CHARACTERISTICS OF PHYSICOCHEMICAL AND RHEOLOGICAL PROPERTIES OF CHITOSAN HYDROGELS BASED ON SELECTED HYDROXY ACIDS

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Abstract

Chitosan is a natural cationic polymer that dissolves in an acidic environment and forms gels. Its properties depend on the degree of deacetylation and molecular weight. It is a bioactive compound with antibacterial and film-forming properties that allow to increase the regenerative capacity of the skin. Moreover, it is biodegradable, biocompatible, non-toxic, and stable. In this research, chitosan was combined with mandelic and lactobionic acids which are characterized by biological activity and low toxicity. This combination not only has a positive effect on the chitosan solubility, but it also allows to obtain new biomaterials whose positive features of the base ingredients are enhanced by their synergistic effect. The obtained hydrogels were assessed regarding the interaction of chitosan and hydroxy acid molecules, and the stability of the resulting structures was examined. The research was performed by using rheological methods and IR spectroscopy.

Chitosan hydrogels made with mandelic acid are characterized by higher viscosity values, as compared to hydrogels containing lactobionic acid. The samples of the obtained hydrogels stored for 7 days showed no signs of degradation and their viscosity values were constantly increasing, which proves the ongoing process of creating new bonds between hydroxy acid molecules and chitosan chains. After this time, the hydrogels with mandelic acid revealed higher viscosity values in comparison to hydrogels made with lactobionic acid. Based on the obtained IR spectra, the shifts of the characteristic chitosan bands as a result of interaction with the tested hydroxy acids were analyzed.

Keywords: *chitosan, lactobionic acid, mandelic acid, rheology, FTIR spectra*

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Introduction

In recent years, there has been a growing trend in tissue engineering to use natural biopolymers from various sources. These include, for example, proteins, lipids and polysaccharides which can be obtained, among others, from marine organisms. They are represented by collagen (so-called marine), chitin and its derivative - chitosan. These compounds have found application in biomaterials due to their biocompatibility, non-toxicity and biodegradability. Stable hydrogels are formed through the process of self-assembly or as a result of chemical or physical cross-linking, which helps to restore natural tissues [1,2].

Chitosan (FIG. 1) is produced by chemical or enzymatic deacetylation of chitin. It dissolves only in acidic solutions at a pH lower than 6 due to the presence of intermolecular hydrogen bonds that prevent the dissolution of chitosan in water or in organic solvents [1,3]. As a result of the protonation of the amino groups in the acidic environment, chitosan behaves like a cationic polymer. The presence of positively charged amino groups is closely associated with the antimicrobial activity of this compound. These groups interact with the negatively charged cell wall surface of the microorganism. Then, the membrane is damaged and the internal structures of the pathogen are destroyed. In addition, there is another mechanism of the antimicrobial action of chitosan. It is related to its ability to chelate metal ions necessary for the proper functioning of microorganisms, and thus chitosan contributes to their death [4-8]. Of all the known natural polymers, chitosan has the highest chelating capacity [2].

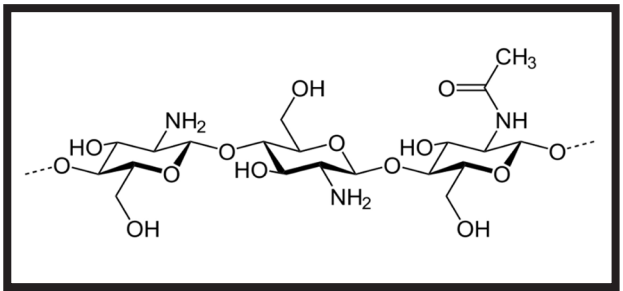

FIG. 1. Structure of a chitosan molecule.

The positively charged chitosan molecule adheres to other negatively charged surfaces, including mucous membranes. This can facilitate drug delivery by various routes - oral, nasal, and direct application to the eye [2].

The degree of deacetylation and molecular weight have a large influence on the physicochemical properties and bioactivity of chitosan. There are three types of chitosan: low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW). Chitosan with the low molecular weight and high deacetylation has been shown to have better antibacterial properties and better solubility than the one with the high molecular weight and low deacetylation [6-9].

