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Conjugation of kojic acid with chitosan

Andriy Synytsya [a,*], Petra Blafková [a], Alla Synytsya [b], Jana Čopíková [a], Jiři Spěváček [c], Michal Uher [d]

[a] Department of Carbohydrate Chemistry and Technology, Institute of Chemical Technology in Prague, Technická 5, 166 28 Prague 6, Czech Republic
[b] Department of Analytical Chemistry, Institute of Chemical Technology in Prague, Technická 5, 166 28 Prague 6, Czech Republic
[c] Institute of Macromolecular Chemistry, Academy of Science of the Czech Republic, Heyrovského 2, 162 06 Prague 6, Czech Republic
[d] Department of Organic Chemistry, Slovak Technical University Bratislava, Radlinského 9, 412 37 Bratislava 1, Slovakia

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Abstract

Direct and non-direct conjugation of kojic acid with chitosan was carried out according to the following approaches: (a) covalent binding of chlorokojic acid to chitosan via free amino groups, and (b) complexation of kojic acid with iron(III) bound to chitosan. Obtained products were analysed by organic elemental analysis and spectroscopic methods. The maximal degree of substitution was ~0.2 mol/mol for the chitosan–kojic acid conjugates. The triple chitosan–FeO(OH)–kojate complex contained these components in the molar ratio of 1:0.48:0.07. UV–vis absorption, FT-IR and Raman spectra of the obtained products showed characteristic bands of bound kojic acid and thus confirmed that both ways of kojic acid binding were efficient. Solution ^{1}H NMR and solid state ^{13}C CP-MAS NMR spectra of the chitosan–kojic acid conjugates supported the predicted way of 4-oxypyranone binding to chitosan. UV–vis absorption spectra of the chitosan films subsequently modified with ferric chloride and kojic acid indicated that the ligand molecules are bound to peripheral iron(III) cations of FeO(OH) subcolloidal particles arranged on the surfaces of a film.

Keywords: Kojic acid; Chitosan; Iron(III); Chlorokojic acid

1. Introduction

Chitosan is a β-(1,4)-linked polymer of partially *N*-acetylated D-glucosamine units, and it can be defined as mostly or completely deacetylated chitin. In contrast to water insoluble chitin, chitosan is soluble in water at acid pHs owing to the protonation of its free amino groups ($pK_a = 6.3$ for chitosan) (Park, Choi, & Park, 1983). Chitosan has been employed in cosmetics, pharmaceuticals, food industry, agriculture and biotechnology (Ravi Kumar, 2000). In the food industry it has been used in preservation of foods from microbial deterioration, formation of w/o/w emulsions (Del Blanco, Rodríguez, Schulz, & Agulló, 1999; Schulz, Rodríguez, Del Blanco, Pistonesi, & Agulló, 1998) and edible films (Arvanitoyannis, Nakayama, & Aiba, 1998; Kittir, Kumar, & Tharanathan, 1998), purification of water, clarification and deacidification of fruit juices; in cosmetics it is a component of toothpaste, hand and body creams, shampoo, toiletries (Shahidi, Arachchi, & Jeon, 1999). Chitosan is used in pharmaceutics as a carrier material in drug and gene delivery systems (Dodane & Vilivalam, 1998; Hejazi & Amiji, 2003; Lorenzo-Lamosa, Remuñán-López, Vila-Jato, & Alonso, 1998; RaviKumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Such biodegradable chitosan-based forms as nanoparticles, microspheres (RaviKumar et al., 2004), hydrogels (Berger et al., 2004a, 2004b), blends (Arvanitoyannis, 1999), fibres and tablets (RaviKumar et al., 2004) have been prepared for various biomedical applications (Khor & Lim, 2003).

Chitin and chitosan have been widely used for preparation of various derivatives that significantly enlarge the area of their applications (Kurita, 1998; Kurita, 2001;

* Corresponding author. Tel.: +420 220443116; fax: +420 220445130.
E-mail address: Andrej.Sinica@vscht.cz (A. Synytsya).

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Morimoto, Saimoto, & Shigemasa, 2002; Sashiwa & Aiba, 2004; Sashiwa, Fujishima, & Yamano, 2003). Partially, chitosan-complexing agent conjugates showed good bioadhesive properties (Il'ina & Varlamov, 2005; Li, Ramay, Hauch, Xiao, & Zhang, 2005), inhibition of various enzymes (Bernkop-Schnürch, 2000; Bernkop-Schnürch & Freudl, 1999; Bernkop-Schnürch & Kast, 2001) and high affinity to metal cations (Varma, Deshpande, & Kennedy, 2004).

Chitosan and its derivatives are able to bind selectively metal cations involving ion exchange, sorption and chelation processes (Guibal, 2004; Schmuhl, Krieg, & Keizer, 2001; Sipos, Berkesi, Tombácz, St Pierre, & Webb, 2003; Varma et al., 2004; Wang, Du, & Liu, 2004). This feature of chitosan has been applied to heavy metal uptake from contaminated water (Babel & Kurniawan, 2003). Recently the interaction between chitosan and iron(III) received considerable attention due to the potential applications of chitosan as an iron(III) binding agent (Burke, Yilmaz, & Hasirci, 2000; Sipos et al., 2003). To describe the structure of such adducts, there are two main approaches in the literature. The first is based on site specific interaction between cations and polysaccharide moieties (site binding model), the second proposes the formation of FeO(OH) precipitate on the surface of chitosan (colloidal model) (Sipos et al., 2003). It has been elsewhere reported that chitosan macromolecules acts as steric stabiliser of the subcolloidal FeO(OH) particles and inhibits their further aggregation (Sipos et al., 2003).

Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one) is an antibiotic produced by many species of *Aspergillus*, *Acetobacter* and *Penicillium* in an aerobic process from a wide range of carbon sources (Uher, Chalabala, & Čižmárik, 2000; Uher et al., 1989). This compound has been observed during investigation of steamed rice fermentation, but it is also a component of some other fermented foodstuffs and oriental beverages (saké, soybean paste and soy sauce). Kojic acid is commonly used in the food industry as a food additive for preventing enzymatic browning (Burdock, Soni, & Carabin, 2001) and in cosmetics due to its inhibition of melanin production (Uher et al., 2000). As a component of cosmetic preparations, it possesses bleaching skin effects and prevents skin against sun tan and ultraviolet radiation. Kojic acid derivatives are promising compounds of potential usage in human and/or veterinary medicine (Brtko et al., 2004).

