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The photochemical stability of collagen–chitosan blends

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Abstract

The photochemical stability of collagen–chitosan blends in solution and film form was investigated using viscosimetry measurements, UV-Vis spectrophotometry, FTIR spectroscopy and wide angle X-ray diffraction. It was found that the relative viscosity of collagen decreased upon UV irradiation. The initial relative viscosity of collagen–chitosan blends were greater than the viscosity of collagen; upon UV irradiation the viscosity of the blends decreased rapidly. The absorption/scattering of collagen in solution increased during irradiation of the sample as shown by UV-Vis, indicating a conformational transition in the sample. FTIR showed that the amide A, B, I and II bands from collagen are shifted after UV irradiation to lower wave numbers; these shifts in collagen–chitosan blends are less well pronounced. Wide angle X-ray diffraction indicated that collagen and collagen–chitosan blends in film form retain much of their structural characteristics after irradiation.

The viscosimetry and UV-Vis spectrophotometry results have shown that solutions of collagen–chitosan blends are less stable photochemically than a pure collagen solution. FTIR spectra have shown, that collagen–chitosan blended films are also less stable photochemically than pure collagen films. Wide angle X-ray diffraction indicates that collagen and collagen–chitosan blend samples in film form are less susceptible to conformational change than equivalent samples in solution.

Keywords: Collagen; Chitosan; UV radiation; X-ray diffraction; Structure

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1. Introduction

1.1. Collagen

Collagen is one of the most important biopolymers in animals [1]. The abundance and structural properties of collagen has led to a wide range of uses, including leather, sutures and haemostatics [2]. Collagen possesses characteristics as a biomaterial distinct from those of synthetic polymers—collagen is capable of acting as the basis for biomaterials that can be used to interact with living tissue as it possesses commensurate biomechanical and biochemical properties to living tissue. Collagen is subject to the action of solar radiation; the solar spectrum is a complex band of radiation including UVC (220–290 nm), UVB (290–320 nm) and UVA (320–400 nm) as well as visible light and infrared radiation. Moreover, collagen based biomaterials are sometimes subject to the action of UVC radiation from artificial sources during sterilisation. Collagen contains aromatic amino acids (phenylalanine and tyrosine) absorbing in the far-ultraviolet (250–280 nm) range. Many studies have demonstrated modification of collagen induced by UV radiation—it has been shown that in solution collagen loses the ability to form natural fibrils after irradiation [3]. In addition, the fluorescence observed after UV irradiation is due to the presence of photo-products of phenylalanine and tyrosine residues the collagen amino acid sequence [4–9]. Photo-crosslinking [10–12] and photo-degradation [6,13–15] of collagen may also occur on exposure to UV radiation.

The type of photochemical process and its efficiency depend on the presence of additives to collagen [16–20]. Modifications of photochemical stability of collagen in the presence of β-carotene [15–17], riboflavin [18], melanin [19], methylene blue [21], H_2O_2 and thiourea [12] have been reported.

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1.2. Chitosan

Chitosan is a natural polymer prepared from chitin by deacetylation. Physical and chemical properties of chitosan depend strongly on the molecular weight and the degree of deacetylation [22]. Chitosan is sensitive to various types of degradation such as oxidative, hydrolytic, thermo-, photo- and ultrasonic degradation [23].

1.3. Collagen–chitosan blends

Collagen and chitosan do not exist together as blends in nature, but the specific properties of each may be used to produce synthetic blends that confer unique structural and mechanical properties. The uses of relatively low cost low pollution biomaterials with specific properties has great potential, for instance in developing a new generation of prosthetic implants [24–26].

In our previous study we have shown that collagen–chitosan blends are miscible and interact at the molecular level, new hydrogen bonding networks appear to alter the collagen helical character and therefore the overall physical parameters of the blend [27].

Blends of collagen and chitosan have been used for design of polymeric scaffolds for the in vitro culture of human epidermoid carcinoma cells [28], as membrane for controlled release [29–31], and as implant fibres [32,33].

From the foregoing it can be concluded that blends between collagen and chitosan are of increasing importance in the field of biomaterials as the blends possess characteristics that allow them to be used in a number of medically important ways. The interaction of UV light and the blends is of importance, as the behaviour of the blends needs to be understood under potentially harsh conditions, and UV light may be used to refine the blend preparation process to produce blends of specific structural and chemical characteristics. The aim of this study was to determine the influence of chitosan on the photochemical stability of collagen solution and films.

2. Materials and methods

2.1. Sample preparation

Collagen was obtained in our laboratory from tail tendons of young albinos rats. Tendons were blended in a Waring blender in 0.5 M acetic acid, then spun at 10,000 rpm in a Sorvall centrifuge and the soluble fraction decanted and lyophilised. The method used was the same as previously employed [15,16]. Chitosan (360,000 molecular weight) was obtained from Fluka, Switzerland.

The samples, in the form of the solutions for UV-Vis spectra and films for FTIR and X-ray diffraction were prepared. Polymeric blends were prepared by mixing of appropriate volumes of collagen and chitosan in acetic acid such that a series of 11 solutions were produced in duplicate containing collagen–chitosan blends with a final concentration of 1 g l^{-1}. Films were obtained by casting the solution onto glass plates or CaF_2 spectrophotometric windows. After solvent evaporation, the samples were dried in vacuum at 20 °C.