Chitosan has the ability to increase the influx of phagocytic cells to the infection site and it affects the proliferation of fibroblasts. Additionally, it is able to aggregate platelets at the site of damaged tissues. This shortens the bleeding in the initial stage of wound healing and contributes to the formation of a fibrin clot. Chitosan also stimulates the production of cytokines and activates macrophages and neutrophils, which results in the cleansing of a wound. Literature data indicate that the polymer contributes to the formation of granulation tissue and contributes to the correct course of epithelization.

In addition, it stimulates angiogenesis and reduces scar visibility. Chitosan is also an inhibitor of the metalloproteinase 2 (MMP-2) present in skin fibroblasts and hydrolysing type IV collagen. The inhibition of this process facilitates the correct reconstruction of damaged tissues in the case of chronic wounds [4,10-14].

All the previously mentioned properties of chitosan make it a very desirable component of biomaterials, and the positive effects of its use have been confirmed by many scientific studies. Chitosan can take various forms in biomedical materials, for example hydrogels [15], nanofibers [16], microparticles [17], nanoparticles [18] or scaffolds [19]. Chitosan biomaterials have been extensively studied for the treatment of wounds of various etiologies in the creation of dressing materials, including burn wounds and pressure ulcers. In combination with collagen, a nanocomposite membrane was created that promotes wound healing and induces cell migration and proliferation [20]. Chitosan-alginate nanofibers act in a similar way [21-23]. Fibers made of carboxymethyl chitosan and polyvinyl alcohol constitute the scaffold in the skin regeneration process [16,24]. Chitosan hydrogels, showing similar physical properties to the extracellular matrix, have become a promising dressing material enabling diffusion and stimulation of cell proliferation. They also have the appropriate sorption capacity and antibacterial properties. In the form of hydrogels, chitosan is combined with silver nanoparticles or minocycline which belongs to antibiotics. It is also a promising material for use in carriers for the delivery of analgesic or anti-cancer drugs to improve their performance [4,10,15].

Chitosan has also been studied in the engineering of bone, cartilage and nervous tissues. It is part of special scaffolds (in combination with nanoceramics - hydroxyapatite, silicon dioxide or bioactive glass-ceramics) that support regeneration, increase cell adhesion, proliferation and differentiation. Such scaffolds induce only a minimal foreign body response. The compound was also tested in combination with polycaprolactone or whitlockite in an *in vivo* experiment to repair skull defects. Such combination increase the activity of the compounds and improve the mechanical properties of the structures. Chitosan-based hydrogels can be also administered by intra-articular injection to cause the regeneration of cartilage tissue. There have been also experiments on the effects of chitosan scaffolds on nervous tissue. In the studies carried out on rats, it was possible to obtain positive effects in the regeneration of nerves [1,4,10,25].

Moreover, chitosan biocomposites can be used in dentistry. They effectively counteract bacteria that are responsible for the formation of caries and periodontal diseases. Chitosan can also be an element influencing the differentiation of pulp stem cells. Moreover, it is a potential replacement for some antibiotics that work against drug-resistant bacteria [1].

Research on the use of chitosan in biomaterials focuses on finding ingredients that will work synergistically. A good example are hydroxy acids (for example, lactobionic acid and mandelic acid), since chitosan is soluble only in acidic solutions. These two acids are characterized by good biological activity and low toxicity, therefore they create the required acidic environment to dissolve the polymer [26-28].

Lactobionic acid (4-O-β-D-galactopyranosyl-D-gluconic acid, $C_{12}H_{22}O_{12}$) is a derivative of lactose. It belongs to the group of polyhydroxy acids, its pKa is 3.8. A molecule of lactobionic acid is a combination of gluconic acid with galactose (FIG. 2) with a molecular weight of 358.3 g/mol.

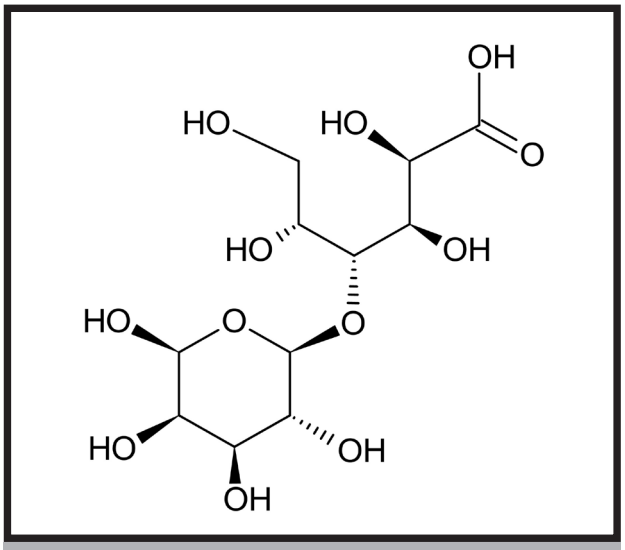

FIG. 2. Structural formula of lactobionic acid.