Kojic acid is also known as an effective metal chelation agent forming complexes with metal cations and organometallic compounds (Marwaha, Kaur, & Sohdi, 1994; Sallam, Haggag, & Masoud, 1990). Anions of kojic acid and its derivatives behave as bidentate ligands coordinated to the iron(III) via the carbonyl and phenolic hydroxyl oxygens (Šima et al., 1993). Chelating ability of kojic acid and its derivatives has been used for analytical purposes, but also plays a significant role in their antimicrobial, antifungal and antineoplastic activity (Baláž et al., 1993; Bransova, Brtko, Uher, & Novotny, 1995; Novotny, Rauko, Abdel-Hamid, & Vachalkova, 1999; Uher et al., 2000). Like some other natural antibiotics, these compounds contain a specific siderophore structure (hydroxypyranone), which is able to sequester iron cations.

The preparation of kojic acid–chitosan conjugates could be interesting for some applications in food industry, cosmetics or pharmacy because such products may combine the advantages of both these compounds. The aim of the present paper is preparation of such conjugates by using two different approaches: (a) covalent binding of chlorokojic acid to chitosan via free amino groups, and (b) complexation of kojic acid with iron(III) bound to chitosan as nanoparticles of subcolloidal FeO(OH). Obtained products were analysed by elemental analysis and spectroscopic methods.

2. Experimental

2.1. Materials

Chitosan lactates originated from mycelium *Aspergillus niger* were obtained from Contipro s.r.o., Czech Republic. A proportion of the chitosans was converted to NH_2 (free base) and NH_3^+ (hydrochloride) forms by washing with sodium hydroxide (0.05 mol l^{-1}) and hydrochloric acid (0.05 mol l^{-1}) solutions in aqueous ethanol (50% v/v), respectively. Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one) was prepared according to Uher et al., 1989. Chlorokojic acid (5-hydroxy-2-chloromethyl-4*H*-pyran-4-one) was prepared by the reaction of kojic acid with tionylchloride in chloroform Uher et al. (1989). Ferric chloride hexahydrate was purchased from Winlab limited, United Kingdom. Ferric kojate complex was prepared by crystallisation from ethanolic solution of ferric chloride hexahydrate and kojic acid (molar ratio 1:3). The complex is highly hygroscopic, so it was stored under P_2O_5.

2.2. Preparation of the chitosan–kojic acid conjugates

Six chitosan–kojic acid conjugates assigned as chit/koj1–6 were prepared by the reaction of initial or free base chitosan with chlorokojic acid in dimethylsulfoxide (DMSO) or dimethylformamide (DMF) (Fig. 1(a)). Reaction conditions are summarised in Table 1. Chlorokojic acid (0.5 g) was dissolved in 20 ml of the reaction medium. By stirring, 0.25–0.5 g of chitosan (lactate or free base form) was added into the solution. Chitosan dissolved completely in DMSO and formed a suspension in DMFA, so the reaction was homogeneous in the former case and heterogeneous in the latter. The reaction mixtures were stirred at room temperature for 7 days. Then an excess of acetone was poured into the DMSO solutions and the

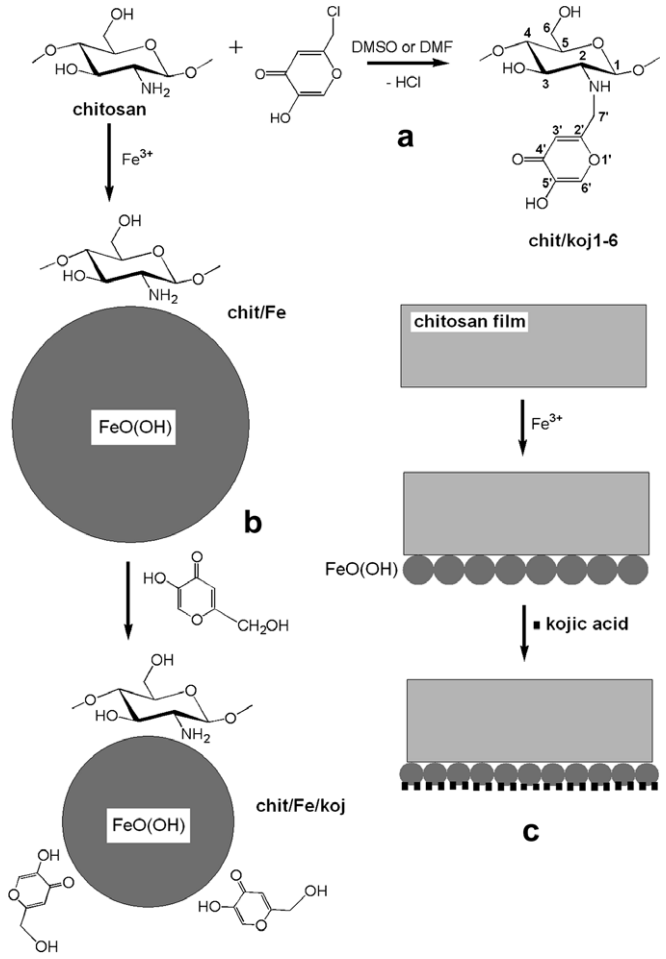

Fig. 1. (a) *N*-alkylation of chitosan with chlorokojic acid; (b) two-step preparation of chit/Fe/koj; (c) two-step surface modification of chitosan film with $FeCl_3$ and kojic acid.

product were precipitated. The solids were isolated, repeatedly washed with acetone and finally oven-dried at 40 °C.

2.3. Film preparation

Small volumes (ca. 2 ml) of chitosan and chitosan/kojic acid conjugate solutions (0.5% m/m) were poured over a food folia stretched between two plastic rings and put into a desiccator at 50 °C for 10 h. Dried films were separated from folia and stored under P_2O_5.

2.4. Preparation of the chitosan–FeO(OH) composite and chitosan–FeO(OH)–kojate complex

In addition to direct linkage onto chitosan, kojic acid was attached to this polysaccharide via FeO(OH) nanoparticles (Fig. 1(b and c)). In the first step the chitosan–FeO(OH) composite was formed by reaction of chitosan (solution or film) with $FeCl_3$ and in the second step kojic acid was chelated to peripheral iron(III) cations of the FeO(OH) particles bound to chitosan.

