

Interactions of fish gelatin and chitosan in uncrosslinked and crosslinked with EDC films: FT-IR study

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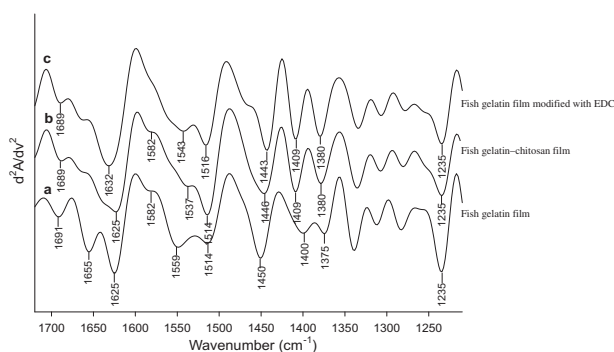
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h i g h l i g h t s

- Interactions of fish gelatin and chitosan in two-component films have been examined.
- Hydrogen bonds involving CO, OH and NH₂ form within and between polymer chains.
- Electrostatic interactions occur involving the COO⁻ of gelatin and NH₃⁺ of chitosan.
- EDC crosslinks of the film components and provides new iso-peptide bonds formation.

g r a p h i c a l a b s t r a c t



a b s t r a c t

Films based on fish gelatin, chitosan and blend of fish gelatin and chitosan before and after cross-linking with EDC have been characterized by FT-IR spectroscopy. The FT-IR spectrum of fish gelatin film showed the characteristic amide I, amide II and amide III bands, and the FT-IR spectrum of chitosan film con-firmed that the polymer was only a partially deacetylated product, and included CH₃AC=O and NH₂ groups, the latter both in their free -NH₂ and protonated -NH₃⁺ form. Analysis of FT-IR spectra of two-component, fish gelatin-chitosan film revealed the formation not only of hydrogen bonds within and between chains of polymers, but also of electrostatic interactions between -COO⁻ of gelatin and -NH₃⁺ of chitosan. Modification with EDC provided cross-linking of composites of the film. New iso-peptide bonds formed between activated carboxylic acid groups of glutamic or aspartic acid residue of gelatin and amine groups of gelatin or/and chitosan.

Keywords:
Polymers
Thin films
Coatings
FT-IR

Introduction

Gelatin and chitosan are natural, non-toxic, biodegradable polymers, with unique structures and interesting properties. Due to their good film-forming ability, they can be used to form edible films which may provide an alternative to synthetic materials, so far most often used in food packaging, and can contribute in this way to the reduction of environmental pollution caused by the

latter [1]. Highly hydrophilic nature of proteins and polysaccharides makes the gelatin and chitosan films effective barriers against oxygen, CO₂, aromas and lipids, but their mechanical and barrier properties against water are poorer than those of synthetic films [2,3]. As it has already been reported, polymeric blends can result in an improvement of the mechanical properties of one-component films [4-7], and cross-linking of the components with chemical and enzymatic agents can bring about limitation of their excessive solubility in water [8,9]. Our previous studies showed that the presence of chitosan in fish gelatin film in concentration four times lower than that of gelatin increased tensile strength of the film

from about 20 to 46 MPa [10,11]. Further, the chemical and enzymatic cross-linking of the components with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and transglutaminase (TGase), respectively, effectively decreased the film solubility in aqueous medium at different pH [12].

In the formation of composite film it is important to characterize the compatibility of its components and intermolecular interactions that may occur between them, since they finally affect the film structure and determine the film properties. It is well-known that chitosan and collagen, gelatin parent protein, may form multiple complexes. In the formation of them, first of all $-OH$ and $-NH_2$ groups of both polymers are involved. The hydroxyl, amine, and also carboxyl groups of collagen are capable of forming hydrogen bonds with hydroxyl and amine groups of chitosan [4,13]. On the other hand the $-NH_2$ groups of chitosan are easily protonated in acidic solutions and converted to $-NH_3^+$. Hence, its flexible chains are able to attain the suitable configuration and form complexes with opposite charged anionic materials. The formation of polyanion-polycation complex between calf-skin collagen and fully deacetylated chitosan has also been observed [14].

