

SYNTHESIS OF CHITOSAN COMPOSITES WITH METHYLENE BLUE, MALACHITE GREEN AND ACID FUCHSIN DYES FOR ENHANCEMENT OF THEIR ANTIMICROBIAL ACTION

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Abstract

*The effect of chitosan oxidation with sodium periodate on the ability to form complexes with the dyes methylene blue, malachite green and acid fuchsin, which have an antiseptic effect, was investigated. A non-covalent interaction occurred for methylene blue and malachite green, while a covalent interaction occurred for acid fuchsin. Fourier-transform infrared spectroscopy confirmed the formation of chitosan composites with these dyes. The antimicrobial effect of these dyes complexed with oxidised chitosan against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* was investigated. The non-covalent attachment of malachite green and methylene blue to chitosan preserved their antimicrobial effect; hence, complexes of these dyes with oxidised chitosan provide a more convenient form that is less staining. However, the covalent conjugation of acid fuchsin with oxidised chitosan caused a significant loss of the antimicrobial activity. The developed method of conjugation of specific dyes with chitosan can be used to create complexes of this biopolymer with a wide range of biologically active organic substances.*

Keywords: chitosan; periodate oxidation; methylene blue, malachite green, acid fuchsin; conjugates and adducts; FTIR analysis; antimicrobial action

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1. Introduction

In the 19th century, scientists proposed specific dyes for use as histochemical reagents in microscopy. Many of these dyes also possess antimicrobial activity. In 1891, Paul Ehrlich showed that methylene blue was an effective remedy in treatment of malaria [1]. At present, the main areas of application of the dyes methylene blue, acid fuchsin and malachite green are the textile industry as well as microbiological and cytological analysis. They are also used in medicines, albeit to a lesser extent, and exert a wide range of the antimicrobial effects against the infectious agents in skin lesions and mucous membranes, as well as a fungicidal remedy in fungal lesions of the outer coverings (Table 1). For example, fucorcine solution, which contains fuchsin, is prescribed to treat skin diseases caused by various types of dermatomycetes (trichophytons, microsporums and epidermophytons), microbial eczema and oily and mixed forms of seborrhoea.

A major disadvantage of dyes is their intense colour, which can stain clothes and other items that come into contact with them. To mitigate this disadvantage, dyes can be complexed with macromolecules such as chitosan. In addition, one may expect a prolongation of the antimicrobial action of dyes complexed with chitosan. Consistently, we previously showed that after subcutaneous administration to rats, ethacridine lactate complexed with chitosan circulated in the blood for a significantly longer time than the intact ethacridine [2].

The preparation of chitosan iodide composites with methylene blue and fuchsin was described recently [3]. These composites formed porous sponges and films and, therefore, could be used for a variety of applications. They possessed antibacterial activity against antibiotic-resistant gram-positive and gram-negative bacteria. Chitosan obtained from different sources and using different methods can vary in the molecular mass, from ≥ 10 to 1,000 kDa, impurities content, and degree of deacetylation, from 30% to 95% [4, 5].

In this work, we oxidised chitosan with sodium periodate (NaIO_4) and complexed the oxidised product with one of three dyes, namely methylene blue, malachite green and acid fuchsin. We evaluated the physicochemical characteristics of the obtained complexes using viscometric analysis to determine the molecular mass of chitosan and Fourier-transform infrared (FTIR) spectroscopy to determine the structure of chitosan–dye complexes. Finally, we assessed the antimicrobial action of intact and chitosan-conjugated forms of these dyes towards the gram-negative bacteria *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 25922 and the gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 31324.

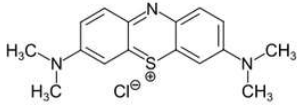
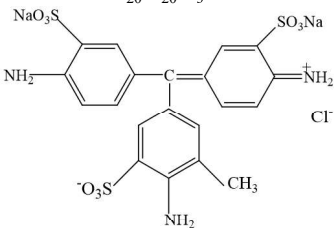
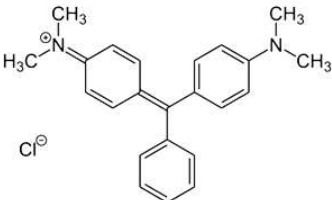
2. Materials and Methods

2.1. Materials

Methylene blue, malachite green and acid fuchsin were obtained from Klebrig (India). Table 1 provides the chemical structure, physicochemical properties and examples of the applications of these dyes.

Shrimp shell chitosan with different molecular masses and degrees of deacetylation – namely, CS-M (502 kDa, 80.8%), CS-S (313 kDa, 85.5%) and CS-L s (739 kDa, 91.3%) – were purchased from Chitopharm (Norway).

Table 1. The physicochemical properties and application of the three dyes used in this work.