The blends were irradiated under air at room temperature using a Philips TUV-30 mercury lamp which emits light at a wavelength of 254 nm. The fluence level of radiation was 0.263 J cm^{-2} min^{-1}. The dose of incident radiation during 1 h exposure was 16 J cm^{-2}. The intensity of the incident light was measured using an IL 1400A Radiometer (International Light, USA). Irradiation experiments were carried out in a quartz cuvette at a distance of 3 cm from the light source. All measurements were performed at 20 °C to avoid any temperature-dependent influence on the physico-chemical properties of collagen films.

2.2. UV-Vis spectrophotometry

The UV-Vis absorption spectra of the collagen solution, before and immediately after UV irradiation were recorded with a Shimadzu spectrophotometer (Model UV-1601PC). Data was collected and plotted using the UVPC program and computer data station supplied by the manufacturer.

2.3. Relative viscosity measurements

The relative viscosity of collagen, chitosan and collagen–chitosan blend solutions were measured at 20 °C in 0.5 M acetic acid (pH 2.7) using a quartz Ubbelohde viscometer.

2.4. FTIR measurements

The FTIR spectra was obtained using a Mattson Genesis (USA) spectrophotometer. This method allows observation of the structural changes that occurred as a results of photochemical reactions. The functional groups in chitosan and their corresponding chemical characteristics can be observed in Table 1. All spectra were recorded at the resolution 4 cm^{-1} and 16 times scanning.

2.5. Wide angle X-ray diffraction

Wide angle X-ray diffraction was carried out at the NanoSTAR facility (Bruker AXS, Karlsruhe) at Stirling University. Thin film samples were loaded in to the sample chamber and scattering profiles were collected at a sample-to-detector distance of 4.5 cm for 2 h per exposure. The data collection procedure used followed that described in detail in Wess et al. [34]. Sample-free images were taken and the data was corrected to remove camera-dependent artefacts. Images were analysed using in-house software. The two-dimensional detector output was converted into one-dimensional profiles in preparation for analysis.

Table 1
The identification of the functional groups in FTIR spectra of chitosan [23]

Band (cm^{-1})	Group	Characteristic
3450	OH	
3360	NH	(Stretching vibration)
2920, 2880, 1430, 1320	CH_2	Symmetric or asymmetric stretching vibration attributed to pyranose ring
1730	C=O	Carbonyl group vibrations
1655	C=O in amide band	Amide I band
1560	NH–	Bending vibrations in amide group
1590	NH_2	In amine group
1380	$-CH_3$	In amide group
1150–1040	–C–O–C–	In glycosidic linkage

Principal components analysis (PCA) was carried out on the WAXS profiles to determine the nature of the changes that occur within blends either as a function of the structural characteristics of a blend or as a function of ultraviolet radiation on the blends. The mathematical basis for PCA is well documented [35,36]; PCA is a technique that is capable of analysing the nature and level of variance in a data set, and can detect underlying trends present in data.

3. Results and discussion

3.1. UV-Vis spectrophotometry

The absorption spectra of a solution of acid soluble type I rat tail tendon collagen in acetic acid reveals absorption in the 250–280 nm region. The UV-Vis spectra which characterise the collagen solution was significantly altered after UV irradiation (Fig. 1); irradiation of the collagen solution at a wavelength of 254 nm leads to a minor increase in overall absorption, most notably between 240 and 300 nm. After irradiation the peak at 275 nm is less well pronounced, becoming more shoulder-like with increasing irradiation time.

This may be an effect of increasing of turbidity in irradiated solution, as the UV irradiation causes the changes in conformation of the collagen molecule (helix-coil transition). The increased presence of a coil structure and the progressive loss of the helical character of collagen bring about an overall increase in the scattering level of the sample [11–14]. After 1 h and 2 h of UV irradiation the maximum of absorption/scattering is not further altered.

The absorption spectra of chitosan are shown in Fig. 2. After UV irradiation the absorption spectrum was altered with the development of a distinct absorption band at 300 nm being formed, and the loss of a shoulder at 250 nm. The absorption of irradiated samples was consistently lower than that of the control samples. The chromophores responsible for the absorption band at 250 nm are apparently destroyed by UV irradiation and new chromophores are generated that absorb at approximately 300 nm wavelength. A study by Andrady et al. [37] suggested that the increase of absorbance is due to an increase in the carbonyl and amino groups in chitosan after irradiation.