TGase and EDC provide cross-linking of protein chains. The formation of the covalent linkages, between $-NH_2$ groups of polymer and either its γ -carboxamide groups of peptide-bound glutamine residues, or activated carboxylic acid groups of glutamic or aspartic acid residues in the case of using enzyme or carbodiimide, respectively, results in the formation the same iso-peptide bonds [15]. However, in the two-component, gelatin-chitosan film in the presence of cross-linking agent covalent bonds can be formed not only between structural units of gelatin, but also between units of gelatin and chitosan, as chitosan, depending on the degree of deacetylation, possesses a smaller or a greater number of NH_2 groups.

Since there is a view that EDC is not safe enough to be used to modify films for foodstuffs, in our previous investigations it was used only for comparative purposes [10,11]. However, there is no consistent evidence about the toxicity of EDC. Contrary to many other cross-linking compounds, e.g. toxic aldehydes (glutaraldehyde, polyepoxides), EDC is not included in a formed bond, but is simply transformed into water-soluble urea derivatives, which do not reveal toxicity, and potential depolymerization of the material does not release residual toxic reagents. Therefore, according to Yunoki, Nagai, Suzuki, and Munekata [16], EDC cross-linked films are not toxic, unlike materials modified with aldehydes.

This paper is a continuation of our study of fish gelatin-chitosan film [11,17] and is focused on investigation of intermolecular interactions of the components of the film before and after cross-linking with EDC, by using Fourier Transform Infrared Spectroscopy (FT-IR). FT-IR is a powerful technique used to determine the molecular structure of proteins and polysaccharides, including collagen/gelatin [18–21] and chitosan [22–24], as well as to reveal protein-polysaccharide interactions in the case of their blends. Knowledge of these interactions is crucial to understanding of film structure-property relationships. Although several studies of collagen/gelatin interactions with chitosan have been already reported, most of them concerned protein obtained from mammalian sources [4,13,14,25–27]. To our knowledge similar studies of gelatin from alternative source, namely from fish skins, are lacking, and it is well-known that the functional properties of the gelatin films strongly depend on the source of gelatin [28].

Materials and methods

Materials

Fish gelatin was obtained from skins of Baltic cod (*Gadus morhua*) as described by Kołodziejska, Kaczorowski, Piotrowska and

Sadowska [29]. Chitosan of 73% deacetylation degree was obtained from krill chitin in the Sea Fisheries Institute in Gdynia according to Kołodziejska, Wojtasz-Pajak, Ogonowska, and Sikorski [30]. EDC applied for the chemical modification was purchased from Sigma Chemical Co.

Chemical modification

To prepare one-component solutions, fish gelatin was dissolved in deionized water, and chitosan in deionized water in presence of 0.5 M HCl, to achieve the final concentration of 5% and 2% (w/w), respectively. The pH of fish gelatin solution was about 6.6.

Two-component fish gelatin-chitosan (4:1, w/w) solution was obtained by mixing a 2% solution of chitosan with a 25% solution of fish gelatin. The resulting mixture of pH 5 was occasionally stirred during 2 h of incubation at 50 °C and centrifuged at 2000g and 20 °C for 15 min.

For chemical modification of two-component film, fish gelatin-chitosan solution was cooled down to room temperature, and EDC was added to the final concentration of 30 mM. The film was formed immediately after adding of EDC, as described below. A chemical reaction was occurring during that process.

Film formation

In all experiments, 20 g of solutions were cast on a rectangle of sides 9.5 and 13.5 cm of a polyester surface and spread manually to the outside borders. The films were obtained after water evaporation (24–48 h) at room temperature and at 35–45% relative humidity (RH). The thickness of the films was measured at five random locations with a hand-held micrometer. The average thickness of the films ranged from 0.10 to 0.12 mm.