For the preparation of the chitosan–FeO(OH) composite, an alkali washed powder chitosan (0.2 g) was dissolved in an aqueous solution of $FeCl_3$ (0.1 mol l^{-1}). The mixture was continuously stirred for 10 h, then an excess of acetone (4:1 v/v) was added. Precipitated chitosan–FeO(OH) composite (chit/Fe) was repeatedly washed with acetone and dried. A part of the precipitate was used for the analysis and the rest was dissolved in aqueous solution of kojic acid (0.1 mol l^{-1}). After 24 h of continuous stirring an excess of acetone (4:1 v/v) was added to the mixture. The precipitated chitosan–FeO(OH)–kojate (chit/Fe/koj) was collected by filtration, washed repeatedly with acetone and finally dried. The filtrate was evaporated in a crystallising dish and obtained residue containing kojic acid and some amount of ferric kojate (koj/Fe) was milled and dried.

Chitosan films were immersed into the solution of $FeCl_3$ (0.1 mol l^{-1}) in 50% (v/v) aqueous ethanol for 22 h. Then films were subsequently washed with 50% and 96% (v/v) aqueous ethanol for an hour in each solvent to remove an access of iron(III) cations. The chitosan/FeO(OH) films were dried between sheets of filtration paper to avoid the deformation. These composite films were immersed into the solution of kojic acid (0.1 mol l^{-1}) in 50% (v/v) aqueous ethanol for 68 h. Obtained chitosan/FeO(OH)/kojate films were washed and dried by the same way like the initial chitosan/FeO(OH) ones.

2.5. Analytical methods

Organic elemental analysis (C, H, N and S) was performed on Elementar vario EL III elemental analyser. Iron content was determined by spectrophotometric method with thiocyanate (Ivšić & Tamhina, 2003) using Unicam UV 4 UV–vis spectrophotometer (Unicam, Great Britain). The degrees of chitosan substitution with

Table 1
Reaction conditions for *N*-alkylation of chitosan with chlorokojic acid and degrees of substitution (DS, mol %) and acetylation (DA, mol %) for the derivatives

Sample	Initial chitosan	Mass ratio[a]	Reaction medium	Temperature (°C)	Time (days)	Yield (mol %)
chit/koj1	1 (lactate)	1:1	DMSO	20	7	7
chit/koj2	1 (lactate)	2:1	DMSO	20	7	25
chit/koj3	1 (free base)	2:1	DMSO	20	7	27
chit/koj4	1 (lactate)	2:1	DMSO	40	7	16
chit/koj5	1 (lactate)	2:1	DMSO	60	7	10
chit/koj6	2 (lactate)	2:1	DMF	20	7	22

[a] Chlorokojic acid to chitosan.

kojic acid residues (DS, mol/mol) and of acetylation (DA, mol/mol) were calculated from the results of ^{1}H NMR and organic elemental analysis. The content of glucan in the initial chitosan lactates was determined using a Megazyme enzymatic kit K-YBGL 10/2005 (Magazyme, Ireland).

Absorption UV–vis spectra (spectral region 190–600 nm, beam width 2 nm) of chitosan films were measured by Unicam UV 4 UV–vis spectrophotometer (Unicam, Great Britain). FT-IR spectra (spectral region 4000–400 cm^{-1}, resolution 2 cm^{-1}) of the samples in the form of KBr tablets were recorded on FT-IR spectrophotometer 1760× (Perkin-Elmer, USA). FT-Raman spectra (spectral region 4000–100 cm^{-1}, resolution 2 cm^{-1}) of powder samples were recorded by using Bruker FT-Raman (FRA 106/S, Equinox 55/S) spectrometer equipped with a Nd: YAG laser ($\lambda_{ex} = 1064$ nm), a quartz beam splitter and a liquid nitrogen cooled germanium detector. The laser power was set at 100 mW, and 256 scans were accumulated with a spectral resolution of 2.0 cm^{-1}.

Proton NMR spectra were measured by Varian Mercury Plus 300 BB (Varian, USA) Fourier transform NMR spectrometer. Temperature of measurement was 22 °C. The samples (5% w/v) were dissolved in D_2O solution of 0.1 mol l^{-1} DCl. Obtained NMR spectra of initial chitosan and conjugates were used to estimate the degrees of acetylation (DA) and substitution with kojic acid (DK). High-resolution ^{13}C CP/MAS spectra were measured by NMR spectrometer BRUKER DSX 200 in 7 mm ZrO_2 rotors at the frequency 50.33 MHz (Bruker Instruments Inc., USA). Number of data point was 2k, magic angle spinning (MAS) frequency 4 kHz, strength of B1 field was 50.0 kHz. The number of scans for accumulation of NMR spectra was 1200–22,000 depending on the signal-to-noise ratio, spectral width was 25 kHz, repetition delay and spin lock pulse length were 3 s and 2 ms, respectively. ^{13}C scale was calibrated by external standard glycine (176.03 ppm – low field carbonyl signal).

3. Results and discussion

3.1. Characterisation of the initial chitosan lactates

The chitosan lactates 1 and 2 used for the synthesis of chit/koj1-6 and chit/Fe/koj were previously analysed. These polysaccharides originated from the cell wall chitin–glucan complex of mycelium *A. niger*. Subsequent alkali and lactic acid treatment of this complex led to deacetylation and removal of glucan, with some residual chitosan lactate if the acidic hydrolysis was not complete. It was found small amount of bound β-glucan (7.8% m/m) were found in chitosan lactate 1, while chitosan lactate 2 contained no glucan. Acetylation degrees (DA) were 17.4 mol % for 1 and 17.8 mol % for 2; lactic acid 10.6% m/m for 1 and 6.0% m/m for 2; moisture 10.7% m/m for 1 and 10.1% m/m for 2. The presence of β-glucan and dissimilarity in DA and lactic acid content caused some differences in the C/N ratios obtained by organic elemental analysis (Table 2). Spectroscopic data summarised below were quite similar for both chitosan lactates.

