Effect of UV Irradiation on the Physicochemical Properties of Collagen Stabilized Using Aldehydes

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ABSTRACT: Collagen is an important biomaterial, finding immense application in the field of wound healing. In this study, effect of UV irradiation on aldehydes crosslinked collagen has been carried out. Aldehydes find predominant application in crosslinking of collagen for various end uses. The physical and optical properties of aldehydes crosslinked collagen affected by UV irradiation have been detailed. Viscosity measurements have shown that aldehydes crosslinked collagen has better stability against UV radiation than native collagen. Circular dichroic studies showed that prolonged exposure to UV radiation changes the triple helical structure of collagen into random coil conformation. The difference spectra for both emission and absorption show that formaldehyde brings about more stability to collagen against UV irradiation than gluteraldehyde. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 3642–3648, 2007

Key words: collagen; UV radiation; conformational analysis; viscosity; fluorescence; absorption

INTRODUCTION

Collagen is an important connective tissue protein. The collagen family of proteins constitute 25% of the total protein mass of the body. In addition to being of great physiological importance, collagen is also the basic raw material for many biological and industrial applications. The native tropocollagen molecule (molecular mass 300 kDa) consists of three polypeptide chains of about 1000 amino acid residues each, wound around one another to form a triple helix. The stability of the triple helix in collagen depends on inter- and intramolecular hydrogen bonds.[1,2] The problem of effect of UV radiations on collagen has evoked interest owing to the use of radiations in medicine, industry, and research. Effect of UV radiation on collagen has been studied because of the negative effects of UV radiation on skin, which predominantly contains collagen.[3]

It has been reported that the process of UV irradiation can induce crosslinks into collagen fibrils. Molecular scission through free radical mechanisms has also been reported.[4,5] UV radiation has been shown to induce both chemical and physical changes in collagen. The thermal helix-coil transition of UV irradiated collagen in rat tail tendon has been investigated by differential scanning calorimetry.[6] The aromatic groups, phenyl alanine and tyrosine, of collagen have been found to be affected because of UV irradiation.[7] Fujimori[8] has shown that collagen underwent photopolymerization under irradiation and that this took place at the telopeptide regions of the molecule. An increase in the intensity of fluorescence spectra with increasing radiation dosage has been observed earlier.[9] Recently, Sionkowska et al.[10–12] have carried out extensive work on the effect of UV radiation on collagen.

Studies on the effect of UV irradiation on stabilized collagen are important in that stabilized collagen has wide application in various fields including cosmetics, wound healing, drug delivery, etc. In applications where increased thermal and enzymatic stability is required, collagen is treated with small molecules like aldehydes. Gluteraldehyde-treated collagen is widely used in wound healing application.[13] The effect of gluteraldehyde crosslinking on the dielectric behavior of collagen has been studied recently.[14] This article records the experiments that investigate the effect of UV irradiation on the physicochemical properties of collagen treated with aldehydes. Two aldehydes, both a mono- and a dialdehyde viz., formaldehyde (monoaldehyde) and gluteraldehyde (dialdehyde), have been chosen for this study.

EXPERIMENTAL

Collagen solutions

Collagen solutions were prepared from tendons freshly dissected from 6-month-old male albino rat

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tails frozen at −20°C by acetic acid extraction and salting out with NaCl.[15] The purity of collagen preparation was confirmed by SDS–polyacrylamide gel electrophoresis. The collagen concentration in the solutions was determined from the hydroxyproline content.[16] The average molecular weight of collagen is 300,000 Da,[17] based on which the molar concentration was determined.

Ultraviolet irradiation

Solutions were irradiated under air at room temperature using a quantum yield photoreactor (model 2001, Applied photophysics, London), with 250 W medium pressure mercury lamp, which emits light mainly at a wavelength of 330 nm. Irradiation experiments were carried out in a quartz cuvette at a distance of 20 cm from the light source for various time intervals. All measurements were performed in the same conditions of temperature and humidity to avoid any influence on the physicochemical properties of collagen.