Attenuated total reflectance Fourier transformation infrared (ATR FT-IR) spectroscopy

The ATR FT-IR spectra of one- and two-component film samples were recorded on a Nicolet 8700 spectrometer (Thermo Electron Scientific Inc., Waltham, MA), using a Golden Gate ATR accessory (Specac) equipped with a single-reflection diamond crystal. The temperature during measurements was kept at 25 ± 0.1 °C using an electronic temperature controller (Specac). For each spectrum, 128 scans were collected with a resolution of 4 cm^{-1} . The spectrometer's EverGlo source was on turbo mode during measurements. The spectrometer and ATR accessory were purged with dry nitrogen to reduce water-vapor contamination of the spectra. All samples were conditioned before their analysis for 7 days in a desiccator containing silica gel.

Results and discussion

Fish gelatin and chitosan films

The FT-IR spectra of fish gelatin and chitosan films are presented in Fig. 1, and band assignment in these spectra are listed in Table 1 [13,19,26,31]. The both spectra demonstrated a broad band in the range of $3600\text{--}2700 \text{ cm}^{-1}$, attributed to ν_{NH} and ν_{OH} vibrations. Furthermore, the spectrum of fish gelatin film showed characteristic absorption peaks at 1633 , 1538 and 1238 cm^{-1} , corresponding, respectively, to $\nu_{C=O}$ and ν_{NH} vibrations in amide I, to δ_{NH} and ν_{CN} vibrations in amide II, and to ν_{CN} and δ_{NH} vibrations in amide III band. The peaks at 1633 , 1518 and 1315 cm^{-1} , assigned to amide I, amide II and amide III bands of chitosan film, confirmed that the polymer was only a partially deacetylated product. Especially, the amide I band, present in the spectrum due to $\nu_{C=O}$

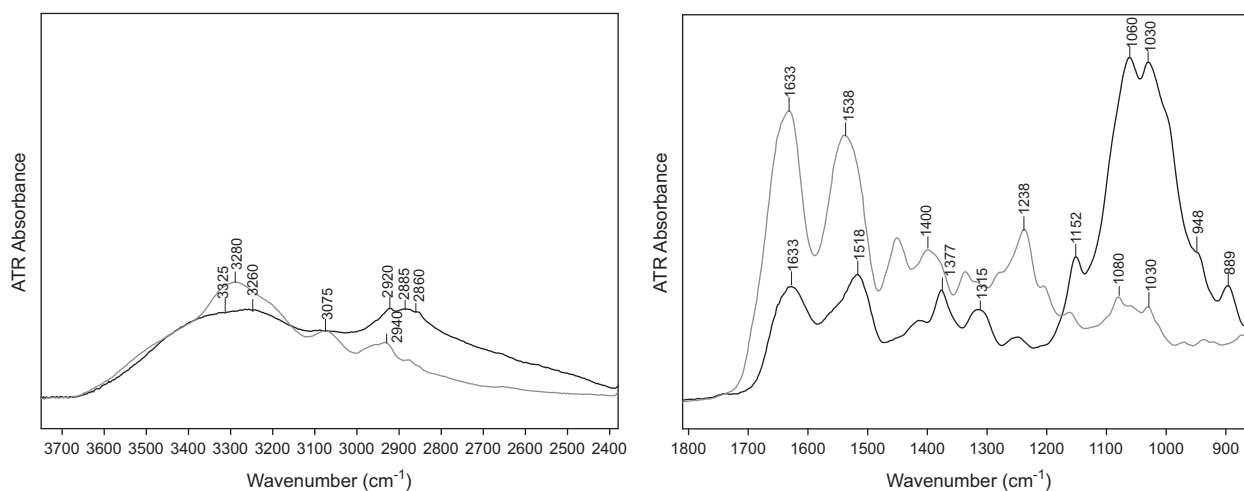


Fig. 1. FT-IR spectra of fish gelatin and (–) chitosan (–) film.

Table 1
FT-IR spectra characteristics of fish gelatin and chitosan films.