Name, structure and physicochemical properties	Application
<p>Methylene blue $C_{16}H_{18}ClN_3S \cdot 3H_2O$</p>  <p>Dark green crystals with a bronze lustre; easily soluble in alcohol or hot water, but less soluble in cold water</p>	<p>Used as an antiseptic in the form of a water–alcohol solution to treat burns, pyoderma, folliculitis, etc. [1].</p>
<p>Fuch sine (rosaniline hydrochloride) $C_{20}H_{20}N_3Cl$</p>  <p>Green crystals with a metallic lustre; aqueous solutions are purple-red; poorly soluble in water, but good solubility in alcohol</p>	<p>Treatment of skin diseases, a component of some antiseptics, along with other aniline dyes – diamond green and methylene blue – which are active against staphylococci [6, 7].</p>
<p>Malachite green $C_{23}H_{25}ClN_2$</p>  <p>Dark green crystals with a metallic sheen; good solubility in water and ethanol</p>	<p>A classic remedy to treat diseases in fish; in most European countries, it is allowed to treat infections of only aquarium fish; corals and other invertebrates in sea and fresh water do not tolerate it [8, 9]</p>

2.2. Methods

The viscosity of chitosan solutions in a mixture of 0.1 M acetic acid and 0.2 M sodium chloride (NaCl) (1:2, v/v) was measured at 25°C with a Ubbelohd VPZh-4 viscometer (Soyuznauchpribor, USSR) with a capillary of diameter = 0.82 mm. The molecular mass of chitosan was determined from this measurement.

2.2.1. Fourier-Transform Infrared Spectroscopy

The FTIR spectra of the samples (chitosan, dyes and chitosan–dye complexes) were recorded on a Spectrum Two spectrometer (PerkinElmer, USA) using a diamond universal attenuated total reflectance (UATR) accessory. The PerkinElmer Spectrum 10 software was used to draw the spectra. The spectra (16 scans per spectrum) of the solutions were collected in the mid-infrared wavenumber range from 4000 to 400 cm^{-1} , with a spectral resolution of 0.4 cm^{-1} .

2.2.2. Preparation of Oxidised Chitosan and Chitosan–Dye Complexes

Finely ground chitosan powder (50 mg) was placed in a centrifuge tube; then, 3.0 ml of 1% sodium bicarbonate (NaHCO_3) solution was added and the mixture was incubated for 30 min. After swelling and impregnation of the chitosan powder, the NaHCO_3 solution was carefully removed with a pipette and 0.4 ml of 5% NaIO_4 was added. The mixture was incubated for 30 min at room temperature. The oxidised chitosan was washed three times with 10 ml of water. Next, 1–2 ml of a 1% dye solution in 0.1 M phosphate buffer (pH 7.0–7.5) was added to the washed oxidised chitosan. The mixture was incubated for 4–16 h, after which time unbound dye was washed away (8–10 times with 10 ml of 6% NaCl solution, and then 2 times with 10 ml of distilled water until complete discoloration of the washing water). In the control, the dye solution was added to the same amount of unoxidised chitosan; the incubation and washing were performed as described above.

2.2.3. Determination of the Bound Dye Content

The washed chitosan powder with dye was dried in an oven at 50–60°C, weighed and then dissolved in 1% acetic acid to a concentration of 1%. The pH of the solution was adjusted to 6.0. Then, the content of the dye in complexes was determined using a colorimetric or spectrophotometric method.

2.2.4. Determination of the Rate of Diffusion in Agar Gel

Filter paper discs (diameter = 5 mm) were impregnated with dye solution (control) or a chitosan–dye complex and dried in an oven at 60°C. Petri dishes were filled with 1% Sabouraud medium agar gel. A disc was placed on the solidified agar gel; after 2, 4 and 10 h, the diameter of the stained zone was measured. The ratio of the diffusion rate of the chitosan–dye complex to the diffusion rate of the pure dye was determined.

2.2.5. Measurement of Antimicrobial Activity

Filter paper discs (diameter = 5 mm) were impregnated with a dye solution (control) or a chitosan–dye complex and dried in an oven at 60°C. Microbial cultures were sown in 100-mm diameter Petri dishes filled with 1% Sabouraud medium agar gel; their growth was monitored and photographed. The experiments were carried out with the strains *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 25923, *B. subtilis* ATCC 31324 and *E. coli* ATCC 25922 obtained from the Collection of microorganisms at the Faculty of Biology of Ivan Franko Lviv National University (Ukraine).

3. Results and Discussion

Table 2 shows the total mass and molecular mass of chitosan before and after oxidation with NaIO_4 .

Table 2. Reduction in the total mass and molecular mass of chitosan after its oxidation with sodium periodate.

Average molecular mass of chitosan [kDa]		Total mass of chitosan [mg]	
Before oxidation	After oxidation	Before oxidation	After oxidation
313	40	50	28 ± 3
502	70	50	32 ± 3
739	110	50	35 ± 3

These results demonstrate that during the treatment of chitosan with NaIO₄, there was oxidation of hydroxyl groups of glucosamine to aldehyde groups and hydrolysis of chitosan chains. Fragments of low-molecular-mass chitosan are probably lost during washing to remove excess NaIO₄. These losses reached 30%–50% of the mass of the chitosan sample, and the average molecular mass decreased by 5–8 times.

Due to the presence of amino groups, chitosan is capable of adsorbing various acidic substances, including dyes. Although unoxidised chitosan remained coloured after treatment with dyes followed by washing to remove unbound dye, the amount of dye it contained was negligible. On the other hand, oxidation of chitosan with NaIO₄ markedly increased the amount of bound dye (Table 3).

Table 3. The content (%) of dye complexed with chitosan (CS) before and after its oxidation with sodium periodate.