Viscosity studies

Viscosity measurements were performed using an Ostwald type viscometer (10 mm diameter) of 2 mL capacity. The viscometer was thermostated at (25 ± 1)°C. The flow times of collagen samples were measured after a thermal equilibrium time of 30 min. The collagen concentration ($0.84 \times 10^{-6} M$) was fixed, and the flow time measurements were carried out in the presence of varying amounts of aldehydes (0.84×10^{-6} to $63 \times 10^{-6} M$) before and after irradiation. The viscosity measurement was based on the flow rate of collagen solution through the capillary of an Ostwald viscometer. In these experiments, the viscosity contribution (η) due to collagen was measured as a function of the concentration of added aldehydes. The flow time was measured with a digital stopwatch at least three times and the average was taken. The viscosity was calculated from the relation, $\eta = t - t^o/t^o$, where t^o is the flow time of buffer (pH 4.2, ionic strength = $2 \times 10^{-2} M$) and t is the flow time for each sample. Plots of relative viscosity, η/η_o (η and η_o are the viscosity of collagen in the presence and absence of aldehyde) versus [aldehyde]/[collagen] were constructed.

Circular dichroic studies

CD spectra were measured using Jasco 715 Circular Dichroism spectropolarimeter using a quartz cell with a light path of 1 mm at 25°C, with three scans for each sample. CD spectra were recorded in the far UV region (190–250 nm), under nitrogen, to estimate the conformational changes of native and aldehyde-treated collagen samples before and after irradiation. For studying the effect of increasing concentration of aldehydes on the conformation of irradiated collagen, aqueous solution of collagen ($0.6 \times 10^{-6} M$) in acetate buffer (pH 4.0, $I = 2 \times 10^{-2} M$) was incubated with aldehydes, namely formaldehyde and gluteraldehyde (0.6×10^{-6} to $60 \times 10^{-6} M$) solution for 18 h at 25°C. To study the effect of duration of irradiation on the conformation of collagen, an aqueous solution of collagen ($0.84 \times 10^{-6} M$) in acetate buffer (pH 4.0, $I = 2 \times 10^{-2} M$) was incubated with aldehyde ($63 \times 10^{-6} M$) solution in the ratio of 1 : 75 for 18 h at 25°C. The duration of irradiation was varied from 0–2 h.

Fluorescence studies

The emission spectra for native and aldehyde-treated collagen solution before and after irradiation were recorded using Cary Eclipse Fluorescence Spectrophotometer from VARIAN. The solution was excited at 270 nm and emission was measured at 290 nm. The concentration of collagen used was 0.84 μM. The ratio of collagen:aldehyde (formaldehyde and gluteraldehyde) was maintained at 1 : 75.

UV–visible spectral studies

The UV absorption spectra for native and aldehyde-treated collagen solution before and after irradiation were recorded using Lambda 35 spectrophotometer. The concentration of collagen used was 0.84 μM. The ratio of collagen:aldehyde (formaldehyde and gluteraldehyde) was maintained at 1 : 75.

RESULTS

Effect of UV irradiation on viscosity

To study the effect of UV radiation on viscosity of native and aldehyde-treated collagen, viscosity measurements were carried out using Ostwald viscometer. The viscosity for native collagen before irradiation was 2.002 and the same after 15, 30, 45, and 60 min of irradiation was 1.688, 1.421, 1.1765, and 0.956, respectively. The concentration of collagen was kept constant and influence of increasing concentration of aldehydes before and after irradiation was studied. Figure 1(a,b) shows the η/η_o Versus [aldehyde]/[collagen] plot for formaldehyde- and gluteraldehyde-treated collagen, respectively. It is evident that the viscosity of both native and aldehyde crosslinked collagen solution decreases on increasing the time of radiation. It is observed that there is an increase in viscosity for ratio 1 : 40 after 30 min radiation. This could be because of the reason that after prolonged radiation when the concentration of aldehydes is also high, the UV radiation