FT-IR (solid state): 3394 cm^{-1} (OH); 2940, 2891 cm^{-1} (CH); 1634, 1526 cm^{-1} (NH_3^+); 1420, 1380, 1320, 1213 cm^{-1} (in-plane ring def.); 1154 cm^{-1} (COC); 1082, 1034 cm^{-1} (CO, CC); 899, 658, 566 cm^{-1} (skeletal). FT Raman (solid state): 1650 cm^{-1} (CONH); 1461 cm^{-1} (CH_2); 1383 cm^{-1} (CH_3); 1327 cm^{-1} (CONH); 1268, 1115, 958, 900, 799, 437, 363 cm^{-1} (skeletal). The assignment and chemical shifts of the ^{1}H and ^{13}C NMR signals of chitosan lactate are as follows. Chitosan lactate: ^{1}H NMR (DCl/D_2O) $\delta = 4.53$ (H_{-1}), $\delta = 4.30$ (CHOH Lac), $\delta = 3.10$ (H-2), $\delta = 3.67$–3.84 (H-3-6), $\delta = 1.88$ ($NCOCH_3$) and $\delta = 1.33$ (CH_3 Lac); ^{13}C CP-MAS NMR (solid state of the HCl form) $\delta = 98.9$ (C-1), $\delta = 56.8$ (C-2), $\delta = 71.4$ (C-3), $\delta = 81.5$ (C-4), $\delta = 74.9$ (C-5), $\delta = 59.3$ (C-6), $\delta = 174.3$ ($NCOCH_3$) and $\delta = 174.3$ ($NCOCH_3$). Obtained results of spectroscopic measurements are in agreement with the literature (Fernandez-Megia, Novoa-Carballal, Quinoa, & Riviera, 2005; Heux, Brugnerotto, Desbrieres, Versali, &

Table 2
Experimental and calculated contents (in % m/m) of organic elements in the products and the initial chitosan lactates

Sample	Content (% m/m)								C/N
	C		H		N		S		
	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	
Chitosan lactate 1	37.10	37.10	6.78	6.82	5.61	5.61	0	0	6.61
Chitosan lactate 2	36.98	36.90	6.78	6.82	5.99	5.97	0	0	6.18
chit/koj1	35.71	35.44	6.58	6.62	5.36	5.43	1.10	1.18	6.53
chit/koj2	36.57	36.59	6.26	6.32	5.01	5.00	0	Traces	7.32
chit/koj3	37.61	37.62	6.32	6.35	5.07	5.05	0.78	0.82	7.45
chit/koj4	35.24	35.27	6.54	6.60	5.18	5.21	0	Traces	6.77
chit/koj5	34.41	34.52	6.56	6.57	5.33	5.30	0	Traces	6.51
chit/koj6	36.21	36.21	6.84	6.92	5.74	5.63	0	0	6.43
chit/Fe	26.15	26.16	5.86	5.87	4.35	4.40	0	0	5.95
chit/Fe/koj	30.40	30.27	6.23	6.27	4.77	4.80	0	0	6.31
Fe/koj	48.82	48.87	4.07	4.04	0.00	0	0	0	–

Rinaudo, 2000; Lavertu et al., 2003; Paulino, Simionato, Garcia, & Nozaki, 2006).

3.2. Characterisation of the chitosan/kojic acid conjugates

The introduction of the kojic acid moieties into the chitosan macromolecule via the nucleophilic substitution of a primary amine with chlorokojic acid yielded a chitosan derivative with different properties from the initial chitosan lactate. Since both reactants are soluble in DMSO, this solvent goves homogeneous conditions. Chlorokojic acid is poorly soluble in water and methanol, so water–methanol mixtures which have been successfully used for similar *N*-alkylation of chitosan with chlorocarboxylic acids (Kim & Choi, 1998) cannot be used for this purpose. Chitosan is not soluble in DMF but form gentle suspension in this medium that support the reaction at heterogeneous condition. Thus, DMSO and DMF were chosen as the appropriate reaction media.

Generally, the nucleophilic substitution of a primary amine is promoted by the presence of a catalytic base, which binds the acid generated from the reaction and improves the nucleophilic properties of the amine. However, the *N*-alkylation of chitosan was ineffective in this case (Kim & Choi, 1998) as has been explained by chitosan precipitation or the competitive reaction of the base as a nucleophile. The formation of mixed *N*,*O*-substituted chitosan derivatives has been reported for the reaction with chloroacetic acid under strong alkali conditions (Carolan, Blair, Allen, & McKay, 1991; Rinaudo, Dung, Gey, Milas, & Mariotti, 1992), so the presence of a base lead to non-selective alkylation. According to these experiences, we decided to carry out the reactions of chitosan with chlorokojic acid without a base catalyst.

The efficacy of *N*-alkylation could also be improved by the use of an excess of a reagent. The 6- to 12-fold excess of the chlorocarboxylic acids has been applied for the preparation of *N*-carboxylalkylated chitosans (Kim & Choi, 1998). We used the 1:1 and 2:1 chlorokojic acid to chitosan mass ratios owing to the restricted availability of the reagent. This reaction expected to be slow and, indeed, it was required at least 7 days to reach about 0.2 mol/mol degree of substitution. We assume that kojic acid is bound to chitosan mainly as the secondary amine, while the formation of the tertiary amine seems to be improbable owing to steric hindrance.

The results of organic elemental analysis are summarised in Table 2. In addition to carbon, hydrogen and nitrogen contents displayed, sulphur content originated from the reaction medium (DMSO) was also determined and confirmed by ^{1}H NMR. Small or trace amount of sulphur was found in all the products obtained by the reaction in this solvent. Removal of residual DMSO is a difficult procedure that involves multiply washing and drying. The degrees of substitution (DS) and the reaction yields were obtained from the results of organic elemental analysis; the DS values were also calculated from the

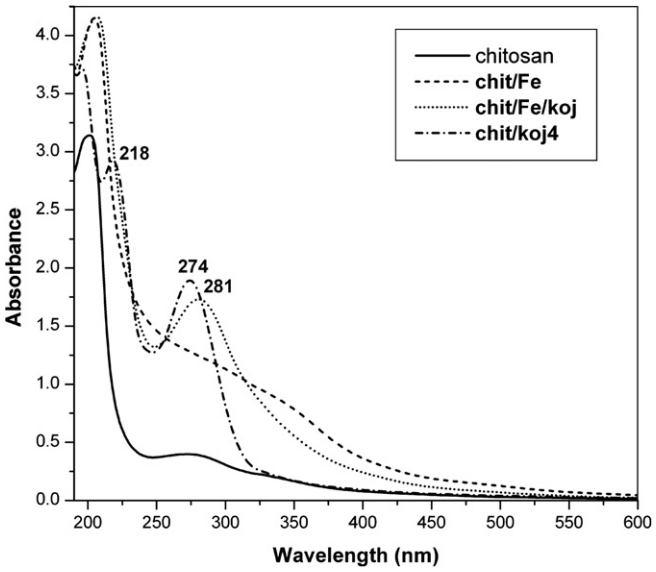

Fig. 2. UV–vis absorption spectra of the initial chitosan film (solid) and the modified chitosan films: chit/Fe (dash), chit/Fe/koj (dot) and chit/koj4 (dash dot).