Sample	The amount of bound methylene blue [%]		The amount of bound malachite green [%]		The amount of bound acid fuchsin [%]	
	Native (313 kDa)	Oxidised (40 kDa)	Native (313 kDa)	Oxidised (40 kDa)	Native (313 kDa)	Oxidised (40 kDa)
CS-S	0.06	5.7	0.08	7.9	0.024	8.7
	0.06	5.0	0.078	7.7	0.018	6.5
CS-M	0.07	3.2	0.09	6.9	0.021	4.8
	0.06	5.0	0.078	7.7	0.018	6.5
CS-L	0.06	5.0	0.078	7.7	0.018	6.5
	0.07	3.2	0.09	6.9	0.021	4.8

Note. See Section 2.1 for the details of each chitosan.

Filter paper discs (diameter = 5 mm) impregnated with a dye solution (control) or a solution of chitosan–dye complex were examined to determine the rate of dye diffusion in 1% Sabouraud medium agar gel at pH 7.0 (Figure 1). There were no significant differences in the diffusion rate of pure methylene blue and malachite green compared with the chitosan–methylene blue and chitosan–malachite green complexes, respectively. However, the diffusion rate of chitosan–acid fuchsin was reduced markedly compared with pure acid fuchsin. These differences could be due to the bonds formed between the dyes and chitosan. Acid fuchsin binds covalently to oxidised chitosan, leading to a marked reduction in its diffusion. On the other hand, methylene blue and malachite green bind non-covalently. Of note, it is unknown exactly how methylene blue binds to chitosan; the bonds are stronger than those formed between chitosan and malachite green, but weaker than those formed between chitosan and acid fuchsin.

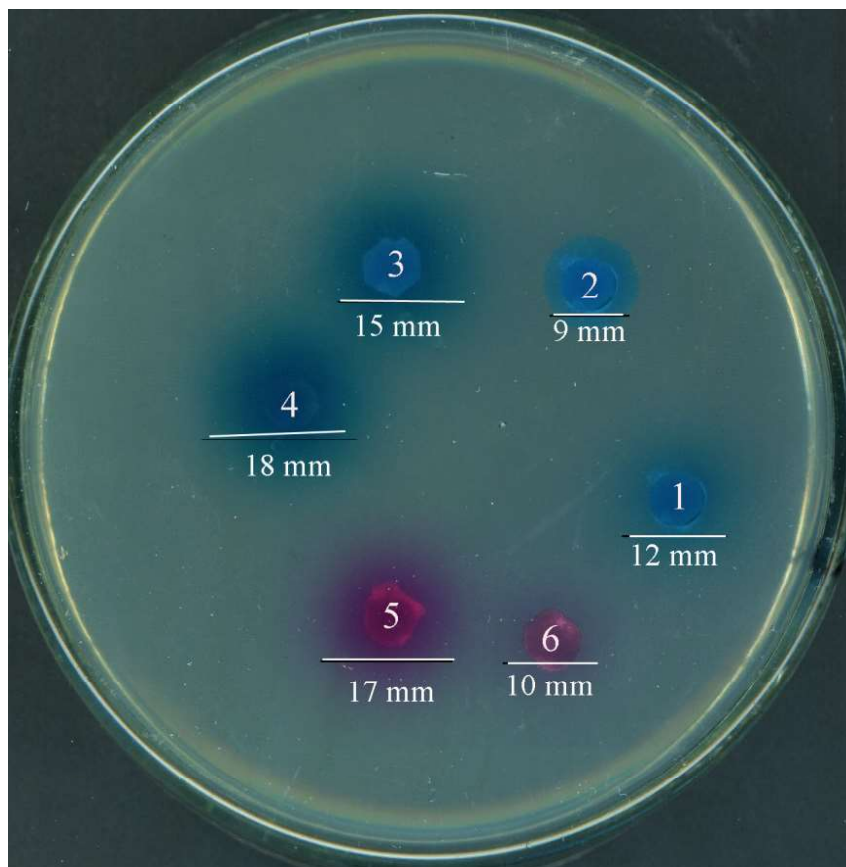


Figure 1. The photograph of a Petri dish containing 1% Sabouraud medium agar gel (pH 7.0) with filter paper discs impregnated with pure dyes of oxidised chitosan–dye complexes incubated for 10 h to allow diffusion. Identities: 1 – methylene blue; 2 – the chitosan–methylene blue complex; 3 – malachite green; 4 – the chitosan–malachite green complex; 5 – acid fuchsin; 6 – the chitosan–acid fuchsin complex.

Next, we compared the antibacterial activities of the pure dyes and the chitosan–dye complexes against gram-negative and gram-positive bacteria (Figure 2). In general, the findings were consistent with the diffusion results: the stronger the interactions between the dye and oxidised chitosan, the weaker the antibacterial activity. Based on the zone of inhibition, for each bacterium pure acid fuchsin (sample 5) presented much stronger antibacterial activity than the chitosan–acid fuchsin complex (sample 6). Pure methylene blue (sample 1) also showed stronger antibacterial activity than the chitosan–methylene blue complex (sample 2), although the difference was not as pronounced as for acid fuchsin. Finally, pure malachite green (sample 3) and the chitosan–malachite green complex (sample 4) showed similar antibacterial activity. Hence, the formation of a covalent complex between chitosan and acid fuchsin (via an unknown mechanism) or a stronger non-covalent complex between chitosan and methylene blue substantially reduces antibacterial activity.