Fish gelatin film			Chitosan film		
Region	Band position in cm^{-1}	Band assignment	Region	Band position in cm^{-1}	Band assignment
Amide A	3280	ν_{NH} , ν_{OH}		3325	ν_{NH} , ν_{OH}
Amide B	3075	ν_{NH}		3260	ν_{NH} , ν_{OH}
	2970–2940	asym and sym ν_{CH_2}		2920–2860	asym and sym ν_{CH_2}
Amide I	1633	$\nu_{\text{C=O}}$, ν_{NH}	Amide I	1633	$\nu_{\text{C=O}}$, ν_{NH}
Amide II	1538	δ_{NH} , $\nu_{\text{C-N}}$, $\nu_{\text{C-C}}$	Amide II	1518	δ_{NH} , $\nu_{\text{C-N}}$, $\nu_{\text{C-C}}$
	1400	sym ν_{COO^-}		1377	δ_{CH_2}
Amide III	1238	$\nu_{\text{C-N}}$, δ_{NH}	Amide III	1315	$\nu_{\text{C-N}}$, δ_{NH}
			Saccharide	1152	asym $\nu_{\text{C-O-C}}$
			Saccharide	1060	Skeletal $\nu_{\text{C-O}}$
			Saccharide	1030	Skeletal $\nu_{\text{C-O}}$
			Saccharide	889	δ_{CH} β -glycosidic bond

vibrations, indicated the presence of $\text{CH}_3\text{-C=O}$ groups in a chitosan molecule. As it has been previously reported, during the *N*-deacetylation of chitin the intensity of its amide I band gradually decreased, while that of amine band, detected usually at around 1590 cm^{-1} , increased, indicating thus the domination of NH_2 on $\text{CH}_3\text{-C=O}$ groups [23]. As could be seen in Fig. 1, the peak at 1518 cm^{-1} , corresponding to δ_{NH} vibrations in amide II band of chitosan, showed slightly greater intensity than that at 1633 cm^{-1} , but the peak was located in the range of lower wave numbers than it usually occurs in the case of δ_{NH} vibrations of the chitosan NH_2 groups [31]. However, the second-derivative procedure that was applied enabled to resolve the chitosan amide II band into its three component peaks, at 1590, 1562 and 1518 cm^{-1} (Fig. 2). The first and the last of them could be related to δ_{NH} vibrations of the amine groups in their free -NH_2 and protonated -NH_3^+ form, respectively [24,32]. The second peak could be associated with δ_{NH} and ν_{CN} vibrations of the acetamide groups of chitosan used in this study. Apart from the above-mentioned amide bands, the spectrum of chitosan film displayed a set of characteristic saccharide bands situated in the range of $1100\text{--}900\text{ cm}^{-1}$ (Fig. 1).

Fish gelatin–chitosan film

The spectrum of the two-component film prepared from solutions of fish gelatin and chitosan (4:1, w/w) retained the pattern of both components (Fig. 3). Fish gelatin, the dominant component of the film, was identified mainly by the amide I, amide II and amide III bands, and chitosan primarily by the saccharide band.

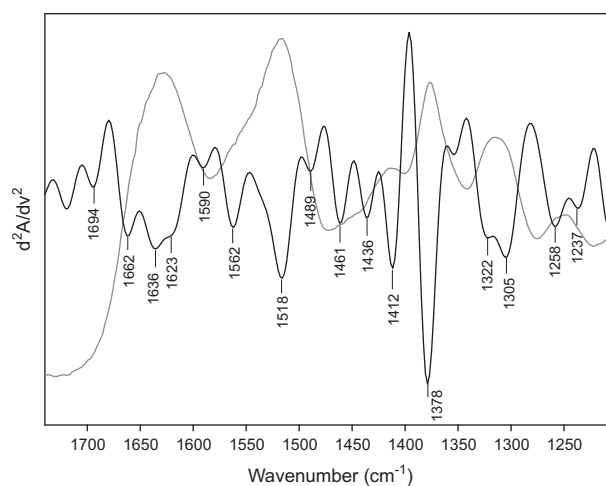


Fig. 2. Absorbance (–) and second-derivative (–) spectra of the chitosan film in the range of $1800\text{--}1100\text{ cm}^{-1}$.