Due to the presence of 8 hydroxyl groups in the molecule, this compound has very good hygroscopic properties and high water solubility. There is another carboxyl group in the molecule that can react with functional groups of other substances (for example, with an amino group). Mostly it is produced by chemical synthesis, in the process of lactose oxidation or by enzymatic or microbiological biosynthesis. Another method of preparation is wet catalytic oxidation and electrochemical catalysis of lactose. However, this process results in the by-products of these reactions and higher costs. Lactobionic acid in combination with chitosan forms stable gels [29-31].

In the last decade, interest in lactobionic acid has increased in fields, such as pharmacy, medicine, cosmetology, the chemical and food industries [31,33-36]. It is also increasing in popularity as a bioactive molecule providing an excellent platform for the synthesis of biocompatible and biodegradable biomaterials, tissue engineering scaffolds and drug delivery carriers [26,32,34].

Studies on the lactobionic acid combined with copper or with micro-capsuled chitosan were conducted regarding the treatment of hepatocellular carcinoma in the liver. The obtained results are the basis for carriers used in drug delivery systems to a specific organ. These compounds have been used as substrates in the synthesis of radiopharmaceuticals that target liver cell imaging receptors. Researchers have also suggested the suitability of lactobionic acid to design nanofiber scaffolds supporting the regeneration of damaged nerves [26,31-33].

Lactobionic acid has also found application in the production of pharmaceutical products and antibiotics. It has a strong antibacterial effect, inter alia, against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Thanks to this, it may be used in antimicrobial drugs that are not antibiotics. Lactobionic acid is also added to solutions intended for the preservation and storage of transplantable organs. In the pharmaceutical industry, it is currently used as a counter ion for the intravenous administration of erythromycin and in mineral supplementation to minimize irritation during the therapy. In addition, it can be used as a component to form nanoparticles. It acts as a stabilizer in pharmaceuticals containing low-stability ingredients. Such a wide application of lactobionic acid is related to the fact that it is non-irritating, non-toxic, biocompatible and biodegradable [31,32,34].

Lactobionic acid, when applied externally, has the ability to stimulate fibroblasts to produce collagen and elastin. It accelerates the wound healing process, has strong antioxidant properties, and strengthens the epidermal barrier function. It has been shown to stop the production of hydroxyl radicals as a result of its chelating properties and to inhibit the action of metalloproteinase enzymes - enzymes that contribute to skin photoaging. Free radicals are also responsible for causing skin cancer and skin autoimmune diseases. Lactobionic acid prevents the formation of wrinkles, sagging skin, dilatation, and cracking of capillaries [31,35,36].

Mandelic acid (2-phenyl-2-hydroxyacetic acid, $C_8H_8O_3$) belongs to the group of optically active α-hydroxy acids, which contain an aromatic ring in their structure (FIG. 3). Its pKa value is 3.4. The structure of the acid allows it to be dissolved in both polar and non-polar solvents. It is well soluble in ethyl alcohol, isopropyl alcohol, and partially in water and fats. The natural source of mandelic acid are almonds, apricots, and cherries. It can be obtained as a result of the hydrolysis of the bitter almond extract [37,38].

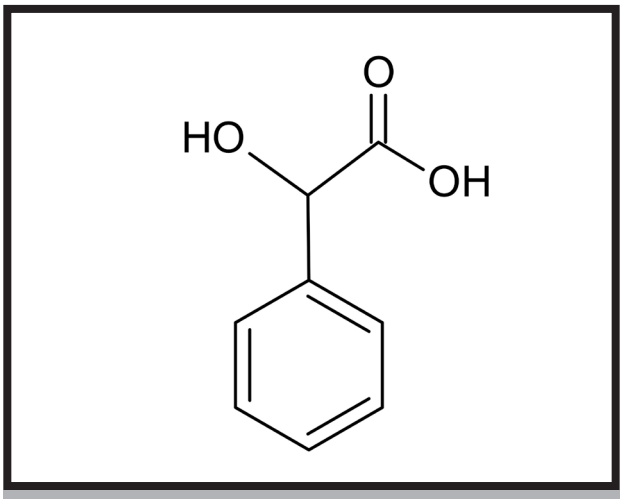

FIG. 3. Structural formula of mandelic acid.