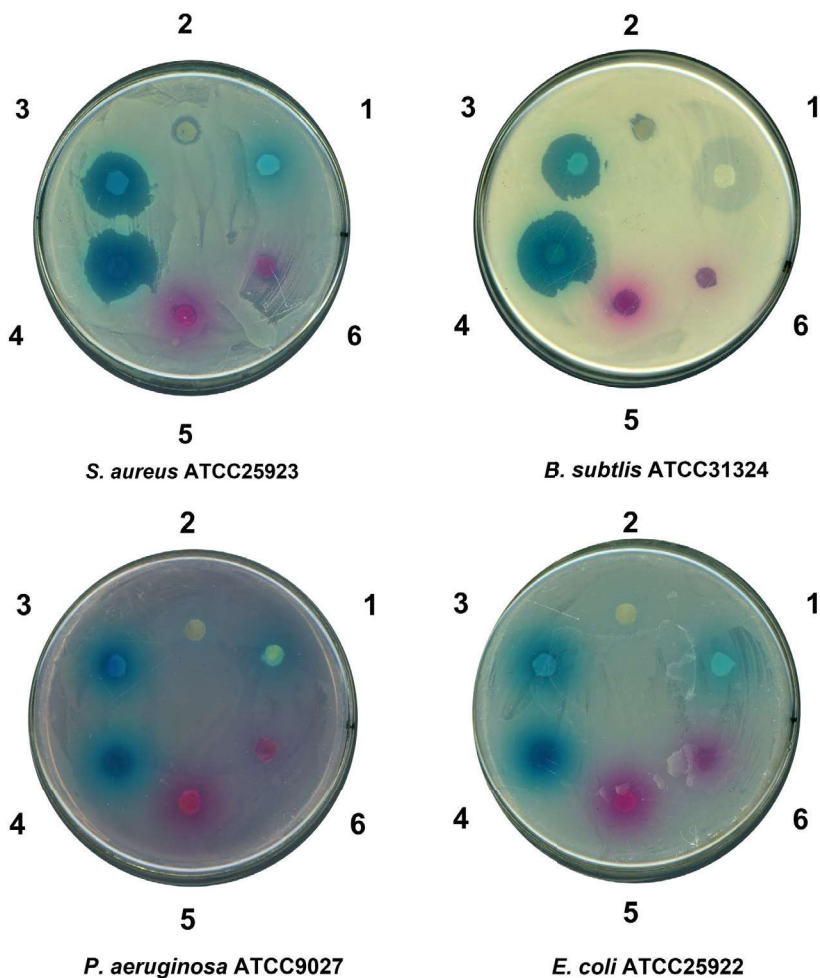


Figure 2. The photographs of the zone of inhibition of the gram-positive bacteria *Staphylococcus aureus* (ATCC25923) and *Bacillus subtilis* (ATCC31324) and the gram-negative bacteria *Pseudomonas aeruginosa* (ATCC9027) and *Escherichia coli* (ATCC25922) in the presence of pure dyes and chitosan–dye complexes. Identities: 1 – methylene blue; 2 – the chitosan–methylene blue complex; 3 – malachite green; 4 – the chitosan–malachite green complex; 5 – acid fuchsin; 6 – the chitosan–acid fuchsin complex.

Next, we used FTIR in an attempt to determine the mechanisms by which the studied dyes form complexes with oxidised chitosan. As shown in Figure 3, we consider that a covalent bond appears between the oxygen atom of oxidised chitosan and the nitrogen atom of the primary amino group of acid fuchsin. Such a change might affect its antimicrobial activity. The possibility of the formation of such a covalent bond does not exist in the case of malachite green (see Table 1). Finally, when methylene blue is added to oxidised chitosan, the aldehyde groups may oxidise to an acid, creating an opportunity for increased binding of methylene blue to oxidised chitosan.

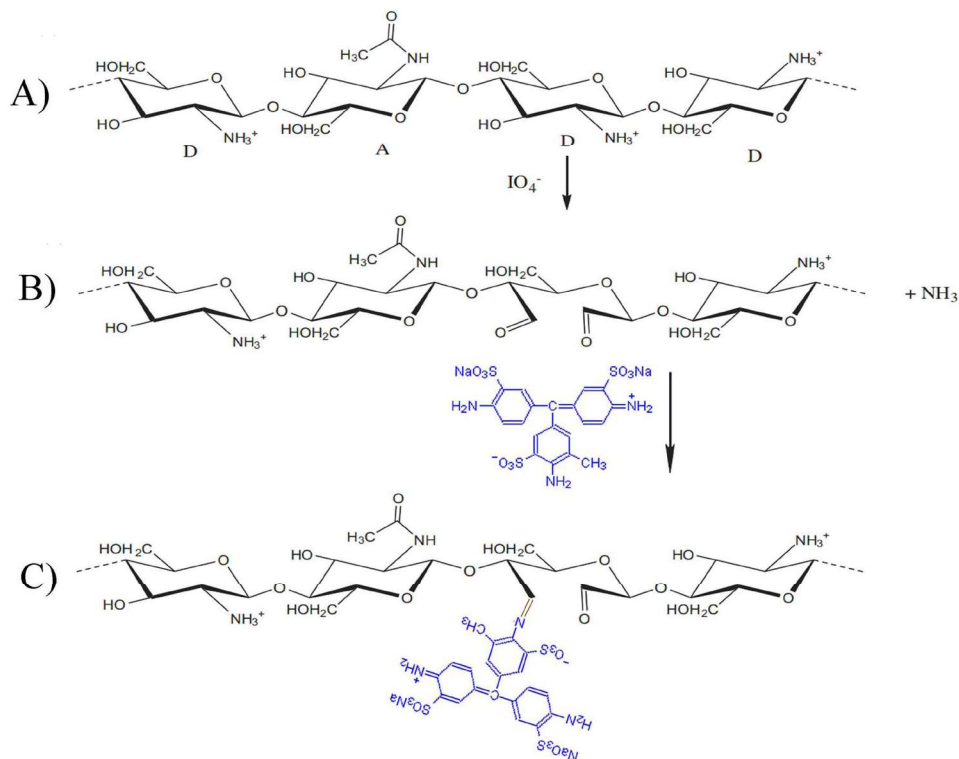


Figure 3. A schematic representation of the interaction between sodium periodate–oxidised chitosan and acid fuchsin.