However, subtle differences in the intensity and position of all individual bands, observed both in the region between $3600\text{ and }2700\text{ cm}^{-1}$ and between $1700\text{ and }900\text{ cm}^{-1}$, clearly indicated bonding of both components of the film. In the first region, between $3600\text{ and }2700\text{ cm}^{-1}$, as a result of the formation of the two-component film, the absorption bands at $3280\text{ and }2940\text{ cm}^{-1}$ of fish gelatin decreased in intensity and moved by 5 and 10 cm^{-1} , respectively, to lower wave numbers, and the band

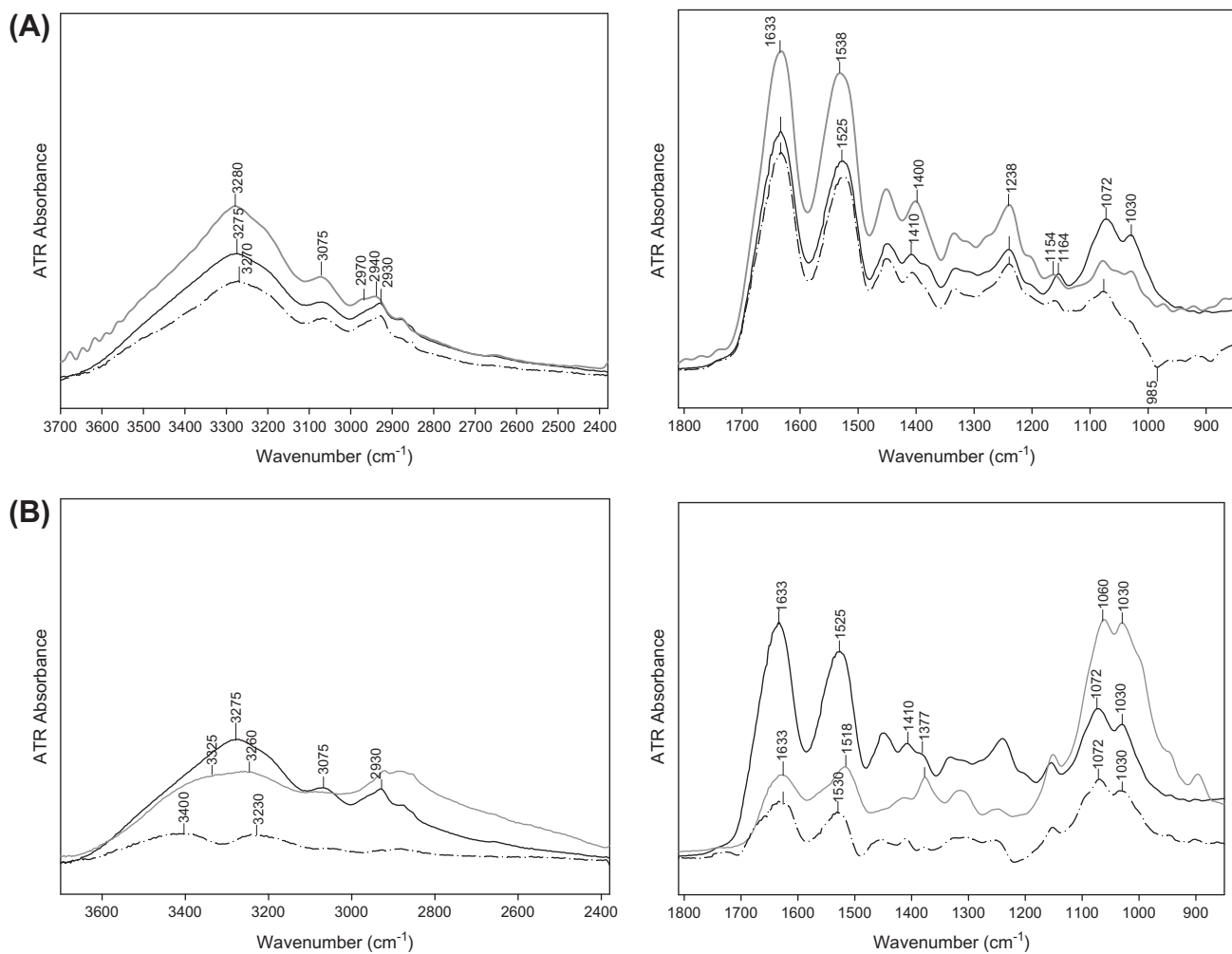


Fig. 3. (A) FT-IR spectra of the fish gelatin film (—), fish gelatin-chitosan film (---) and difference spectrum of the fish gelatin-chitosan film from which the spectrum of chitosan was subtracted (-.-); (B) FT-IR spectrum of the chitosan film (—), fish gelatin-chitosan film (---) and difference spectrum of the fish gelatin-chitosan film from which the spectrum of fish gelatin was subtracted (-.-).

at 2970 cm⁻¹ disappeared (Fig. 3A). These changes were accompanied by the appearance of the peaks in the difference spectrum at 3270 and 2930 cm⁻¹, that is at wave numbers by 10 cm⁻¹ lower than in the fish gelatin film. Typically, a shift of the broad 3600–3000 cm⁻¹ band towards lower wave numbers has been described as indicative of water-mediated hydrogen bonding [33,34]. Thus, it was likely that the energy of interactions involving NH and OH groups in hydrogen-bonding with H₂O decreased, because the chains of both polymers required conformational changes to be able to form a two-component film. The modified band around 2940 cm⁻¹ might reflect these conformational changes of the fish gelatin chains. In the second region, between 1700 and 900 cm⁻¹, due to the formation of the two-component film, the amide I, amide II and amide III bands of fish gelatin decreased in intensity but the saccharide band at 1100–900 cm⁻¹ increased (Fig. 3A). That trend followed former findings revealed by Taravel and Domard [14] for calf-skin collagen complexed with chitosan. Further, the amide I and