For collagen–chitosan blends (ratio 50:50), the change in absorption/scattering occurs after a shorter irradiation time than for collagen alone (Fig. 3). With UV irradiation, there is a large increase in the absorbance level of the samples; greater than that of pure collagen or a theoretical blend based on weighted collagen and chitosan absorbance profiles. The

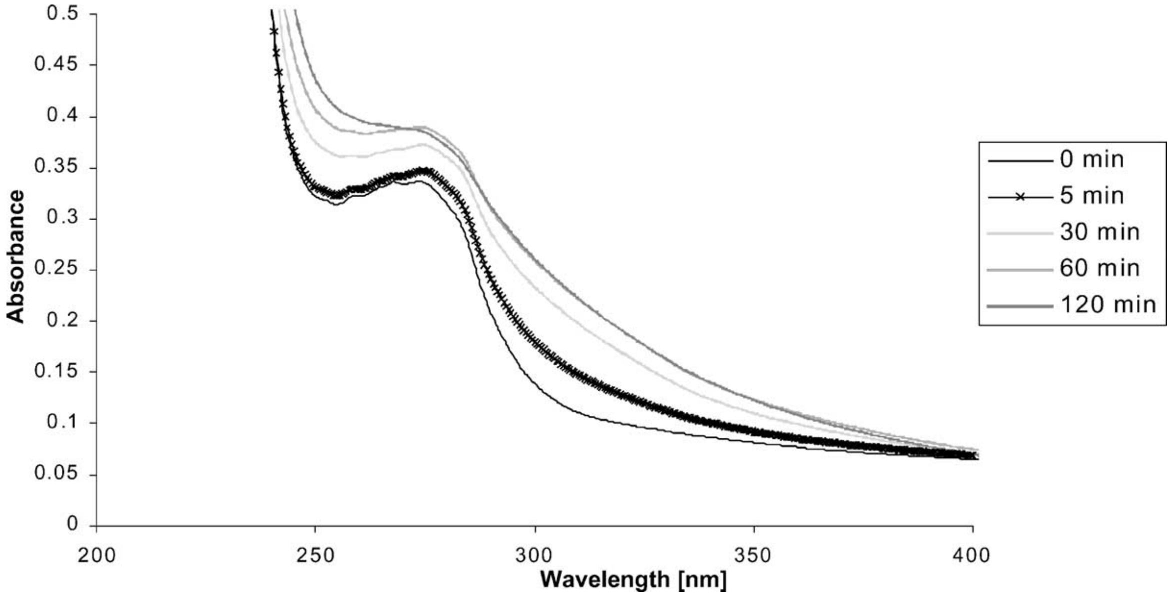


Fig. 1. UV-Vis spectra of collagen before and after UV irradiation (key to time on chart). The features of the chart change with time: after irradiation the peak at 275 nm is less well pronounced, becoming more shoulder-like with increasing irradiation time.

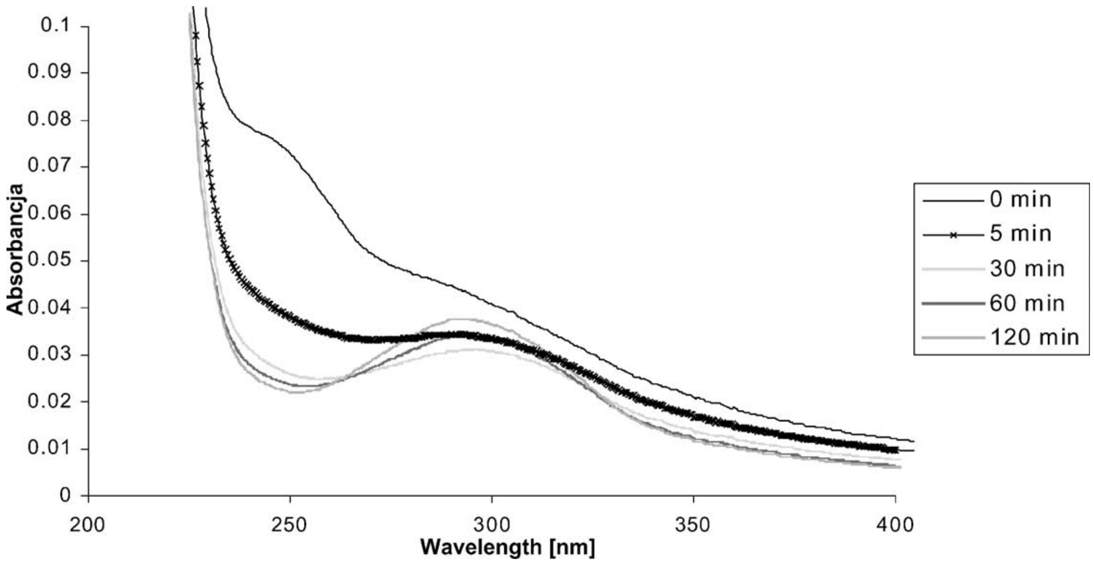


Fig. 2. UV-Vis spectra of chitosan before and after UV irradiation (key shown on chart). With increased irradiation time, the shoulder-like peak at 250 nm disappears and is replaced with a peak at 300 nm.

difference between absorption/scattering after 1 and 2 h irradiation is greater than in pure collagen for the blends with other compositions as well (Fig. 4). As the level of chitosan increases in the sample, and the level of collagen decreases, the absorbance at 275 nm increases also. In addition, the pure collagen sample shows no further increase in absorbance between 60 and 120 min, whereas the blended samples show an increase in absorbance at this time period.