Acid fuchsin has three amino groups that could interact with oxidised chitosan, while methylene blue and malachite green do not have such chemical groups. However, while malachite green looks to be totally inert for any conjugation, methylene blue has an S(+) atom in its heterocyclic ring. Therefore, it is possible that the aldehyde group of the modified chitosan chain forms a non-covalent bond with the free electron pair of the sulfur atom in methylene blue. It is also possible that when methylene blue is added to oxidised chitosan, there is intramolecular rearrangement of two adjacent aldehyde groups and the formation of hydroxyl and carboxyl groups in their place. This would create an opportunity for enhanced binding of methylene blue to oxidised chitosan through the attraction of sulfur in the phenothiazine ring (positive charge) to carboxyl groups in chitosan (negative charge). Figure 4 presents a scheme of such transformations.

Chitosan oxidation reduces its molecular mass (Table 2). That may increase the number of loci that can interact with other substances. As reported previously, chitosan with a medium molecular mass (135 kDa) and a high degree of deacetylation binds more effectively to substances than chitosan with a high molecular mass [10].

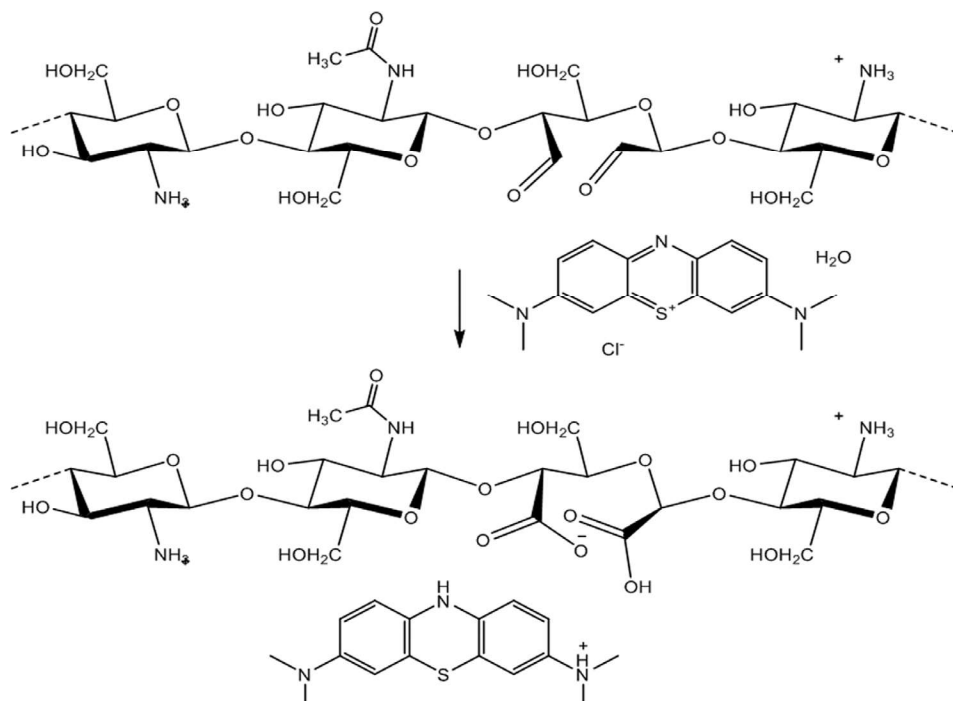


Figure 4. A schematic representation of the possible interaction between sodium periodate–oxidised chitosan and methylene blue.

If the interaction between oxidised chitosan and a dye occurs non-covalently, then the dye can be detached from chitosan with long-term washing with a suitable solvent, or by increasing the temperature or changing the pH or the ionic strength of the solutions. The three dyes attached to oxidised chitosan much better (3.2%–8.7%) than to unoxidised chitosan (0.018%–0.09%; Table 3). As one can see in Figure 1, malachite green was totally released from the chitosan–malachite green complex, while acid fuchsin was almost totally captured by oxidised chitosan. A repeated, more intensive washing of oxidised CS–M containing the absorbed dyes with acidified and alkalisied 80% ethanol led to almost total release of malachite green and methylene blue, which lost their colour, while the release of the attached acid fuchsin was not complete. The dye content after the washing procedure revealed a mean concentration of 0.06% for methylene blue, 0.04% for malachite green and 0.87% for acid fuchsin. Based on these results, we hypothesised that the chitosan–acid fuchsin complex is a mixture of acid fuchsin attached covalently and non-covalently. We tested this assumption by recorded FTIR spectra of chitosan alone and chitosan complexed with the studied dyes.