^{1}H NMR spectra (Tables 1 and 2). The highest substitution was achieved in the case of chit/koj3. The low chlorokojic acid to chitosan ratio (1:1) was ineffective and led to reaction yields of 7 mol %. Free base chitosan (chit/koj3) and chitosan lactate (chit/koj2) showed similar reactivity at the same conditions. Thus, the state of free amino groups in chitosan (NH_2 or NH_3^+) slightly influenced *N*-alkylation of chitosan with chlorokojic acid. Surprisingly, reaction at higher temperatures (40 and 60 °C) reached significantly lower reaction yields than could be explained by the occurrence of side reactions (Kim & Choi, 1998). The product chit/koj6 prepared in DMF showed similar DS like chit/koj2 prepared at the similar conditions in DMSO.

UV–vis absorption spectrum of the film prepared from chit/koj4 is shown in comparing with the spectra of an initial chitosan film before and after subsequent modification with $FeCl_3$ and kojic acid (Fig. 2). Two absorption bands at 218 and 274 nm indicated the presence of 4-oxopyranone groups. These bands were assigned to intraligand $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the chromophoric C=O group (Marwaha et al., 1994; Šima et al., 1993).

FT-IR spectra of chitosan–kojic acid conjugates chit/koj1–6 obtained at different reaction conditions were quite similar to each other (Fig. 3(a)). New IR bands of kojic acid residues were observed at ~1650 cm^{-1} (C=O stretch), 1630–1617 cm^{-1} (C=O, C=C stretch) and 1220–1210 cm^{-1} (aromatic C—O—C stretch) (Marwaha et al., 1994). Corresponding bands of chlorokojic acids are centred at 1658, 1624 and 1230 cm^{-1} (not shown). The intense band at 1218–1212 cm^{-1} and the shoulder at 922 cm^{-1} were broadened and shifted from the corresponding features of chlorokojic acid that could be due to covalent binding of the kojic acid residues onto the polysaccharide. The shoulder

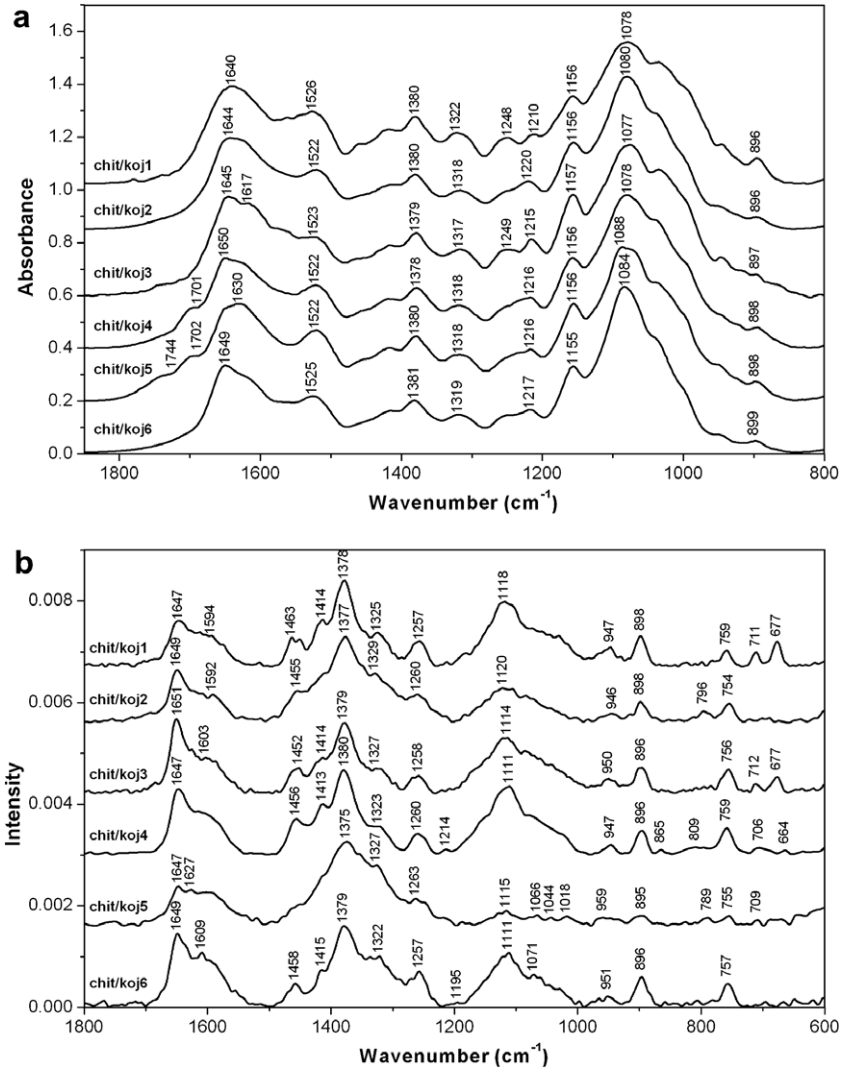

Fig. 3. FT-IR (a) and FT Raman (b) spectra of chit/koj1–6.

near 1242 cm^{-1} in the spectra of chit/koj5 and chit/koj6 prepared at higher temperatures, respectively, 40 and 60 °C, may indicate another way for 4-oxypyranone binding. New bands of the derivatives at 1660–1620 cm^{-1} are highly overlapped by the chitosan bands at 1630 and 1526 cm^{-1} (Paulino et al., 2006), so their identification is difficult. These new bands, however, cause significant absorbance increase near 1630 cm^{-1} in comparison with the neighbouring region around 1530 cm^{-1}, where kojic and chlorokojic acids do not absorb. The products chit/koj4 and chit/koj5 also showed new shoulders at 1744 and 1702 cm^{-1} that could be assigned to difficultly identified products of side reactions, probably carbonyls. These bands were more pronounced in the case of chit/koj5 prepared at higher temperature (60 °C).

