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Physicochemical and Biological Properties of O-carboxymethyl Chitosan Obtained From Chitosan of Dead Bees

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Abstract

Objective: To synthesize carboxymethyl chitosans (CMCS) from local raw material *Apis mellifera*, study their physicochemical properties, determine molecular weight and biological activity, and make CMCS-based antibacterial biodegradable polymer compositions.

Methods: CMCS was synthesized from dead bees; its molecular weight was determined by viscometry, and the degree of acetylation was established by coductometric titration. Its physicochemical properties were studied using infrared, nuclear magnetic resonance, and X-ray spectroscopy, and the results obtained were compared with quantum chemical calculations. The study presents the results of histological analysis to investigate the biological activity of CMCS.

Results: For the first time, O-CMCS was synthesized from *Apis mellifera* chitosan, and we determined its optimal conditions: sodium hydroxide concentration, alkylating agent ratio, process temperature and duration, and kinetic parameters.

For the first time, ointments for burn wounds based on *Apis mellifera* O-CMCS were prepared and tested on white rats.

The sodium hydroxide concentration was found to be 30%; the required temperature for the reaction was 650 °C; the polymer formation reaction process lasted 3 hours; the ratio of chitosan to monochloroacetic acid was 1:1, and the ratio of chitosan to isopropyl alcohol was 1:50.

Conclusions: The CMCS-based ointment has an antimicrobial effect and a unique antiseptic property. It does not disrupt the physiological functions of the skin or cause allergic reactions and toxicological problems.

Keywords: chitosan, carboxymethyl chitosan, degree of substitution, infrared spectroscopy, nuclear magnetic resonance spectroscopy, X-ray spectroscopy, quantum chemical calculation, histological examination

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Изучение физико-химических и биологических свойств О-карбоксиметилхитозана, полученного из хитозана пчелиного подмора

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Резюме

Цель: Синтез карбоксиметилхитозанов из местного сырья *Apis Mellifera*, изучение их физико-химических свойств, определение молекулярной массы и биологической активности и создание на их основе антибактериальных биоразлагаемых полимерных композиций.

Методы: Синтезирован карбоксиметилхитозан из подмора пчел, определена его молекулярная масса методом вискозиметрии, степень ацетилирования методом кодуктометрического титрования. Изучены его физико-химические свойства методами ИК-, ЯМР- и РСА-спектроскопии, полученные результаты сопоставлены с квантово-химическими расчетами. В работе представлены результаты гистологического анализа для изучения биологической активности карбоксиметилхитозана.

Результаты: Впервые синтезирован О-карбоксиметилхитозан из хитозана *Apis Mellifera* и определены оптимальные условия: концентрация гидроксида натрия, соотношение алкилирующего агента, температура и продолжительность процесса, кинетические параметры.

Впервые приготовлены и испытаны на белых крысах мази для ожоговых ран на основе О-карбоксиметилхитозана *Apis Mellifera*. Определена концентрация гидроксида натрия 30%, необходимая температура для проведения реакции 650 °С, продолжительность процесса реакции полимерообразования 3 часа, соотношение хитозана к монохлоруксусной кислоте 1:1, а соотношение хитозана к алкилирующему агенту ИПС 1:50.

Заключение: Мазь на основе карбоксиметилхитозана оказывает противомикробное действие, обладает фармакологической активностью в виде уникального антисептического свойства, препарат не нарушает физиологические функции кожи, не вызывает аллергического состояния организма, не вызывая в нем токсикологических проблем.