This suggests that chitosan decreases the photochemical stability of this protein and accelerates the processes which lead to the increase of the absorption i.e. photo-degradation (with scission of bonds in the main chains) or photo-transformation (with changes in the conformation of collagen molecules). Moreover, the increase of absorption for collagen-chitosan blends with UV irradiation time is due to the new absorption band which is formed in irradiated chitosan (see Fig. 2).

3.2. Relative viscosity measurements

On UV irradiation with 254 nm wavelength the relative viscosity (η_{rel}) of collagen decreases rapidly, and then remains at a stable low level at 1.1 without any changes with further irradiation (Fig. 5).

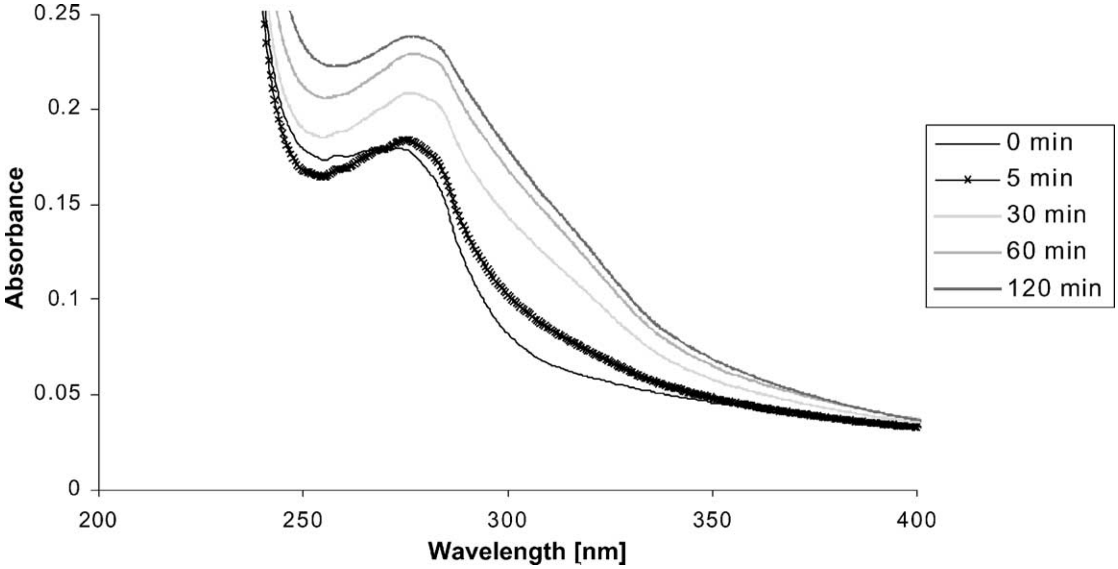


Fig. 3. UV-Vis spectra of a collagen–chitosan blend (50:50 proportions of each). Upon irradiation, there is a large increase in the absorbance level of the samples; greater than that of pure collagen.

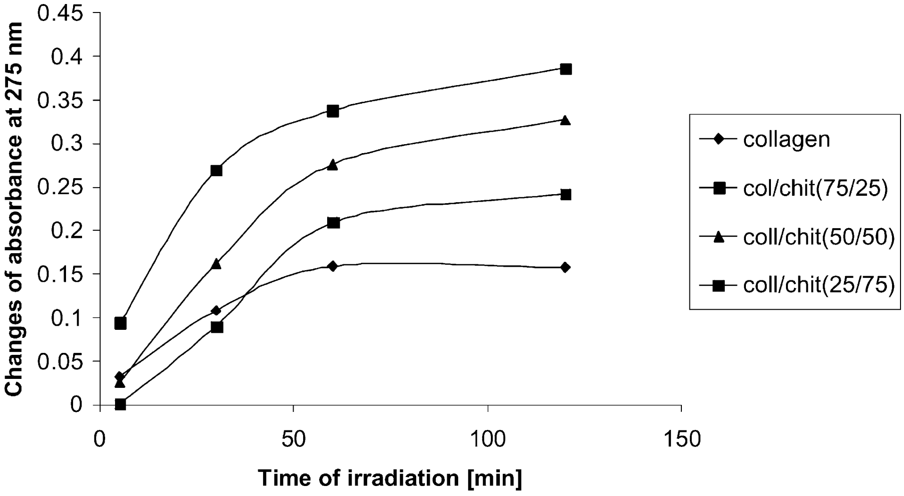


Fig. 4. Changes in the level of absorbance of samples at 275 nm ($A_t - A_0/A_0$) upon irradiation. As the level of chitosan increases in the sample, and the level of collagen decreases, the absorbance at this wavelength increases also. In addition, the pure collagen sample shows no further increase in absorbance between 60 and 120 min, whereas the blended samples show an increase in absorbance at this time period.