FT-Raman spectra of chitosan–kojic acid conjugates (Fig. 3(b)) contained several bands assigned to the substituted kojic acid groups. The intense sharp band at 1647–1651 cm^{-1} and several smaller bands at 1609–1592 cm^{-1} were assigned to C═O and C═C stretch in the kojic acid moieties; the band at 754–759 cm^{-1} to skeletal vibration of the 4-pyronone ring. Corresponding Raman bands of chlorokojic acid were found at 1648 and 1589 cm^{-1} (not shown). The first group of bands is overlapped by the chitosan bands of similar position (1657 cm^{-1} – amide I, 1598 cm^{-1} – in-plane deformation of $–NH_2$). The ratio between the intensities of the bands at 1647–1651 and 896–898 cm^{-1} could be used for the estimation of the substitution degree (Table 3). It is evident that this ratio correlated well with the DS value obtained by elemental analysis or ^{1}H NMR. Raman spectrum of chit/koj5 showed weak features and a strong fluorescence background due to the presence of products of chitosan oxidation, so the intensity ratio did not calculated for this product. Nevertheless, the marker bands of kojic acid were definitely present even in this case. Two bands at 711 and 677 cm^{-1} present in chit/koj1 and chit/koj3 were assigned to residual DMSO (Bianco et al., 1986).

Proton NMR spectrum of chit/koj4, which is similar to those of the other derivatives, is shown in Fig. 4(a) together

Table 3
Characterisation of the chitin–kojic acid conjugates and the initial chitosan lactates

Sample	I_{1650}/I_{898}	Content (mol/mol)[a]							
		DS		DA	Lactate	Glucan		DMSO	Water
		Elem.	^{1}H NMR	^{1}H NMR	^{1}H NMR	Elem.	GC	^{1}H NMR	Elem.
Chitosan lactate 1	–	–	–	0.17	0.28	0.11	0.11	–	1.4
Chitosan lactate 2	–	–	–	0.18	0.17	0	0	–	1.4
chit/koj1	1.5	0.06	0.09	0.17	0.02	0.11	ND	0.08	1.3
chit/koj2	2.5	0.21	0.18	0.16	Traces	0.11	ND	Traces	1.5
chit/koj3	3.0	0.22	0.20	0.13	Traces	0.11	ND	0.06	1.3
chit/koj4	1.7	0.13	0.13	0.14	0	0.11	ND	Traces	1.7
chit/koj5	ND	0.08	0.08	0.11	0	0.11	ND	Traces	1.6
chit/koj6	2.5	0.18	0.19	0.14	0	0	ND	–	2

[a] Calculated on the monomeric unit of chitosan.

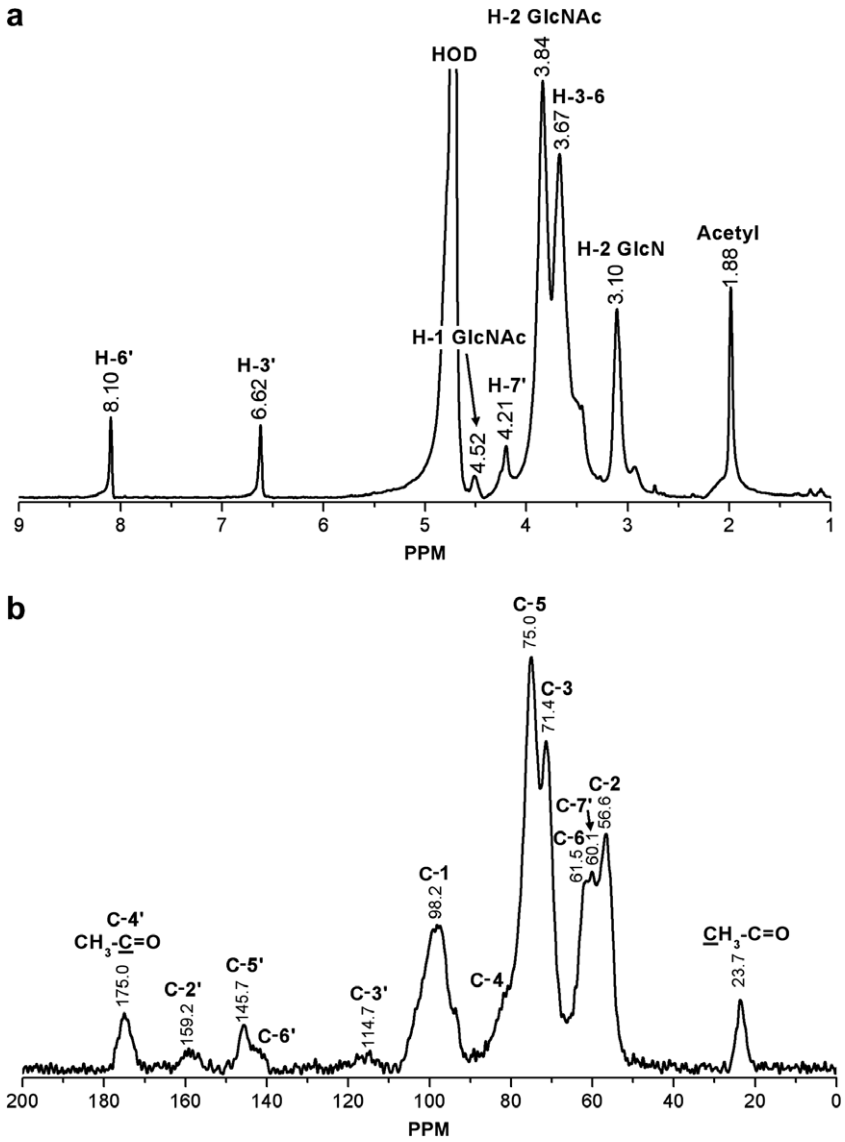

Fig. 4. ^{1}H NMR (a) and ^{13}C CPMAS NMR (b) spectra of chit/koj4.