Ключевые слова: хитозан, карбоксиметилхитозан, степень замещения, ИК- и ЯМР-спектроскопия, рентгеноструктурный анализ, квантово-химический расчет, гистологическое исследование

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Introduction

Chitosan is a natural biopolymer of the 21st century, which distinctive properties attract many researchers. Biopolymers play an important role in our daily lives, in the chemical and food industries, pharmacy and medicine, and agriculture.[1]

Chemistry and chemical technology have been taking a lot of interest in natural polymers—chitin, chitosan, and its derivatives, chemically, physically, or enzymatically modified chitosan. Thus, it is thought to be pertinent to conduct both scientific and applied research to enhance the chitosan modification[2,3] in order to produce chitosan carboxymethyl ethers. Over the past 10 years, interest in research and application of chitosan and carboxymethyl chitosan (CMCS) has significantly grown.[4,5]

Chitin and chitosan are useful materials because they are nontoxic, biocompatible, and biodegradable into innocuous products.[6] CMCS, a carboxymethylated derivative of chitosan, exhibits better water solubility and biocompatibility compared with chitosan and possesses superior microbiological properties.[7]

Methods

Chitosan with molecular weight of 162 kDa and deacetylation degree of 85% was used for chemical synthesis of O-CMCS (Figure 1). Dead honeybees were a source of chitin utilized for deacytylation.[8] Monochloroacetic acid ($ClCH_2COOH$), double-distilled water (18 $M\Omega \cdot cm^{-1}$), isopropyl alcohol (C_3H_7OH), sodium hydroxide (NaOH), acetic acid (CH_3COOH), and ethanol (C_2H_5OH) were used to create CMCS from chitosan. Every intermediate that formed during the chemical reaction and was a part of the final product did not undergo further purification.[9] Table 1 compares characteristics of chitosan synthesized from *Bombyx mori*[10] and chitosan from dead bees.

Infrared Spectroscopy

The IRTracer-100 (Shimadzu Corp, Japan) Fourier transform infrared spectrometer was used to perform infrared spectroscopic studies of the synthesized biopolymers. It had the MIRacle-10 attenuated total reflection attachment with a diamond/ZnSe prism, a spectral range of 4000 ± 400 cm^{-1} on the wavenumber scale, a resolution of 4 cm^{-1}, a signal-to-noise ratio of 60 000:1, and a scanning speed of 20 spectra per second.

X-ray phase analysis was conducted using CuK radiation and the Rotaflex RU-200 diffractometer (Rigaku Corp, Japan).

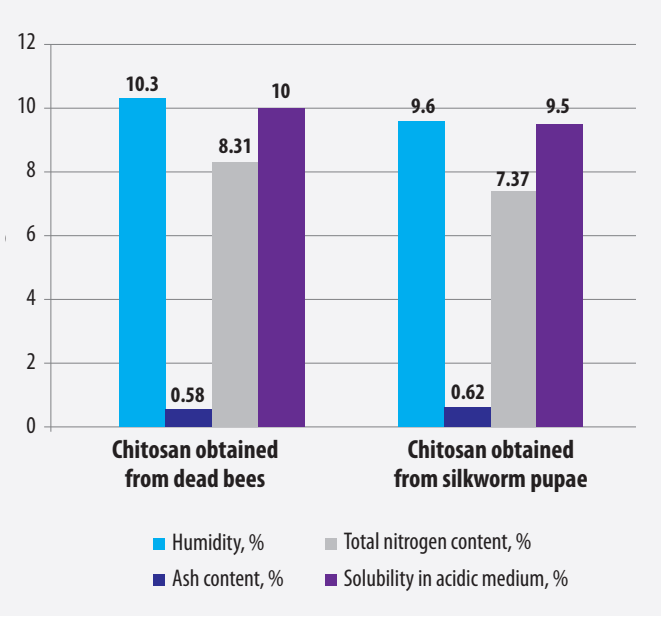


Figure 1. Certain physical characteristics of chitosan obtained from dead bees

Рисунок 1. Некоторые физические характеристики хитозана, полученного из подмора пчел

A pulsed nuclear magnetic resonance (NMR) relaxometer (Bruker Corp, USA) was used for NMR spectroscopy. NMR characteristics were tested using phase detection at 298 K and an operating frequency of 20 MHz. The duration of the π/2 pulse was 4 μs. The receiver dead time was 3.5 μs. Following a single pulse of 900 μs duration, free induction decay was used to obtain T2 measurements. The Carr-Purcell pulse sequence was applied when the T2 values were high. A model function, comprising a sum of terms representing different sample components, was used for heterogeneous systems:

$$M(t) = M_a(0) \cdot (P_a \cdot e^{(\frac{-t}{T_{2a}})}) + M_b(0) \cdot (P_b \cdot e^{(\frac{-t}{T_{2b}})}),$$

where P_a and P_b are the apparent populations for phases *a* and *b*, respectively.