amide III bands of fish gelatin retained their position, but the amide II band moved from 1538 to 1525 cm⁻¹, indicating the formation of hydrogen bonds in which –NH groups of this protein were involved. Simultaneously, the position of the saccharide band centered in the spectrum of the chitosan film at 1060 cm⁻¹, in the spectrum of the fish gelatin-chitosan film shifted by 12 cm⁻¹ towards higher wave numbers (Fig. 3A and B). A similar shift observed in the difference spectrum (Fig. 3B) confirmed the

involvement of the hydroxyl and ether groups of chitosan in the formation of the two-component film. Moreover, a band at 1377 cm⁻¹ of chitosan, in the spectrum of the fish gelatin-chitosan film could be recognized as a shoulder of the peak at 1410 cm⁻¹ of fish gelatin (Fig. 3B). The shift of the latter by 10 cm⁻¹ towards higher wave numbers compared with its location in the spectrum of the fish gelatin film pointed at participation of the –COO⁻ group of gelatin in the formation of the composite film. As the amide II band of chitosan also shifted, from 1518 to 1525 cm⁻¹, thus the carboxyl groups of fish gelatin likely participated in electrostatic interactions with oppositely charged –NH₃⁺ groups of chitosan.

As the intensity of the bands is controlled by the dominating component of film, some bands in the spectrum of the fish gelatin-chitosan film overlapped. Therefore, the second-derivative procedure was applied to resolve overlapping peaks in their components. Fig. 4 compares the second-derivative spectrum of the fish gelatin film to that of fish gelatin-chitosan film. While in the former three peaks in the amide I band could be clearly observed, at 1691, 1655 and 1625 cm⁻¹ corresponding, respectively, to –COOH groups, free-fold α -helix and imide residues [14,19], in the latter the peak at about 1660 cm⁻¹ was barely recognizable as a shoulder of the dominant peak located at 1625 cm⁻¹. Also in the amide II band region, the peak at 1549 cm⁻¹ of fish gelatin became a shoulder of the dominant peak at 1514 cm⁻¹ in the second-derivative spectrum of the fish gelatin-chitosan film. Dis-