with band assignments based on previous reports (Fernandez-Megia et al., 2005; Lavertu et al., 2003; Marwaha et al., 1994). The signals of chitosan protons at 3.83, 3.67, 3.10 and 1.88 ppm did not change significantly their chemical shifts after the reaction with chlorokojic acid, while lactate hydrogen peaks at 4.20 and 1.23 ppm vanished from the

spectrum and three resonance signals appeared at ca. 4.2, 6.6 and 8.1 ppm. These new peaks were assigned to the protons of kojic acid residues bound to the polysaccharide. Corresponding resonance signals of chlorokojic acid in D_2O solution were found at 4.40 and 4.65 ppm (CH_2), 6.53 ppm (H-3′) and 7.97 ppm (H-6′). Therefore, the signals of aromatic proton H-3′ and H-6′ in chit/koj4 were downfield shifted by 0.1 ppm in accordance to the initial chlorokojic acid, while the CH_2 signal was upfield shifted by 0.2–0.5 ppm. Aromatic protons of the 4-pyron ring are much less sensitive to the chlorine substitution than the methylene protons, so observed shifts confirm that the kojic acid moiety is bound to chitosan via the CH_2 group.

^{13}C CP-MAS NMR spectrum of chit/koj4 with the assignment of the resonance signals (Heux et al., 2000; Marwaha et al., 1994; Paulino et al., 2006) is shown in Fig. 4(b). Spectrum of the product contained broad signals of all the carbons of kojic acid residues bound to chitosan. The signals of C-2′ (159.2 ppm), C-3′ (114.7 ppm) and CH_2 (61.5 ppm) carbons of the substituents are markedly shifted in comparison with the corresponding carbon signals of chlorokojic acid (respectively, 162.7, 113.9 and 40.9–43.4 ppm). Chemical shift of the last one is nearly identical with that of chlorokojic acid. According to ^{13}C CP-MAS NMR the degree of substitution DS was 0.12 mol/mol which is close to the value obtained by ^{1}H NMR (0.13 mol/mol) (Table 3).

3.3. Characterisation of the chitosan–FeO(OH) composites and the chitosan–FeO(OH)–kojate complexes

Subsequent reactions of chitosan with $FeCl_3$ and kojic acid were carried out both in homogeneous (aqueous solutions) and heterogeneous (polysaccharide films) conditions. Obtained products were precipitates and modified films. The precipitate chit/Fe was yellow coloured, so it contained FeO(OH) bound to chitosan. The reaction with kojic acid led to a change in colour of the product chit/Fe/koj from yellow to reddish brown that could be an evidence of complexation. During the reaction of a chitosan film with $FeCl_3$ the subcolloid particles of FeO(OH) are formed and assembled into thick layers on the opposite surfaces of the film; further reaction with kojic acid led to partial dissociation of the particles, while the thinned FeO(OH) layers carrying bound kojate retained on the film surfaces (Fig. 1(c)). Chitosan films became yellow coloured after interaction with iron(III) salt, and this colour was more intense for longer reaction times. Further reaction of modified film with kojic acid induced dissolution of some FeO(OH) that led to colouring of the reaction medium. This fact could be clarified by appearing of soluble ferric kojate complex. A fraction of FeO(OH), however, was still bound to chitosan film. The colour of the modified films was stable after washing.

Table 4 represents molar relations between iron(III), kojic acid and monomeric units of chitosan in the precipitates calculated from the results of organic elemental and iron(III) analysis. A large amount of iron(III) bound to chitosan (~24% m/m or 1.39 mol/mol of chitosan units) in chit/Fe obtained by first step reaction of chitosan with $FeCl_3$ was significantly reduced by the second step reaction with kojic acid. Nevertheless, a significant part of kojic acid molecules (0.17 mol/mol of chitosan units) was retained by binding to some peripheral iron(III) cations of chit/Fe. As a result, a triple complex chit/Fe/koj was formed. For this complex the molar ratio of chitosan units:iron(III):kojate is about 2:1:0.34.

Table 4
Characterisation of the iron(III) contained products

Sample	Fe (%)		Content (mol/mol)[a]		
	Calc.	Exp.	Kojic acid	Fe	Water
chit/Fe	24.14	24.36		1.39[b]	2[b]
chit/Fe/koj	9.18	9.23	0.07[b]	0.48[b]	2[b]
Fe/koj	2.42	2.41		0.06[c]	0

[a] Obtained by elemental analysis and photometry.
[b] Calculated in moles per a mole of monomeric units of chitosan.
[c] Calculated in moles per a mole of kojic acid.

UV–vis absorption spectra of modified chitosan films are shown in Fig. 2. Absorption increase in the case of the chit/Fe film indicated the presence of iron(III), that led to appearance of a broad shoulder in UV and partially visible region due to the charge transfer in FeO(OH). Absorption spectrum of the chit/Fe/koj film had an intense band with absorption maximum at 281 nm assigned to $\pi \rightarrow \pi^*$ transitions of the chromophoric C=O group in the ligand. This band is shifted towards the red in comparison to the corresponding band of free ligand at 272 nm that is attributive to the involvement of carbonyl group into the complexation (Marwaha et al., 1994). The absence of the characteristic ferric kojate band at 490 nm that has been assigned to the LMCT transition (02p → Fe3d) (Šima et al., 1993) could be explained by weaker interaction between kojic acid and metal on the film surface than in ferric kojate. The ligand is able to bind to the peripheral iron(III) cation on the surface of the FeO(OH) coating of the chitosan film. This reaction may lead to the elimination of the cation or to the addition of the kojate onto the composite depending on that how strong the cations are connected inside the FeO(OH) particle. The metal cation connecting to other ones inside a subcolloid particle cannot possess enough binding sites to form chelates with kojic acid like in ferric kojate.