Quantum Chemical Calculations

Quantum chemical calculations were performed using HyperChem 8.0 (Hypercube Inc, Canada) to determine the molecular geometry and electronic structure of O-CMCS.

Histological Analysis

Following laboratory experiments on white rats, skin tissue was removed, preserved in a 10% formaldehyde solution, and stained with hematoxylin and eosin. Microslides (sizes x=4×10, 10×10, 20×10, 40×40, 60×10, and 80×10) were photographed through a microscope.[11]

Results and Discussion

It is well known that the amount of chitin in raw materials varies depending on the organisms' age and environment in addition to their kind.[12] The composition of the bee-derived chitosan used in this study is presented in Figure 1, alongside literature data for raw materials of different origins. The results indicate that the chitosan composition from the dead bees is comparable to that of mulberry moth pupae.[13]

A carboxymethyl group was introduced into the chitosan structure using carboxymethyl chitosan (CMCS). This modification enhances solubility in neutral and basic solutions without compromising other essential properties. Carboxymethylation of chitosan's hydroxyl and amine groups produces CMCS.[14,15] Key factors influencing the carboxymethylation process include temperature, reaction duration, and the molar ratio of monochloroacetic acid (MCAA) to chitosan. Variations in these parameters result in CMCS samples with differing concentrations of carboxymethyl groups.

By adjusting the CMCS:MCAA ratio, the carboxymethylation process was optimized (Table 1).

The ionic carboxymethyl groups in CMCS enhance its solubility. Increasing the proportion of the alkylating agent (MCAA) in the reaction medium increases the number of carboxymethyl groups and, consequently, the solubility of the samples. However, a higher molar concentration of MCAA generates significant amounts of low molecular weight salts, leading to excessive ethyl alcohol consumption during CMCS purification.

Table 1

Dependence of the degree of substitution in CMCS samples on the CMCS:MCAA ratio (T = 65 °C, τ = 3 h)

Таблица 1

Зависимость степени замещения образцов карбоксиметилхитозана от соотношения карбоксиметилхитозан:монохлоруксусная кислота, T=650C, τ=3 ч

MCAA conc., mol/L	CMCS: MCAA ratio	N_{amin}, %	Degree of substitution	Solvent, %
0.05	1:0.5	4.9	80	78.5
0.1	1:1	4.75	91.5	90.2
0.15	1:2	4.50	94	93.4
0.2	1:3	3.8	96	96.2

Note: CMCS, carboxymethyl chitosan; conc., concentration; MCAA, monochloroacetic acid

Прим.: CMCS – карбоксиметилхитозан; conc. – концентрация; MCAA – монохлоруксусная кислота

A chitosan:MCAA molar ratio of 1:1 was determined to be optimal for the carboxymethylation process, as it achieves sufficient solubility for evaluating biological activity while minimizing purification challenges.

Reaction rate calculations were performed for CMCS samples prepared at various CMCS:MCAA ratios.

By adjusting the chitosan:MCAA ratio, the carboxymethylation process was optimized in terms of temperature, alkaline solution concentration, reaction duration, and MCAA quantity. Notably, the solubility of chitosan samples subjected to alkaline treatment increased from 70%-75% to 85% when the NaOH concentration was raised from 20% to 30%.[9]

The kinetic parameters for the carboxymethylation of O-CMCS derived from dead bees were established as follows: a NaOH concentration of 30%, a reaction temperature of 65 °C, a reaction duration of 3 hours, a chitosan:MCAA ratio of 1:1, and a chitosan:isopropanol (CS:IPA) ratio of 1:50. Under these conditions, O-CMCS was synthesized from chitosan for the first time, and its optimal production parameters were defined (Figure 2).