Mandelic acid exists in the form of two enantiomers, S and R, which determine its properties. (R)- mandelic acid is widely used as an intermediate product for the production of semi-synthetic cephalosporins, penicillin and anticancer agents, while (S)-mandelic acid is a component of intermediates used in the production of pharmaceuticals. Both enantiomers are effective resolving agents which are used in the resolution of racemic amines and alcohols [39,40].

Mandelic acid is used in many medical and peri-medical fields, including dermatology, pharmacy, and cosmetology. It usually occurs in a racemic form. It has keratolytic properties that regulate the work of sebaceous glands, therefore it supports the therapy of acne or excessive actinic keratosis. It gives good results in combating discoloration of various etiologies (for example freckles, acne scars or drug discoloration). That is why, it is widely used in cosmetology. Due to the relatively large size of the molecule, it is absorbed slowly through the skin, thus showing a low irritating potential, acting gently and safely, but slower than other α-hydroxy acids [38,41].

The described compound is distinguished by a strong disinfecting and antibacterial effect. In an acidic environment, it has a bacteriostatic and bactericidal effect on, among others, strains of *Staphylococcus aureus* or *Escherichia coli*. This property allows it to be used in the case of pharmaceuticals, antibiotics or external agents [41].

The properties of lactobionic and mandelic acids allow their use in combination with chitosan in biomedical materials. These acids will beneficially interact with the polymer, enhancing its biological activity and creating a suitable acidic environment for its dissolution. The safety of their use will be maintained because the combined compounds will not cause irritation and will be non-toxic, biocompatible, and biodegradable.

The aim of the presented work was to investigate the physicochemical and rheological properties of chitosan hydrogels obtained by dissolving low molecular weight chitosan in solutions of mandelic or lactobionic acids. In addition to rheological studies, infrared spectroscopy was also performed.

Materials and Methods

Materials

Chitosan powder (low molecular weight, degree of deacetylation DD = 78%, $M_v = 1.4 \times 10^6$ g/mol [42]), lactobionic and mandelic acids were obtained from Merck (Poznań, Poland) and used without further purification.

Preparation of chitosan gels

Hydrogels were prepared by dissolving chitosan (2.6% w/v) in 30 ml of aqueous solutions of mandelic acid and lactobionic acid. The content of hydroxyacids was 0.002 mol. The samples were mixed for an hour at the temperature of 25°C on a magnetic stirrer until clear solutions were obtained.

Viscosity measurements

After 24 hours of incubation, viscosity measurements were taken at the temperature of 25 ± 0.1°C in the range of the shear rate from 0.1 s^{-1} to 35 s^{-1}. The measurements were repeated 5 times per conditions. The rotational viscometer SMART series (Fungilab, Warsaw, Poland) and a set of appropriate spindles were used for the measurements.

FT-IR analysis

The structure of chitosan, mandelic and lactobionic acids as well as the interaction between them were confirmed by infrared spectroscopy, using Nicolet iS10 device (Shimadzu, Kyoto, Japan). All the spectra were recorded by absorption mode at 4 cm^{-1} intervals and 64-times scanning. After the 24 h incubation, the obtained hydrogels were poured in the amount of 25 g into square Petri dishes (10 cm x 10 cm) and allowed to dry. The finished films were carefully removed from the plates and submitted to the FT-IR analysis. The measurements were repeated 3 times per conditions on the different parts of the film.

Results and Discussions

In the presented work, the viscosity characteristics of chitosan solutions obtained by dissolving low-molecular-weight chitosan in solutions of lactobionic acid and mandelic acid were compared, and the changes of these characteristics over time were analyzed. The rheological tests were performed after 24, 48, 72, 168 and 312 hours from the preparation of the samples. The samples were stored at 8°C. Before the measurements, the samples were thermostated to 25°C.

As a result of the rheological studies, the dependence of dynamic viscosity on the shear rate (viscosity curves) was obtained. It allowed to conclude that hydrogels based on chitosan and mandelic acid are characterized by higher viscosity values, as compared to those containing lactobionic acid.