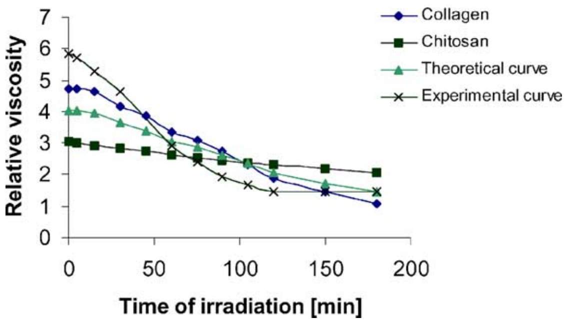


Fig. 5. Relative viscosity for collagen–chitosan blend (60/40). The theoretical curve in the graph is the curve calculated by the appropriately scaled addition of collagen (0.6) and chitosan (0.4) in a theoretical blend. The experimantal results are markedly different from the theoretical blend in that the initial relative viscosity is higher even than that of pure collagen, and shows a sharper decline than the collagen, chitosan or theoretical blend.

In our previous study [27], we have shown that the viscosity of the blend of collagen–chitosan is much higher than the viscosity of single components. Our explanation of the changes in viscosity is through a triphasic system where collagen–chitosan blends contain a third phase, possibly a pre-gelatinous state. The third component was responsible for the enhanced viscosity of blends with an optimum at around 50% collagen and 50% chitosan (by weight). The experimental relative viscosity for collagen–chitosan blends was higher than viscosity of the blends predicted in theoretical calculations (5.8 and 4.1, respectively, for the blend 60% collagen and 40% chitosan, as shown in Fig. 5). After UV irradiation of collagen the relative viscosity decrease rapidly and reaches the lowest value 1.1 without any changes with further irradiation. The relative viscosity of chitosan decreases much slower with UV irradiation time than viscosity of collagen solution.

After UV irradiation of the collagen–chitosan blends the stable low level is reached earlier than for pure collagen (Figs. 5 and 6). For pure collagen the stable level was reached

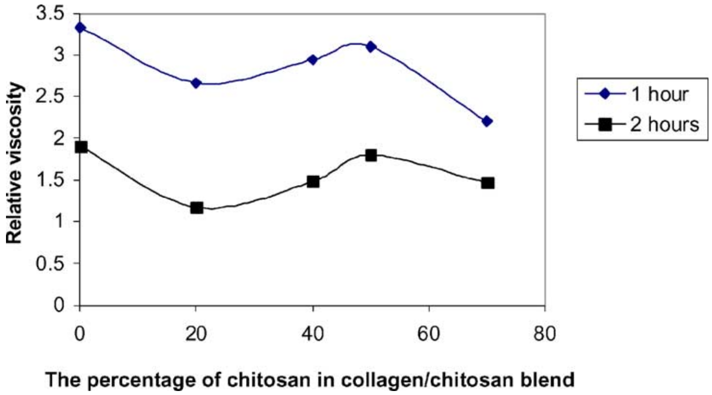


Fig. 6. The relative viscosity of collagen–chitosan blends after irradiation at 1 and 2 h. There is a sharp drop in the viscosity of all samples between 1 and 2 h irradiation time.

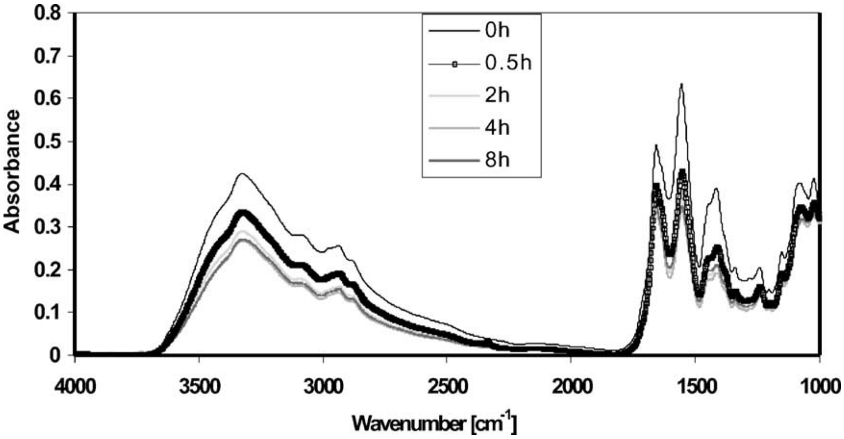


Fig. 7. FTIR spectra of a collagen–chitosan film (60/40) before and after UV irradiation. The amide A, B, I and II bands appear shifted to lower wave numbers after UV irradiation.

after 3 h of UV irradiation, whereas for collagen–chitosan blends it was reached after 2 h of UV irradiation. When the blend contain 80% of collagen and 20% of chitosan the relative viscosity is 1.2 after 2 h of UV light, whereas for pure collagen such low viscosity is obtained after 3 h of UV light. When the blend contains 30% of collagen and 70% of chitosan the relative viscosity is 1.5 after 1 h of UV irradiation, whereas for pure collagen after 1 h is about 2. These results suggests that the third phase in the blends is very sensitive to UV irradiation. It is attractive to speculate that after UV irradiation this third phase was decomposed and again the viscosity was very low. The viscosity results may suggest again that collagen–chitosan blends in solution are less stable photochemically than collagen alone.

