionalization while preserving the main polysaccharide backbone.

Biological evaluation demonstrated that the synthesized starch derivatives are capable of delaying the blood coagulation process. In vitro assays (APTT, TT, and PT) showed significant prolongation of clotting times, which depended on both sulfate group content and molecular weight. Increasing charge density and decreasing molecular weight were associated with enhanced anticoagulant activity.

In vivo experiments using a thromboplastin-induced model confirmed the anticoagulant effect of Conjugate-65. Acute toxicity studies following intravenous administration indicated LD_{50} > 2,000 mg/kg, suggesting low toxicity; however, it should be noted that this value was obtained using a limit test design and requires further detailed toxicological evaluation.

Overall, the obtained results suggest that sulfated starch derivatives are promising candidates for the development of anticoagulant biomaterials, although further mechanistic and hemocompatibility studies are required to fully establish their biomedical potential.

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Declaration of Generative AI in Scientific Writing

The authors declare that generative tools were used exclusively for language editing and grammatical refinement during manuscript preparation. These tools were not involved in the generation of scientific content, data analysis, or interpretation of the results. All research findings, analyses, and conclusions are solely the responsibility of the authors, who have fully reviewed and approved the final manuscript.

CRediT Author Statement

Jamoliddin Abdurakhmanov: Writing - Original draft; Supervision; Formal analysis; Data curation. **Shavkat Shomurotov**: Writing - review & editing; Methodology; Conceptualization. **Oliy Akhmedov**: Writing - review & editing; Formal analysis. **Abbaskhan Turaev**: Resources; Methodology; Conceptualization.

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