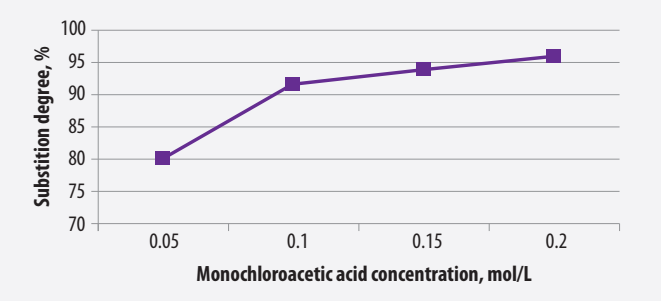


Figure 2. Dependence of the degree of substitution of CMCS on the MCAA concentration

Рисунок 2. Зависимость степени замещения карбоксиметилхитозана от концентрации монохлоруксусной кислоты

The nitrogen mass fraction decreased from 8.31% in chitosan to 4.75% in CMCS following carboxymethylation, reflecting structural changes in the polymer. Conductometric titration revealed a degree of substitution ranging from 80% to 96%. The molecular weight of CMCS, determined by viscometry, was approximately 46 700 g/mol.[9]

Infrared (IR) spectroscopy was used to investigate the molecular structural differences between chitosan (CS) and CMCS. The IR spectrum of chitin exhibits characteristic absorption bands at approximately 3270 cm^{-1}, attributed to -N-H bond stretching vibrations, at 1375 cm^{-1}, indicating the presence of $-CH_3$ groups, and at 1625 cm^{-1}, characteristic of C=O stretching in amide groups. The IR spectrum of chitosan shows peaks at around 3300 cm^{-1}, corresponding to $-NH_2$ group stretching, and in the 1390-1000 cm^{-1} range, reflecting C–N bond skeletal vibrations associated with amine groups.

All amine types in chitosan exhibit absorption bands in the 1360-1000 cm^{-1} range due to C–N bond vibrations. Additionally, bands at 1433 cm^{-1}, corresponding to $-CH_2$ group bending vibrations, and at 1373 cm^{-1}, indicating -OH bond bending (inflection), were observed in both chitin and chitosan samples. A broad band of moderate intensity in the 1320-1387 cm^{-1} range, attributed to -OH bond vibrations, was also detected in the chitosan sample (Figure 3). In the CMCS IR spectrum, an absorption peak characteristic of hydroxyl groups appears in the 3399-3167 cm^{-1} range. The C=O group is identified by an absorption band at approximately 1552 cm^{-1} (Figure 4).[16]

These results indicate that only the -OH groups in O-CMCS undergo substitution, while the $-NH_2$ groups remain unaffected. Furthermore, the -COOH groups in O-CMCS can form intramolecular and intermolecular interactions with $-NH_2$ and charged $-NH_3^+$ groups. Consequently, O-CMCS contains a higher proportion of $-NH_3^+$ groups than chitosan under identical conditions, which likely contributes to its enhanced antibacterial properties.

After synthesizing O-CMCS from dead bees, its X-ray diffraction (XRD) pattern was obtained and analyzed (Figure 5). A flat profile in the 2θ range of 20-30° indicates an amorphous structure. Compared with the XRD pattern of the original CS, the appearance of a new singlet peak in the 2θ range of 35-40° suggests the formation of a single, pure O-CMCS phase.

The nuclear magnetic resonance (NMR) spectra of O-CMCS and the *Apis mellifera* CS sample were

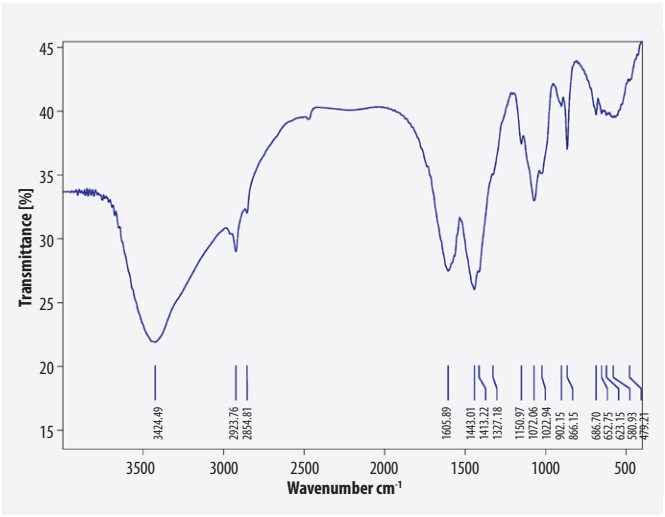


Figure 4. IR spectra of O-CMCS obtained from dead bees
Рисунок 4. ИК-спектры О-карбоксиметилхитозана, полученные из подмора пчел