3.3. FTIR measurements

Table 2
The position of amide A, amide I and amide II bands in collagen–chitosan blends (50/50) after UV irradiation

Amide band	Position (cm^{-1})				
	0[a]	0.5[a]	2[a]	4[a]	8[a]
A	3326	3326	3325	3323	3320
I	1658	1658	1658	1658	1658
II	1556	1556	1556	1556	1556

[a] After time of UV irradiation in hours.

The photochemical stability of collagen–chitosan films was studied using the FTIR spectroscopy. Infrared spectra of proteins and synthetic polypeptides indicate a number of bands attributed to the peptide bond. They may be useful for further characterization of the bound water molecules and hydrogen bonds in collagen. Their position are sensitive to changes of the conformation of the molecules. The amide A band of collagen (associated with the NH-stretching frequency) is usually found at 3325–3330 cm^{-1}. The amide B band of collagen is usually found at 3080 cm^{-1}. The amide I band of collagen is usually found at 1650–1668 cm^{-1}. The amide II band of collagen is centered in range

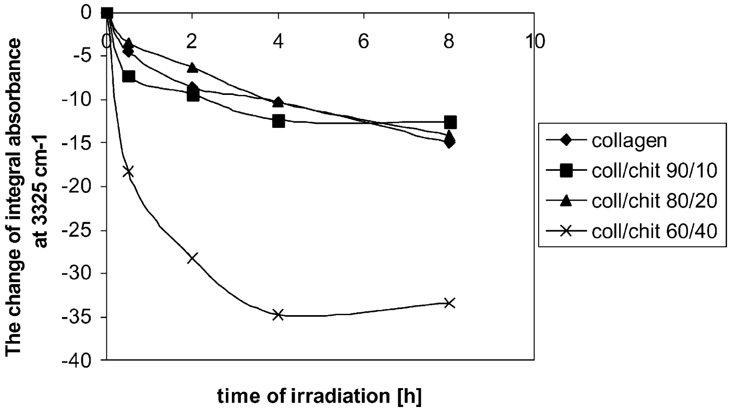


Fig. 8. The integral absorbance of the Amide A band of collagen and a selection of the blends. With increased UV irradiation time the integral absorbance of the peak decreases. At high levels of collagen in the blend there is little variation in the drop on the absorbance of the peak; however as the proportion of chitosan increases and the proportion of collagen decreases in the blend the drop in the absorbance becomes more pronounced.

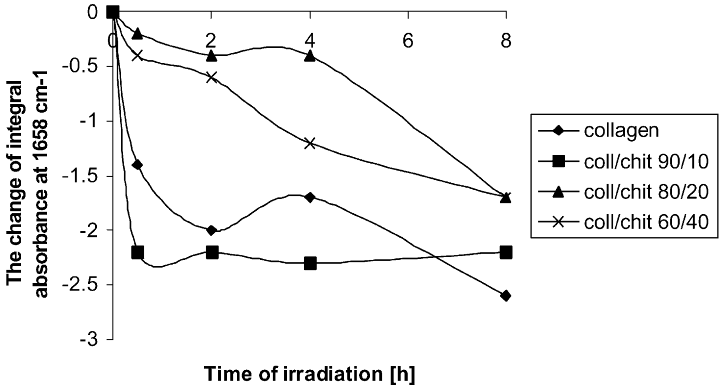


Fig. 9. The change in absorbance of the amide I peak of collagen after irradiation. The drop in the integral absorbance is more pronounced in samples with very high collagen content; samples with less collagen and more chitosan as components show a reduced change in this peak.

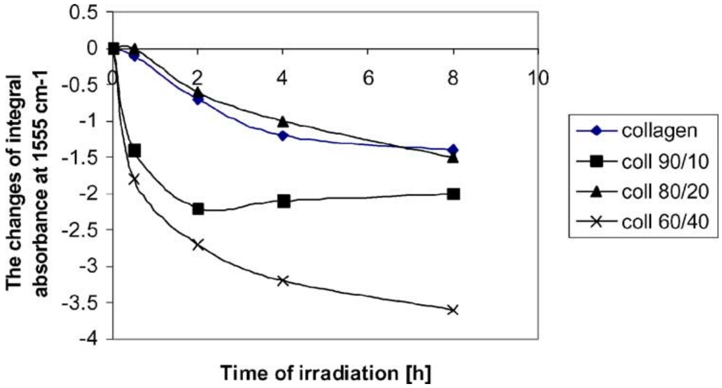


Fig. 10. The changes of integral absorbance of amide II band at 1555 cm^{-1} after UV irradiation of collagen and collagen–chitosan blends.

1530–1555 cm^{-1}, and has a complex nature with its vibrational source in both the NH deformation and CN stretching modes. In our sample the amide bands A are shifted after UV irradiation to lower wave numbers, but amide I and II are not altered (Fig. 7, Table 2). These shifts in collagen–chitosan blends are smaller whereas in collagen films with low concentration of polysaccharide they are greater. These shifts are possible due to the scission of hydrogen bonds on exposure to UV irradiation leading to a reduction in their numbers—hydrogen bonds are necessary to maintain the helical structure of collagen [38–40]. With increasing time of irradiation the more distinct changes are observed within the characteristic functional groups.