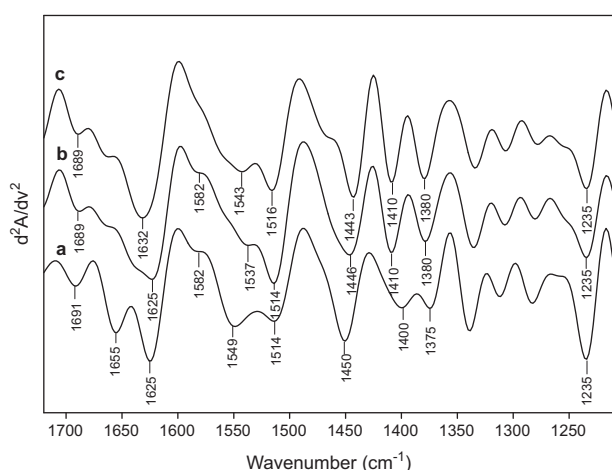


Fig. 4. Second-derivative spectra of the fish gelatin film (a) and unmodified (b) and modified with EDC fish gelatin–chitosan (c) films.

appearance of the characteristic for single α -helix peaks, at around 1660 and 1549 cm^{-1} in the region of amide I and amide II bands, respectively [14,35] indicated that interactions of fish gelatin with chitosan made a helical structure difficult to recover for gelatin. There was no spectral change of unique carboxylic band at about 1690 cm^{-1} which might suggest that these interactions did not involve COO^- groups of gelatin. As it has already been reported, the isoelectric point (pI) of fish gelatin ranges between 7 and 9, and that of cod gelatin amounts to 8.9 [20,36]. pH of the fish gelatin–chitosan film-forming solution amounted to 5. Thus at such a low pH most of the carboxyl groups of fish gelatin could be in an undissociated form, so they could be unavailable for electrostatic interaction with positively charged amino groups of chitosan. But the shift of the band at 1400 cm^{-1} attributed to ν_{COO^-} vibrations in the fish gelatin film (Fig. 3A), and that of the amide II band at 1518 cm^{-1} attributed to δ_{NH} vibrations in the chitosan film (Fig. 3B), by 10 and 7 cm^{-1} towards higher wave numbers, respectively, in the fish gelatin–chitosan film, confirmed participation of COO^- groups of gelatin and NH_3^+ groups of chitosan in these interactions. Thus, electrostatic interactions between fish gelatin and chitosan could occur; however, their number could be restricted. Taravel and Domard [14] also demonstrated an existence of pure electrostatic interactions between calf-skin collagen and

fully deacetylated chitosan, in spite of the presence of the band at 1692 cm^{-1} in the second-derivative spectrum of the collagen–chitosan complex.

Effect of EDC on fish gelatin–chitosan film

Fig. 5 shows the changes in the spectrum of the fish gelatin–chitosan film caused by chemical modification with EDC.