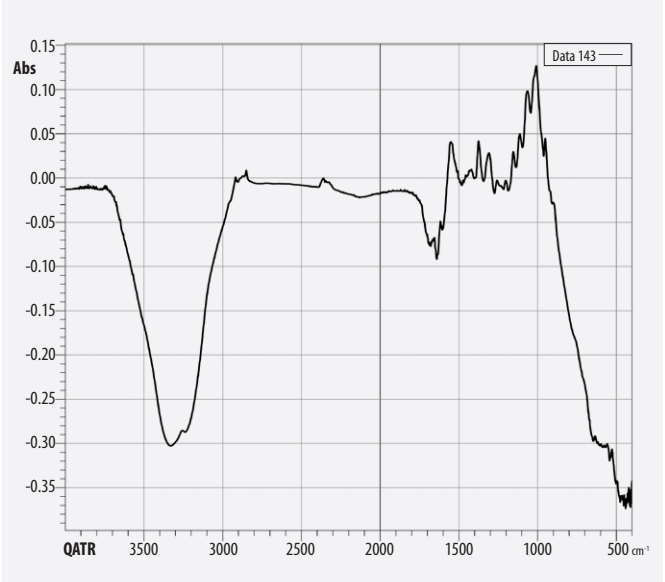


Figure 3. Infrared (IR) spectrum of chitosan obtained from dead bees[17]
Рисунок 3. ИК-спектр хитозана, полученного из подмора пчел[17]

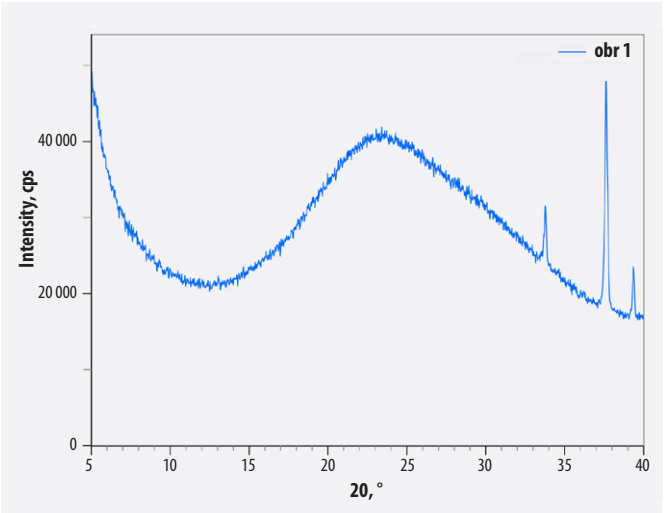


Figure 5. XRD pattern of O-carboxymethyl chitosan derived from chitosan of dead bees
Рисунок 5. Рентгенограмма О-карбоксиметилхитозана, полученного из хитозана пчелиного подмора

acquired and analyzed. In the ^{1}H NMR spectrum of CS, additional signals at 1.89-1.91 ppm correspond to protons on carbon atoms 1, 2, 3, 5, and 6 of the acetamide ring. A peak at 3.01 ppm, attributed to the -NH_2 group protons, appears as a doublet due to coupling with the -OH group protons on the third and sixth carbon atoms of the ring, which resonate at 3.58-3.75 ppm (Table 2 and Table 3).