For collagen–chitosan blends the amide A band is in the same position as the OH group band in chitosan. For the blend with composition 60% of collagen and 40% of chitosan, we observe a noticable decrease in absorbance of this complex band. This suggests that UV irradiation has significantly altered both the collagen and chitosan in the blend.

The integral absorbance of the amide A band in collagen decreases with UV irradiation time (Fig. 8). At high levels of collagen in the blend there is little variation in the drop on the absorbance of the peak; however as the proportion of chitosan increases and the proportion of collagen decreases in the blend the drop in the absorbance becomes more pronounced.

The integral absorbance for the amide I and amide II bands at 1658 and 1555 cm^{-1}, respectively, decreases rapidly after UV irradiation of collagen–chitosan blends (Figs. 9 and 10). The drop in the integral absorbance is more pronounced in samples with very high collagen content; samples with less collagen and more chitosan as components show a reduced change in this peak. This may suggest that collagen–chitosan blended films are less stable photochemically than pure collagen films. Using FTIR method we could observe the changes of integral absorbance of amide bands after 30 min of UV irradiation, whereas using viscosimetry methods and UV-Vis spectra for solution we could observe the changes after 5 min of UV irradiation. We can conclude from this that the photo-degradation of collagen–chitosan blend in solution occurs faster than the photo-degradation of collagen–chitosan films.

3.4. Wide angle X-ray diffraction

Wide angle X-ray diffraction indicates that in the collagen films characteristic peaks at 12 Å and 2.86 Å, which represent lateral packing of the collagen molecules and the rise

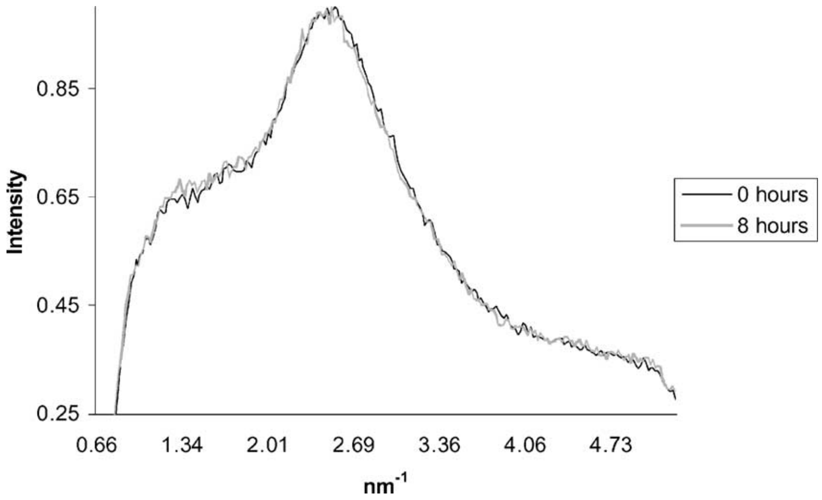


Fig. 11. Wide angle X-ray diffraction normalised linear profiles from a collagen–chitosan blend (50:50 proportions) after 0 and 8 h of UV irradiation. There are no discernible peaks in the profile due to the presence of either collagen or chitosan. The main feature is the 4.5 Å peak that is present in all protein samples.

per residue in the helix, respectively, are present both before and after irradiation. This indicates that the collagen film is more resistant to the helix-coil transition induced by the UV light than collagen in solution. The presence of chitosan in a collagen–chitosan blend has the effect of removing the structural characteristics of collagen [27]. In blends where the relative proportions of collagen and chitosan range from 7:3 to 3:7 no diffraction features from either collagen or chitosan are present (Fig. 11)—UV irradiation therefor has no effect on the collagen peaks in these blends. In pure chitosan films, the characteristic 8.5 Å peak becomes less pronounced in the diffraction profile upon UV irradiation, but remains as a significant feature.

Principal components analysis of the wide angle X-ray diffraction data indicates that the main characteristic of collagen–chitosan blends before and after UV irradiation is dominated by the relative proportions of collagen and chitosan in the blend. Fig. 12 shows the coefficients from PCA from samples ranging from pure collagen films to pure chitosan films after UV irradiation of 0 (reference), 2, 4 and 8 h exposure. The main separation of the data is due to the sample composition as shown by the clustering of the samples in the chart.

3.5. Discussion: mechanisms of photo-degradation

It is generally known that tyrosine and phenylalanine are sensitive chromophores which absorb light below 300 nm and may initiate the photo-degradation of collagen chains and photo-destruction via the cleavage of side groups. On the