In the range of $3600\text{--}2700\text{ cm}^{-1}$, the bands found for the unmodified fish gelatin–chitosan film at 3275 , 3075 and 2930 cm^{-1} retained their position after the modification of components of the film with EDC, but the intensity of the first of them slightly increased. It could reflect an increased number of bounded NH groups, as the cross-linking with EDC results in the formation of iso-peptide bonds between amine groups of gelatin or chitosan, and activated carboxylic acid groups of glutamic or aspartic acid residue of gelatin [15]. Further, in the range of $1700\text{--}900\text{ cm}^{-1}$ the intensity of amide I band increased, and it is in agreement with data of Garcia, Collighan, Griffin and Pandit [21]. These authors found that the intensity of the amide I band of collagen increases with the level of polymer cross-linking, since the strength of $\nu_{\text{C=O}}$ and δ_{NH} vibrations in the new covalent bonds is increased. However, according to Wang et al. [26], due to the change of NH_2 into N-H groups in cross-linked collagen, the intensity of the amide II band decreases because the intensity of NH_2 band is stronger than that of N-H . As could be seen in Fig. 5, the amide II band of fish gelatin–chitosan film did not decrease in intensity as a result of cross-linking, but rather moved to higher wave numbers. However, in the second-derivative spectrum of the modified film (Fig. 4) the component peak of the amide II band at 1582 cm^{-1} , related to δ_{NH} vibrations of the amine groups in their free NH_2 form, totally disappeared, and that at 1543 cm^{-1} associated with δ_{NH} and ν_{CN} vibrations of the amide groups increased in intensity as compared to the spectrum of the unmodified film. These spectral changes evidently confirmed the formation of new N-H bonds, either between fish gelatin and chitosan or within fish gelatin molecules.

Conclusions

The analysis of FT-IR spectra of two-component, fish gelatin–chitosan film showed that beside the hydrogen bonds, formed within and between polymer chains involving their carbonyl, hydroxyl and amino groups, also electrostatic interactions occurred. However, taking into account pI of fish gelatin and pH of fish

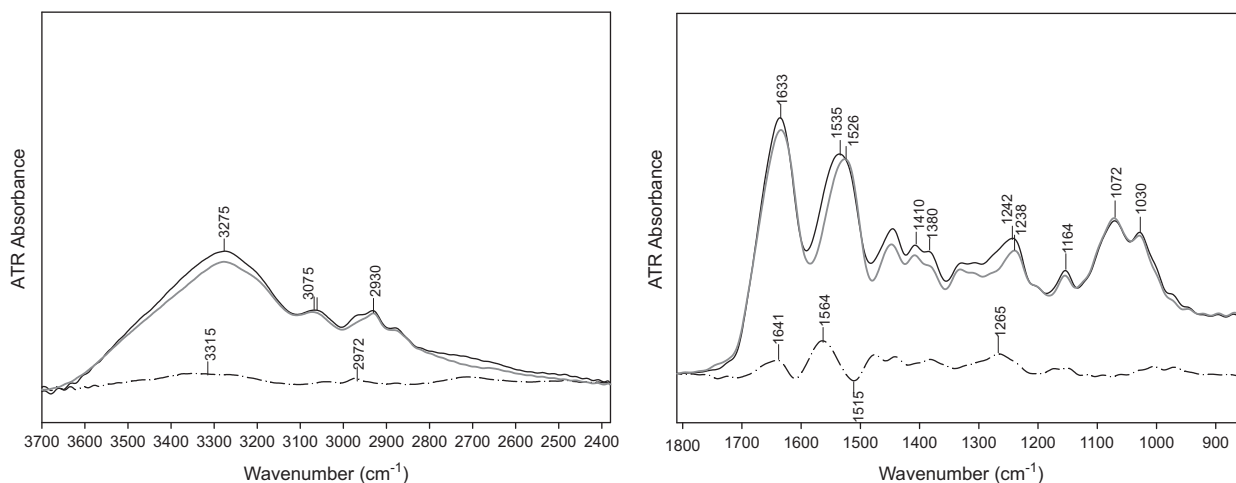


Fig. 5. FT-IR spectrum of the unmodified (—) and modified fish gelatin–chitosan film (---) and difference spectrum of the modified fish gelatin–chitosan film from which spectrum of the unmodified fish gelatin–chitosan film was subtracted (---).

gelatin–chitosan film-forming solution (5), under starting conditions both polymers carried net positive charges and therefore attractive interactions were not expected between them. Nevertheless, FT-IR spectra revealed the formation of electrostatic interactions which involved the COO^- of gelatin and NH_3^+ ions of chitosan. The modification with EDC provided cross-linking of the components of the film. New iso-peptide bonds formed between activated carboxylic acid groups of glutamic or aspartic acid residue of gelatin, and amine groups of gelatin or/and chitosan.

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