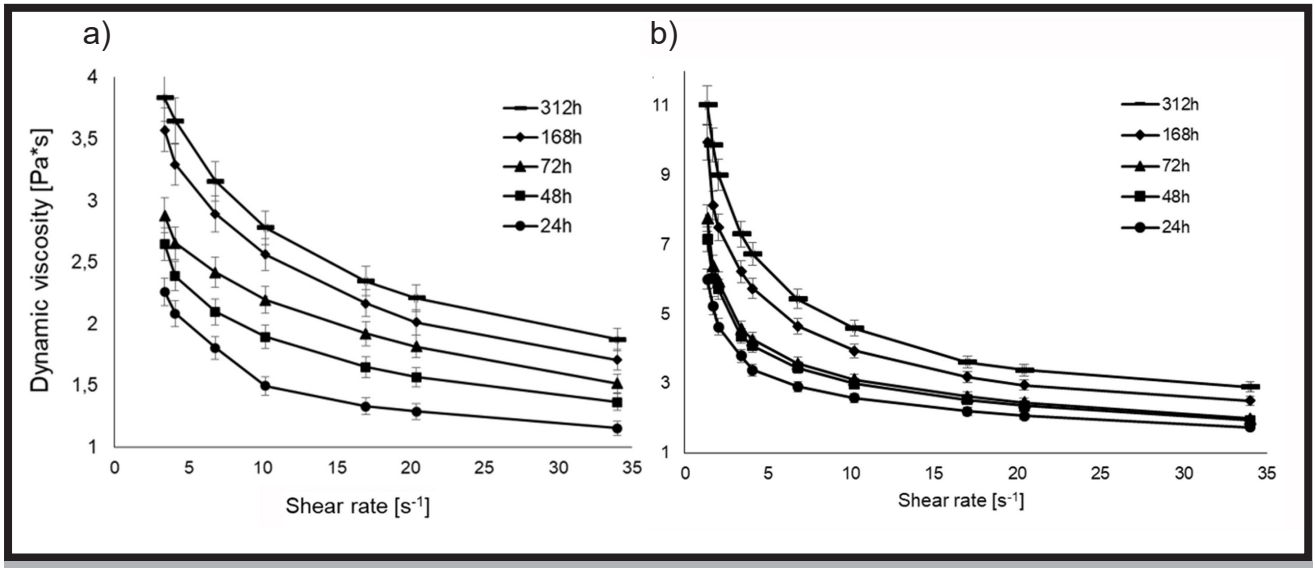

FIG. 4. Comparison of dynamic viscosity of chitosan gel with lactobionic acid (a) and mandelic acid (b) depending on the time.

On the basis of the obtained viscosity curves (FIG. 4), it can be concluded that chitosan gels, characterized by an identical molar ratio of chitosan and acid, prepared with mandelic acid show more than twice higher viscosity than those made with lactobionic acid. The difference in the viscosity parameters of chitosan gels with lactobionic acid and mandelic acid results from the difference in the structure of acids, their strength and the way the polymer interacts with the acid molecules. The interaction of mandelic acid with the chitosan molecule will form an ionic bond between the protonated amino group of chitosan and the dissociated carboxyl group. At the same time, the hydroxyl group of the acid can join another chitosan amino group, but due to the short distance between the carboxyl and hydroxyl groups, most likely belonging to another chitosan chain. The compact flat aryl ring located between the chitosan chains allows them to be closer to each other and interact with the formation of hydrogen bonds, which also contributes to the viscosity increase. The following processes improve the viscosity of the gel formed with mandelic acid gel more than that with lactobionic acid.

Lactobionic acid will interact with the chitosan chains in a similar way, linking the dissociated carboxyl group with the protonated amino group of chitosan, and the hydroxyl groups of the polyhydroxy acid will also form associations with the amino groups of both the same chitosan chain and different chains. It can be assumed that hydroxyl groups of lactobionic acid characterized by different acidity interact with the polymer amino groups, resulting in the formation of association complexes. In this way, the lactobionic acid molecule can bind to the amino groups of both a specific chitosan chain, causing its conformation, and other chains, which increases the resulting hydrogel viscosity However, it can be assumed that these types of bonds will be characterized by lower stability, which in the case of using lactobionic acid results in lower values of dynamic viscosity. A more spatially expanded lactobionic acid molecule causes a looser arrangement of chitosan chains in relation to each other and thus the lower viscosity of the obtained gels. In the case of mandelic acid, the bonds are formed by the interaction of the carboxyl and hydroxyl groups with the amino groups of different chitosan chains, linking them with each other and thus contributing to the higher viscosity than in the case of lactobionic acid.