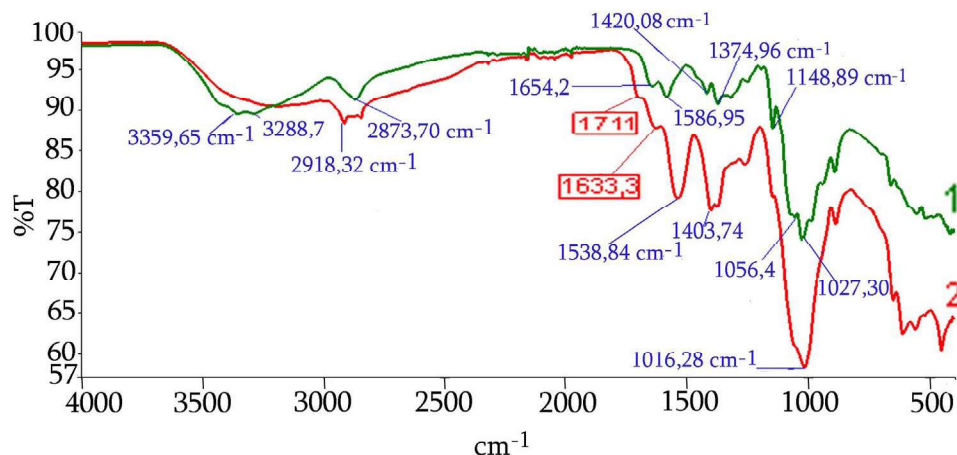


Figure 5. Fourier-transform infrared spectra of chitosan (1, green line) and oxidised chitosan (2, red line).

In general, the FTIR spectra of chitosan, oxidised chitosan and the studied dyes agree with what has been published in the literature [11, 12]. There are three broad absorption bands observed in the FTIR spectra of chitosan and its derivatives (Figure 5). The bands at 1640–1540 cm^{-1} and 1430–1380 cm^{-1} can be attributed to stretching of amide groups and bending of alkyl groups, respectively. The most intense absorption band at 1150–900 cm^{-1} corresponds to C–O–C and C–O–H vibrations. The unmodified chitosan spectrum shows the characteristic absorption bands at 3359 and 3288 cm^{-1} (attributed to O–H and N–H stretching vibrations, respectively), 2873 cm^{-1} (C–H stretching), 1654 cm^{-1} (C=O stretching of amide I), 1586 cm^{-1} (N–H bending of amide II), 1148 cm^{-1} (C–O–C stretching) and 1056 and 1027 cm^{-1} (C–O stretching). The obtained spectrum is consistent with the literature.

The FTIR spectra of chitosan and oxidised chitosan are very similar. The major difference is at 1680–1500 cm^{-1} , where oxidised chitosan shows a shift in the absorption maxima towards short wavelengths and an increase in peak intensity. The only significant difference is the appearance of an absorption band with a low intensity at 1711 cm^{-1} in the FTIR spectrum of oxidised chitosan; it corresponds to stretching vibrations of the aldehyde group formed as a result of oxidation. In addition, a newly formed sharp band at 1633 cm^{-1} derived from a carbonyl group confirms oxidation of chitosan. The changes in the shape and intensity of the stretching vibrations of the hydroxyl band (3200 cm^{-1}) can be explained by the opening of cyclic structure and oxidation of chitosan saccharide units. There are also changes at 1300–1400 cm^{-1} (C–H bending) and 1000–1200 (C–O–C and C–O bending), which prove the effective modification of glucoside rings in chitosan caused by their opening and oxidation at the C2 and C3 positions. Note that in the FTIR spectra of the chitosan–dye complexes, the absorption bands of the dyes are almost absent, probably due to their low concentrations.

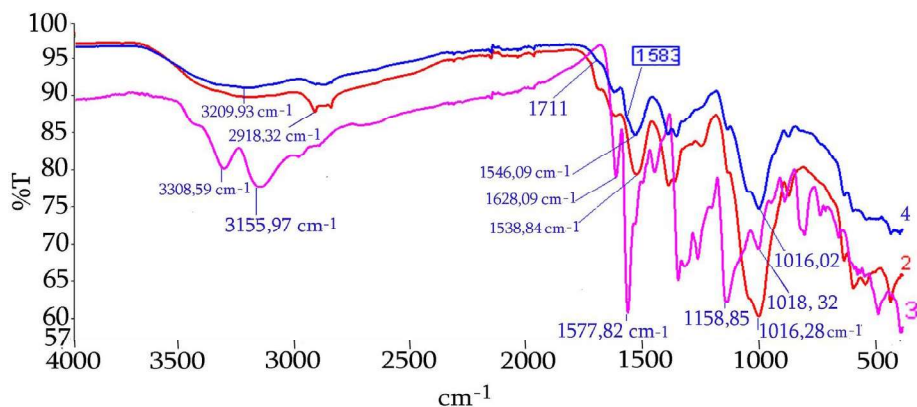


Figure 6. Fourier-transform infrared spectra of oxidised chitosan (2, control, red line), acid fuchsin (3, purple line) and the oxidised chitosan–acid fuchsin complex (4, blue line).