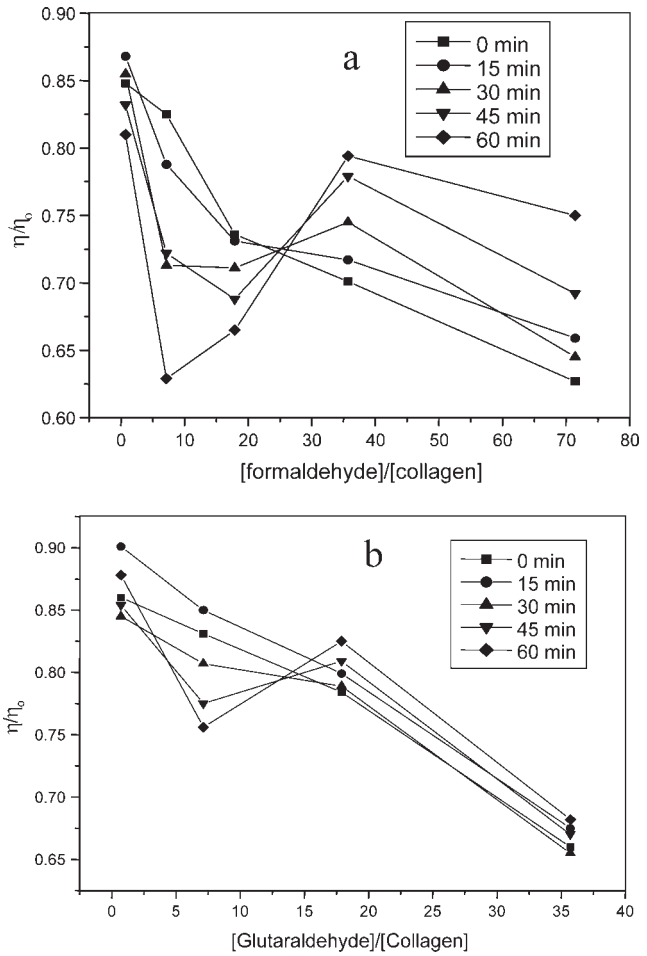

Figure 1 A plot of relative viscosity (η/η_o) against $1/R$ (η and η_o are the viscosity of collagen in the presence of (a) formaldehyde and (b) gluteraldehyde before and after irradiation. R = [collagen]/[aldehyde]).

starts affecting the unbound aldehydes and generates free radicals. These free radicals in turn form additional crosslinks, which leads to increase in viscosity. But, further irradiation causes decrease in viscosity owing to the fact that the free radicals now cause molecular scission, which is breaking the intermolecular bonds. The percentage change in viscosity for formaldehyde- and gluteraldehyde-treated collagen (in ratio 1 : 75) after 1 h irradiation is 42.91% and 50.68%, respectively, whereas the percentage change in viscosity for native collagen after 1 h irradiation is 52.24%.

Effect of UV irradiation on conformation

To investigate whether UV radiation brings about any alterations in the conformation of collagen, CD spectral studies on collagen–aldehyde system were carried out. In the far UV region, collagen exhibits a minimum at 197 nm and a maximum at 220 nm with a crossover point at about 210 nm. The maximum at 220 nm in CD spectrum of native collagen solution is characteristic of triple folded helix.[18] The CD spectra of collagen in the presence of increasing concentrations of formaldehyde and gluteraldehyde after irradiation for 30 min are shown in Figure 2(a,b). The parameter R_{pn} denotes the ratio of positive peak intensity over negative peak intensity. It is a characteristic ratio for the triple helical conformation of collagen and collagen-like peptide.[19,20] The R_{pn} values for native collagen and collagen crosslinked with aldehyde before and after UV radiation are given in Table I.