By examining the changes in viscosity characteristics up to 312 hours of observation, it was found that the viscosity of the systems for both lactobionic and mandelic acid continued to increase (FIG. 4). This fact can only be explained by the formation of new bonds between chitosan and acid molecules. Both mandelic acid (pKa 3.4) and lactobionic acid (pKa 3.8) are weak acids. However, the protonation of amino groups in chitosan in an acidic environment will result in a shift of the equilibrium towards the increasing acid dissociation. This, in turn, creates more bonds between acid molecules and chitosan chains and contributes to the higher viscosity of the obtained gels. The data available in the literature on the interaction of chitosan with hydroxy acids is very limited [43-45], and there is even less information on viscosity tests of such systems.

In order to consider the interaction of individual functional groups of compounds forming the biopolymer, spectroscopic examinations in the infrared range are conducted. In this study, the interactions between the functional groups of chitosan and lactobionic or mandelic acids were tested by Fourier transform infrared spectroscopy.

The FTIR spectra of chitosan, lactobionic acid and mandelic acid are shown in FIG. 5. The characteristic peak at 3280 cm^{-1} (Amide A), 2860 cm^{-1} (C-H), 1645 cm^{-1} (Amide I), 1540 cm^{-1}(Amide II) and 1025 cm^{-1} (C-O-C) are usually observed for chitosan samples. The axial deformation, or stretching bands, for lactobionic acid were observed at: 3320 cm^{-1} ($OH_{alcohol}$), 2885 cm^{-1} (C-H), 870 cm^{-1} (ring vibration), 1110 cm^{-1} and 1220 cm^{-1} (C-O), 1730 cm^{-1} ($C=O_{acid}$). The bands of the lactobionic acid sample corresponded to the ones known from the literature [46]. The following peaks were observed for a crystalline sample of mandelic acid: 3400 cm^{-1} (OH), a wide band in the range 3030-2700 cm^{-1} ($C\text{-}H_{stretch}$), 1720 cm^{-1} ($C=O_{acid}$), 1500-490 cm^{-1} (mainly ring deformations and o-, m-, p-CH bends). These bands of mandelic acid also correspond to the data found in the literature [47].

In the FTIR spectra of films made of chitosan with lactobionic or mandelic acid, we can observe changes in both the location of the observed peaks and their intensity (FIG. 5). After the formation of bonds between chitosan and acids, we do not observe peaks corresponding to $C=O_{acid}$, also the OH peak (3400 cm^{-1}) for mandelic acid disappears, and the corresponding peak for lactobionic acid loses its intensity.