In the ^{1}H NMR spectrum of O-CMCS, the protons attached to the 2, 3, and 6 carbons of the acetamide ring resonate at 3.09 ppm. This value is derived by integrating the signals observed at 3.24 ppm and 3.41 ppm. The methylene protons of the carboxyl group in O-CMCS appear at 2.52 ppm. The protons of the NH_2 group resonate at 3.90 ppm, while the protons of carbon atoms 1 and 4 in the glucosidic bonds of the polymer chain are detected at 3.52-3.54 ppm. The protons associated with the -OH groups contribute to a triplet peak in the spectrum.

The introduction of a carboxymethyl group scatters the chemical shifts of the acetamide ring's protons, as observed in the ^{1}H NMR spectra of O-CMCS. Specifically, protons associated with carbons 1 and 4 are detected at 1.50 ppm. Protons bound to carbon 7 and 6 are observed at 3.24-3.41 ppm, while protons attached to carbon 2 resonate at 2.52 ppm, carbon 3 at 2.73 ppm, and carbon 5 at 3.09 ppm. Due to the presence of the carboxymethyl group, the protons of the NH_2 group shift to 3.52 ppm, and the protons of the -OH group attached to carbon 3 shift to 3.57 ppm. The protons of the carboxyl group in O-CMCS are observed at 3.59 ppm.

Quantum chemistry techniques were employed to investigate the electronic structure of chitosan and its derivative, O-CMCS, focusing on the interactions between their chemical groups. Molecular orbitals of chitosan and structural models of O-CMCS were calculated to evaluate the influence of the carboxymethyl group on the molecule's electrostatic potential and donor-acceptor properties, including ionization potential.

The electrical and spatial structure of O-CMCS was analyzed using computational modeling (Figure 6). Semi-empirical methods, including AM1, CNDO, and MNDO, were applied to the sodium salt of O-CMCS to calculate the energy of the lowest unoccupied molecular orbital (LUMO), the highest occupied molecular orbital (HOMO), the heat of formation, and other energetic properties (Table 4).[18]

The HOMO energy values obtained from these 3 semi-empirical methods are comparable, and the numerical results align with quantum chemical reactivity indices.[19]

These findings indicate that the electronic structure of the chitosan macromolecule is modified by the introduction of substituents. The donor or acceptor properties of electrons in various chemical groups contribute to changes in the effective atomic charge.

Table 2
Results of ^{1}H NMR spectroscopy of chitosan derived from dead bees
Таблица 2
Результаты 1Н-ЯМР-спектроскопии хитозана пчелиного подмора

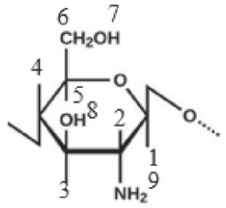
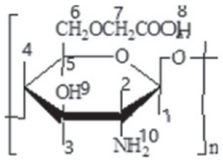
1H, 2H, 3H, 4H, 5H, 6H	(C – H)	1.89-1.91ppm	
7H, 8H	(O – H)	3.58-3.75 ppm	
6H	(NH_2)	3.01 ppm	

Table 3
Results of ^{1}H NMR spectroscopy of CMCS obtained from dead bees
Таблица 3
Результаты 1Н-ЯМР-спектроскопии карбоксиметилхитозана пчелиного подмора

1H, 4H	(C – H)	1.5 ppm	
2H	(C – H)	2.52 ppm	
3H	(C – H)	2.73 ppm	
5H	C – H	3.09 ppm	
7H, 6H	C – H	3.24-3.41ppm	
10H	(-NH_2)	3.52 ppm	
9H	(O – H)	3.57 ppm	
8H	(COO – H)	3.9 ppm	