To study the effect of duration of UV irradiation on the conformation of collagen, CD spectral studies varying the time of irradiation were carried out. The CD spectra of collagen in the absence and presence of formaldehyde and gluteraldehyde after irradiation for 15 min, 30 min, 1 h, and 2 h are shown in Figure 3(a–c), respectively. The R_{pn} values for native collagen solution and collagen solution treated with

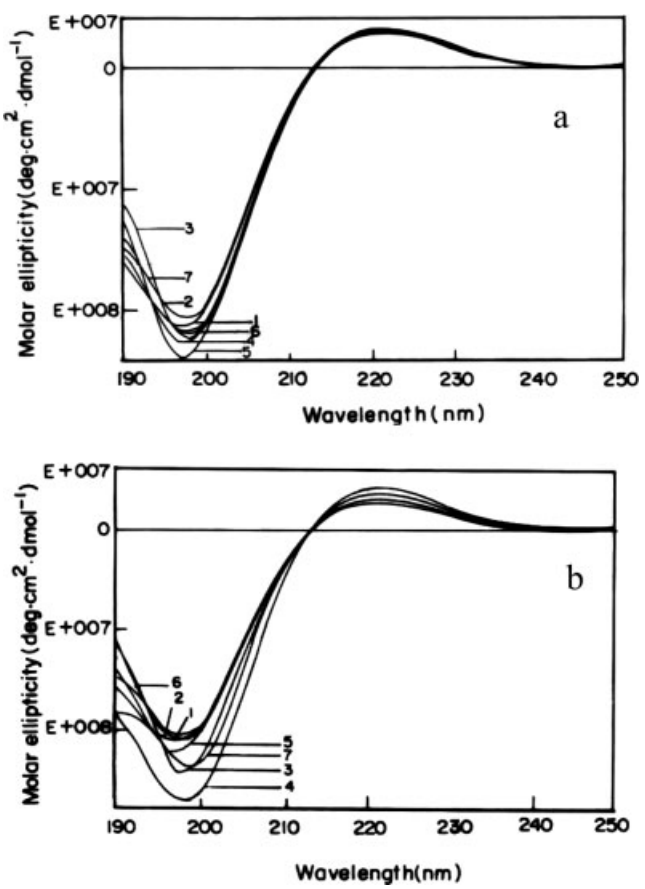

Figure 2 Far-UV CD spectra of collagen (0.6 μM) in the presence of varying concentration of (a) formaldehyde and (b) gluteraldehyde (0.6–60 μM) after irradiating for 30 min (1: collagen nonradiated; 2: collagen irradiated; and 3–7: 0.6–60 μM aldehyde).

TABLE I
R_{pn} Ratio of Native Collagen and Aldehyde-Treated Collagen with Increasing Concentration of Aldehyde Before and After 30 min Irradiation[a]

Concentration of aldehydes (μ*M*)	R_{pn} (characteristic ratio)			
	Formaldehyde		Gluteraldehyde	
	0 min	30 min	0 min	30 min
1	0.139	0.140	0.127	0.139
10	0.121	0.125	0.128	0.158
25	0.128	0.122	0.132	0.129
50	0.141	0.140	0.135	0.131
100	0.143	0.141	0.137	0.135

[a] R_{pn} value for native collagen before and after irradiation is 0.135 and 0.134, respectively.