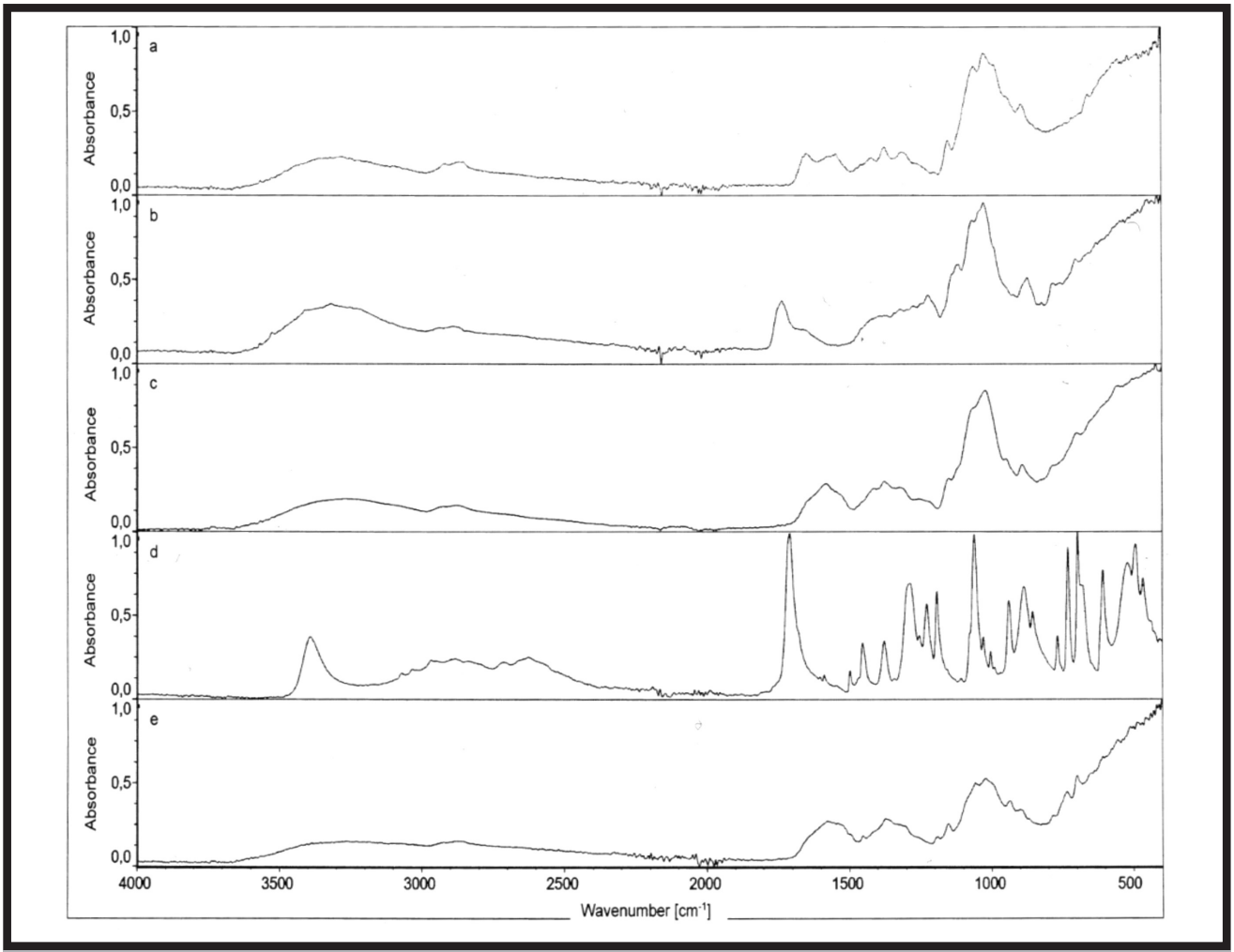

FIG. 5. FTIR spectra of chitosan (a), lactobionic acid (b), mandelic acid (d) and chitosan hydrogels with lactobionic acid (c) and mandelic acid (e).

On the other hand, in the spectrum of both films, new peaks that may belong to the protonated amino group are observed, with the intensity of these peaks for lactobionic acid being slightly higher. The comparison of the bands for pure compounds and the obtained hydrogels shows the participation not only of the carboxyl group of the hydroxy acid but also of the hydroxyl groups that form bonds between chitosan and acids

Conclusions

The viscosity values of chitosan hydrogels (2.6% w/v) based on lactobionic or mandelic acids (each acid content was 0.002 mol) as components of potential application in biomaterials were investigated. The rotational viscometer SMART series (Fungilab, Poland) was used. Viscosity measurements were made at the temperature of 25 ± 0.1°C in the range of the shear rate from 0.1 s^{-1} to 35 s^{-1}. The structure of chitosan, mandelic acid and lactobionic acid as well as the interactions between them were confirmed by infrared spectroscopy using Nicolet iS10 device (Shimadzu, Japan).

The viscosities of hydrogels depending on the structure of the hydroxy acid and the change of hydrogel viscosity with time were analyzed. It was observed that chitosan hydrogels prepared on the basis of mandelic acid showed higher dynamic viscosity than those prepared on the basis of lactobionic acid. It was proved that the dynamic viscosity of the prepared samples for both acids in the time range up to 312 hours was characterized by an upward trend. These phenomena can be explained by the difference in the interaction mechanisms of chitosan and acid molecules, depending on their structure and the difference in acid strength.

Based on the FTIR spectra, it was proved that both carboxyl and hydroxyl groups were involved in the interaction between chitosan and hydroxy acid molecules. The viscosity increased over time due to the progressive process of protonization of the amino groups resulting from the progressive dissociation of hydroxy acids and the increasing number of bonds between chitosan and hydroxy acids.

A combination of the unique properties of chitosan with the bioactivity of hydroxy acids, such as lactobionic acid and mandelic acid, will be useful in the preparation of a biomaterial for wound healing dressing.

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