The FTIR spectra of oxidised chitosan and the oxidised chitosan–acid fuchsin complex practically coincide (Figure 6). The only significant difference is the absence of the absorption band at 1711 cm^{-1} (the aldehyde group in the complex). This can be explained by the formation of a covalent bond between the C=O group of oxidised chitosan and the amino group of acid fuchsin. The formation of this bond is confirmed by the shift in the absorption bands towards longer wavelengths. Thus, N-H bending in oxidised chitosan shifts from 1538 to 1546 cm^{-1} in the complex. In addition, the complex shows a new shoulder in the absorption band at 1583 cm^{-1} , which corresponds to the vibrations of the new C=N covalent bond in the complex. The absorption band at 1546 cm^{-1} is wider due to the intense absorption of acid fuchsin, which entered the adduct.

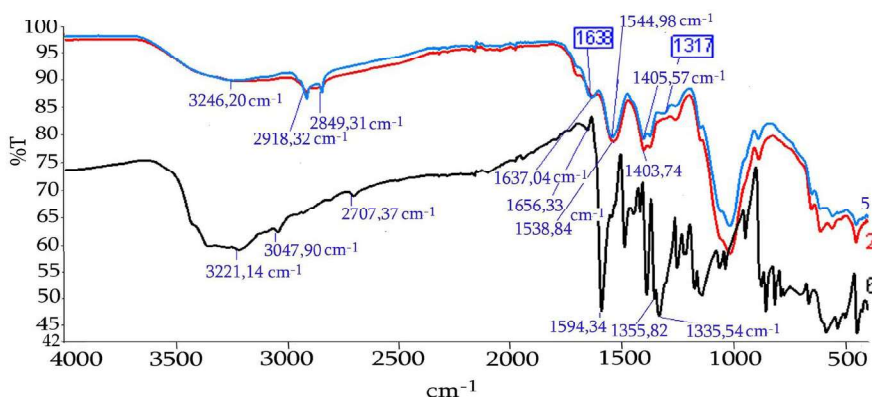


Figure 7. Fourier-transform infrared spectra of oxidised chitosan (2, control, red line), the oxidised chitosan–methylene blue complex (5, blue line) and methylene blue (6, black line).

The FTIR spectra of oxidised chitosan and the oxidised chitosan–methylene blue complex almost coincide (Figure 7). The intensity of the absorption band at 1711 cm^{-1} of the aldehyde group decreases slightly in the complex. It is possible that methylene blue attaches to oxidised chitosan through the free electron pairs of the sulfur atom of the thiazine ring. The formation of such a complex is confirmed in its spectrum by the shift of two absorption bands toward longer wavelengths: N-H bending shifts from 1538 to 1544 cm^{-1} and C-H bending shifts from 1403 to 1405 cm^{-1} . In addition, the complex shows a new absorption band at 1317 cm^{-1} , which corresponds to the vibration of the thiazine ring in methylene blue.

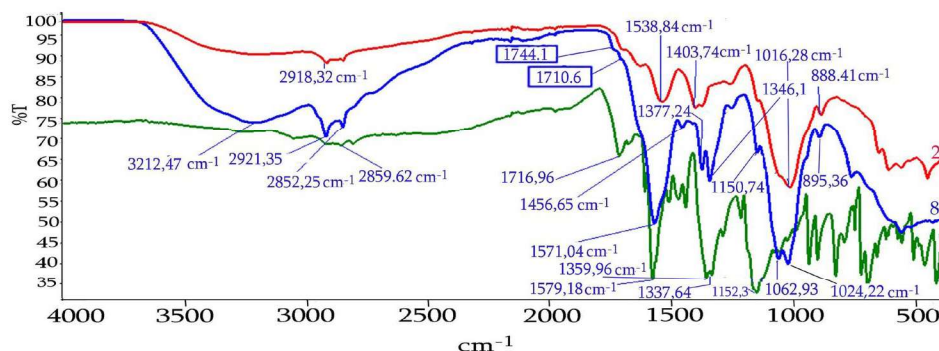


Figure 8. Fourier-transform infrared spectra of oxidised chitosan (2, control, red line), malachite green (7, grey line) and the oxidised chitosan–malachite green complex (8, blue line).

The FTIR spectrum of oxidised chitosan and the oxidised chitosan–malachite green complex are nearly identical (Figure 8). Similarly to the oxidised chitosan–methylene blue complex, the intensity of the absorption band at 1711 cm^{-1} of the aldehyde group decreases slightly. It is possible that the malachite green interacts with oxidised chitosan through the free electron pairs of a nitrogen atom.

Taken together, the technique we developed yields complexes of oxidised chitosan with specific dyes and controls the chemical composition of these complexes. It should be noted that NaIO_4 oxidation of chitosan has been reported previously. The formation of chitosan dialdehyde through NaIO_4 oxidation is particularly interesting. Other conditions of the reaction led to the crosslinking of oxidised chitosan links and an increase in the molecular mass of the formed product, which made it possible to obtain a new material for biometric application [13]. Currently, we are working on the production of chitosan-based hydrogels and ointments with antiseptic dyes to improve wound healing. In addition to such dyes, other biologically active low-molecular-weight compounds, such as the cannabimimetic *N*-stearoyl ethanolamine, will be conjugated with chitosan to enhance its anti-inflammatory effect [14, 15].

4. Conclusions

Based on the results, we drew the following conclusions

1. Oxidation of chitosan with NaIO_4 leads to chitosan hydrolysis. The conditions used in this paper to oxidise chitosan decreased the total mass by 30%–40% and the average molecular mass by 5.3–8 times.