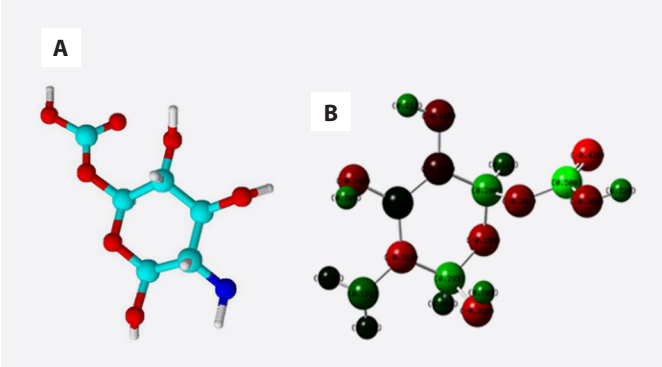


Figure 6. Molecular model (A) and atomic partial charge distribution (B) of O-CMCS derived from Apis mellifera chitosan
Рисунок 6. Молекулярная модель О-карбоксиметилхитозана (А) и атомный заряд (В) О-карбоксиметилхитозана Apis Mellifera

To evaluate the biological activity of O-CMCS derived from chitosan extracted from dead bees, an ointment containing O-CMCS was applied to burn wounds in white rats. Previous studies have demonstrated the efficacy of animal models in assessing treatments for skin wounds.[20]

Histological and morphological analyses were conducted, comparing the O-CMCS ointment to a conventional treatment, Levomecol ointment. The O-CMCS ointment yielded promising results[21] (Figure 7).

Table 4

Energies of the highest occupied E_{HOMO}, lowest unoccupied E_{LUMO} molecular orbitals, heats of formation and energy characteristics calculated by various methods for the structure of O-CMC Na

Таблица 4

Энергии высших занятых E_{HOMO}, низших незанятых E_{LUMO} молекулярных орбиталей, теплоты образования и энергетические характеристики, рассчитанные различными методами для структуры O-CMC Na

Structure	Method	E_{HOMO} (eV)	E_{LUMO} (eV)	Total energy (кDj/mol)	Electronic energy (кDj/mol)	Energy formation (кDj/mol)
O-carboxy methyl chitosan	AM1	7866.2	1300660	334369.2	1635029	76.32
	CNDO	5923.6	139502	516025.8	1911028.6	16858.6
	MNDO	8020.6	1159613	335491.2	1495068.2	78.08

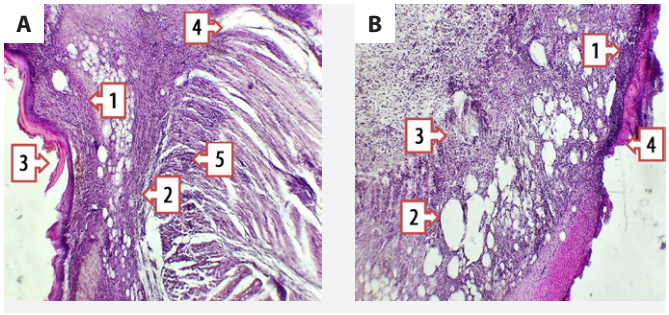


Figure 7. Histological appearance of the burn on the 22nd day. A) CMCS B) Levomecol
A) Healing. Formation of extensive granulation tissue in the dermis (1). Contraction of the wound (2). Eschar (3). Edema (4) with single inflammatory cells (5). Hematoxylin-eosin staining. Lens 10×20 eyepiece.
B) Healing. Cell proliferation (1). Edema (2) with multiple inflammatory cells (3). Eschar (4). Hematoxylin-eosin staining. Lens 10×20 eyepiece.
Рисунок 7. Гистологическая картина ожога на 22-й день. А) Карбоксиметилхитозан Б) Левомеколь
А) Заживление. Образование обширной грануляционной ткани в дерме (1). Контракция раны (2). Струп (3). Отек (4) с единичными воспалительными клетками (5). Окраска гематоксилином и эозином. Окуляр 10×20.
В) Заживление. Пролиферация клеток (1). Отек (2) с множественными воспалительными клетками (3). Струп (4). Окраска гематоксилином и эозином. Окуляр 10×20.

Conclusions

The optimal conditions for producing O-CMCS were investigated, focusing on the carboxymethylation reaction of chitosan derived from *Apis mellifera* and the determination of its kinetic parameters. Physicochemical characterization techniques were employed to study the synthesis of O-CMCS from *Apis mellifera* chitosan. In vivo studies evaluating the biological activity of O-CMCS derived from this novel raw material yielded positive results, supporting its potential use in medical applications.

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