Vibration spectra of the precipitates obtained are shown in Fig. 5 in comparison with those of FeO(OH) and $Fe(koj)_3$. FT-IR spectrum of chit/Fe/koj demonstrates a number of characteristic bands assigned to vibrations of bound ligand (Fig. 5(a)). These are bands and shoulders centred at 1620, 1568, 1478, 920, 860 and 758 cm^{-1}. Two bands at 1568 and 1478 cm^{-1}, which are overlapped by the chitosan bands, are the most intense of them. All these bands as well as an evident change of the broad band at 1620 cm^{-1} due to the ligand contribution confirm the presence of kojic acid in chit/Fe/koj. In contrast, FT-IR

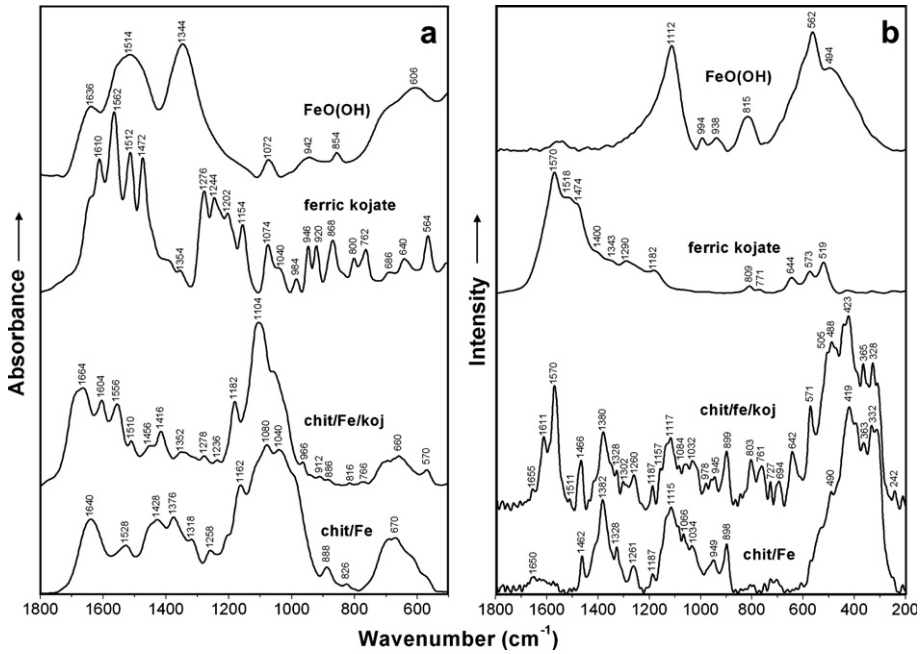

Fig. 5. FT-IR (a) and dispersion Raman (b) spectra of chit/Fe, chit/Fe/koj, $Fe(koj)_3$ and FeO(OH).

spectra of chitosan films did not markedly change after subsequent modification with $FeCl_3$ and kojic acid (Fig. 6). The difference in relative intensity and width of the most intensive polysaccharide bands in the region of 1200–900 cm^{-1} is caused by the variation in thickness across the whole film area; other spectral changes were insignificant. Thus, the surface modification of chitosan films slightly influenced the IR bands because its contribution is negligible in comparison with that of the whole film thickness.

Raman spectra of chit/Fe and chit/Fe/koj (Fig. 5(b)) showed intense band at 1115–1117 cm^{-1} and very broad band at 250–600 cm^{-1}. These bands indicate the presence of FeO(OH). Raman spectrum of chit/Fe/koj showed two intense bands at 1570 and 1511 cm^{-1} assigned to C=O, C=C stretching vibrations of kojic acid bound to iron(III). Several new bands were also found at 1303, 978, 803, 761, 642, 571 and 505 cm^{-1}. These bands arise from skeletal vibrations of kojic acid in ferric complex. Their positions, however, are shifted from those of corresponding ferric kojate bands. Thus, the type of kojate binding to iron(III) is not identical for chit/Fe/koj and ferric kojate.

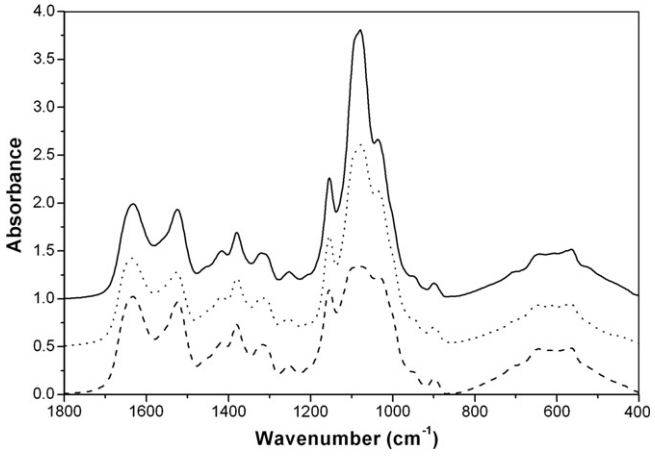

Fig. 6. FT-IR spectra of the chitosan film before (solid) and after subsequent modifications with $FeCl_3$ (dash) and kojic acid (dot).

4. Conclusions

Direct and non-direct conjugation of kojic acid with chitosan was carried out and the products obtained were characterised by elemental analysis and spectroscopic methods. The results of analysis lead us to following conclusions.

(a) *N*-alkylation of chitosan with chlorokojic acid is an effective method of covalent conjugation of 5-hydroxypyranone group to the polysaccharide. The highest substitution degree was obtained for free base chitosan in a homogeneous reaction with an excess of chlorokojic acid in DMSO at the laboratory temperature, while the heterogeneous reaction in the medium of DMF led to comparable yields. A decrease in the substitution degree was observed for the products prepared at higher temperatures that could be due to acceleration of side reactions. The reaction yields were not high (up to 27 mol %) but may be sufficient to enhance antimicrobial properties of chitosan based edible films or other products. Detailed characterisation of such films will be a topic of further investigation. In addition, the presence of 5-hydroxypyranone substituents may improve the ability of chitosan to bind metal cations.

(b) Attachment of kojic acid to chitosan via FeO(OH) was also successive. The product of two-step reaction is triple chitosan–FeO(OH)–kojate complex, in which kojic acid is bound to peripheral iron(III) cations of subcolloidal par-

ticles of FeO(OH) attached to the polysaccharide. This complex is an example of chemically modified nanoparticles assembling on the surface of chitosan fibres or films. Iron oxides have been used as colouring agents in the preparation of edible films for use in pharmaceuticals, confectionery and food (Porter & Woznicki, 1985). Therefore, a combination of FeO(OH) and kojic acid may introduce colour and antimicrobial activity to chitosan-based films.

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