2. NaIO₄-oxidised chitosan shows an increased ability to absorb dyes. The amount of bound dye per unit of chitosan mass increased 70–360 times (from 0.018%–0.09% to 3.2%–8.7%).
3. FTIR spectroscopy revealed that neither malachite green nor methylene blue bind covalently to oxidised chitosan. This was also evidenced by the release of the dyes from their complexes with chitosan under treatment with alcoholic solutions at an acidic pH.
4. FTIR spectroscopy confirmed that acid fuchsin binds covalently to oxidised chitosan through an amino group. This was evidenced by a decrease in the antimicrobial activity of this complex and the formation of insoluble conjugates (especially with CS-L). The product appears to be a mixture of covalent and non-covalent complexes.
5. The oxidised chitosan–acid fuchsin complex exerted much weaker antimicrobial activity compared with pure acid fuchsin. Thus, the amino group of acid fuchsin might be crucial for its antimicrobial effect.
6. The oxidised chitosan–methylene blue complex showed weaker antimicrobial activity compared with pure methylene blue, although the effect was not as pronounced as for acid fuchsin.
7. The oxidised chitosan–malachite green complex and pure malachite green showed similar antimicrobial activity.

5. Acknowledgements

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6. References

- [1] Wainwright M, Crossley KB; (2002) Methylene blue—a therapeutic dye for all seasons? *J Chemotherapy* 14(5), 431–443. **DOI:**10.1179/joc.2002.14.5.431
- [2] Antonyuk V, Manko N, Nektgaev I, Stoika R; (2021) Pharmacokinetics of ethacridine conjugated with chitosan in rats. *Methods Objects Chem* 16(1), 41–47. **DOI:**10.17721/moca.2021.41-47
- [3] Kalinkevich O, Sklyar A, Kalinkevich A, Chivanov V, Zinchenko Y, Trofimenko Y, Holubnycha V; (2021) Preparation and characterisation of new biomaterials based on chitosan iodide with biologically active dyes. *Prog Chem Appl Chitin Deriv* 26, 121–134. **DOI:**10.15259/PCACD.26.011
- [4] Cheung RCF, Bun Ng T, Wong JH, Chan WY; (2015) Chitosan: An update on potential biomedical and pharmaceutical applications. *Mar Drugs* 13, 5156–5186. **DOI:**10.3390/md13085156
- [5] Inmaculada A, Alcántara AR, Concepción CM, Concepción A, Begoña E, Heras CA, Niuris A; (2021) Chitosan: an overview of its properties and applications. *Polymers* 13(19), 3256. **DOI:**10.3390/polym13193256
- [6] Balabanova M, Popova L, Tchipeva R; (2003) Dyes in dermatology. *Clin Dermatol* 21(1), 2–6. **DOI:**10.1016/S0738-081X(01)00330-9
- [7] Sarkar R, Sinha S (Eds.); (2019) *World clinics in dermatology: fungal infections*. Jaypee Brothers Medical Publishers, New Delhi.

- [8] Bojarski B, Jurecka P, Szala L, Kondera E, Gaj-Chucher C, Stonawski B, Rombel-Bryzek A; (2023) The influence of methylene blue and malachite green on common carp (*Cyprinus carpio*) blood parameters. *Anim Genet* 19(3), 27–41. **DOI:**10.5604/01.3001.0053.8652
- [9] Yang CW, Chang YT, Hsieh CY, Chang BV; (2021) Effects of malachite green on the microbiomes of milkfish culture. *Water* 13(4), 411. **DOI:**10.3390/w13040411
- [10] Lim C, Hwang DS, Lee DW; (2021) Intermolecular interactions of chitosan: Degree of acetylation and molecular weight. *Carbohydr Polym* 259, 117782. **DOI:**10.1016/j.carbpol.2021.117782
- [11] Antonyuk V, Manko N, Panchak L, Khomyak S, Stoika R; (2022) Chitosan adduct with tranexamic acid and its hemostatic effect. *Prog Chem Appl Chitin Deriv* 27, 35–42. **DOI:**10.15259/PCACD.27.002
- [12] Sherman Hsu CP; (1997) Infrared spectroscopy. In: Settle F (ed), *Handbook of instrumental techniques for analytical chemistry*. Prentice Hall PTR, New York, 249.
- [13] Wegrzynowska-Drzymalska K, Grebicka P, Mlynarczyk DT, Chelminiak-Dudkiewicz D, Kaczmarek H, Goslinski T, Ziegler-Borowska M; (2020) Crosslinking of chitosan with dialdehyde chitosan as a new approach for biomedical applications. *Materials* 13, 3413. **DOI:**10.3390/ma13153413
- [14] Berdyshev AG, Kosiakova HV, Onopchenko OV, Panchuk RR, Stoika RS, Hula NM; (2015) *N*-Stearoyl-ethanolamine suppresses the pro-inflammatory cytokines production by inhibition of NF- κ B translocation. *Prostaglandins Other Lipid Mediat* 121, 91–96. **DOI:**10.1016/j.prostaglandins.2015.05.001
- [15] Manko N, Lootsik M, Antonyuk O, Ivasechko I, Skorokhyd N, Kosiakova H, Mehed’ O, Horid’ko T, Hula N, Klyuchivska O, Panchuk R, Pokhodylo N, Barabash O, Dumych T, Stoika R; (2024) Multifunctional chitosan-based hydrogels: characterization and evaluation of biocompatibility and biodegradability *in vitro*. *Ukr Biochem J* 96(1), 80–95. **DOI:**10.15407/ubj96.01.080