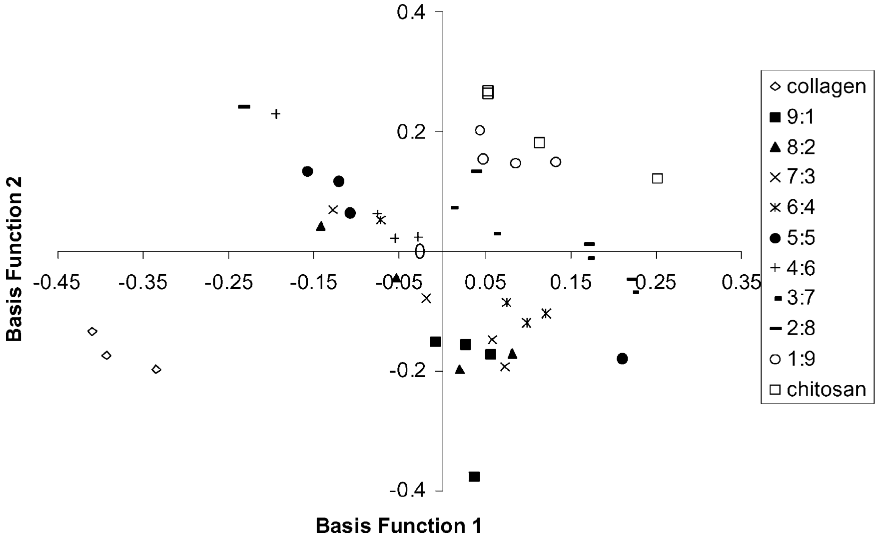
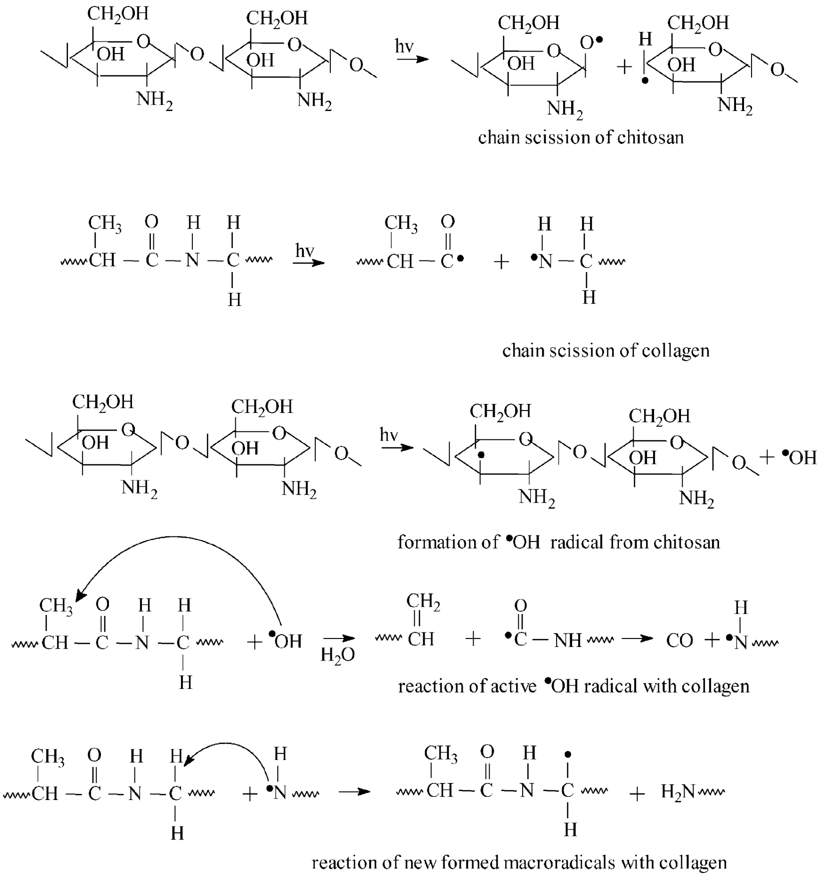


Fig. 12. Coefficients to basis functions 1 and 2 of collagen, chitosan and blend films after 0, 2, 4 and 8 h of UV irradiation from PCA of wide angle X-ray diffraction data. As the proportion of collagen decreases in the samples and the level of chitosan increases, the coefficients for both basis functions change from negative to positive, indicating that the nature of the initial blend is the primary feature in all samples.



Scheme 1. Possible mechanisms of transformations in collagen–chitosan blend induced by UV irradiation.

basis of studies on the viscosity of diluted solutions, UV-Vis spectrophotometry and FTIR spectra we can comment on the photo-degradation of collagen–chitosan blends. A decrease of integral absorbance in the FTIR range is related to scission of collagen and chitosan chains into smaller fragments. This is confirmed by the measurements of viscosity of diluted collagen-chitosan solutions. Collagen and chitosan radicals formed during UV exposure may interact with each other and alter the properties of collagen–chitosan blends (Scheme 1). In particular, the very active •OH radicals derived from irradiated chitosan can interact with the collagen macromolecules and produce new radicals and macroradicals. In chitosan macromolecules there are many OH groups, so many active •OH radicals can be formed.

4. Conclusion

The influence of chitosan on the photochemical stability of collagen depends on the concentration of this polysaccharide in the biopolymer blend. Generally collagen–chitosan blends in solution are less photochemically stable than a pure collagen solution, as has been demonstrated by viscosimetry and UV-Vis spectrophotometry. The FTIR spectra showed that collagen–chitosan films are less photochemically stable than pure collagen films, although a greater length of irradiation time is required to bring about changes to the characteristics of the blends compared to blends in solution. X-ray diffraction confirms that the structural characteristics of collagen and collagen–chitosan blends in film form are not drastically altered after UV irradiation.

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