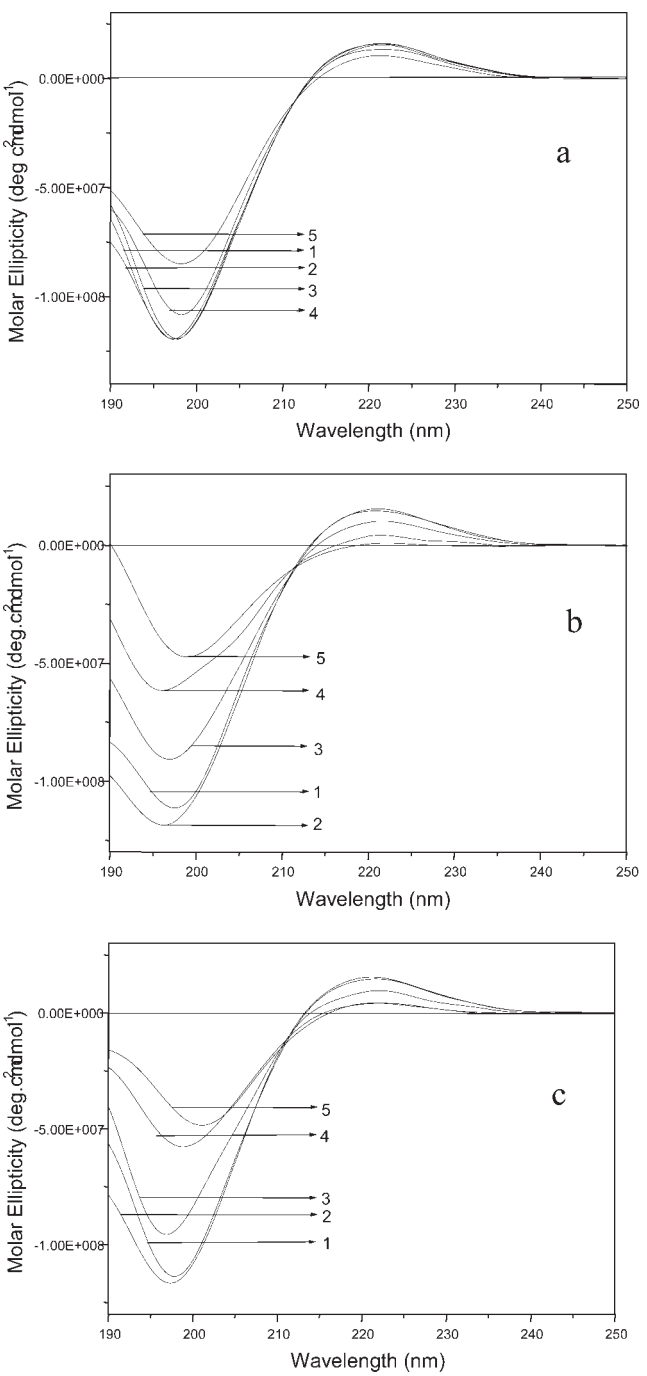

Figure 3 Far-UV CD spectra of (a) native collagen (0.84 μ*M*), (b) formaldehyde, and (c) gluteraldehyde (1 : 75) irradiated for various time intervals. (1: 0 min; 2: 15 min; 3: 30 min; 4: 1 h; and 5: 2 h).

formaldehyde and gluteraldehyde before and after UV radiation are given in Table II. The R_{pn} value decreases with increase in period of irradiation for native collagen. The same is observed for aldehydes-treated collagen.

Effect of UV irradiation on fluorescence

Since the CD studies showed that there is change in the conformation of collagen on UV irradiation, optical measurements were carried out to find out if the aromatic amino acids are involved as UV absorbing centers. The fluorescence emission spectra of collagen and collagen in the presence of formaldehyde and gluteraldehyde before and after irradiation are shown in Figure 4(a–c), respectively. From the figure, it is observed that with increasing the time of irradiation, there is a gradual decrease in the emission maxima at 300 nm when the excitation wavelength is 270 nm. The difference emission spectra for native and aldehydes crosslinked collagen are shown in Figure 5.

Effect of UV irradiation on electronic absorption

The electronic absorption spectra for collagen and collagen in the presence of formaldehyde and gluteraldehyde before and after irradiation are given in Figure 6(a–c), respectively. There is a peak centered at around 275 nm, which is characteristic of tyrosine. The intensity of the peak is found to increase on increasing the time of irradiation. The difference absorption spectra for native and aldehyde crosslinked collagen are shown in Figure 7. The peak at around 275 nm is found to be less intense in the case of formaldehyde-treated collagen. Gluteralde-

TABLE II
R_{pn} Ratio of Native Collagen (0.84 μ*M*) and Aldehyde-Treated Collagen (1 : 75) After Various Durations of UV Irradiation

Time of irradiation (min)	R_{pn} ratio (characteristic ratio)		
	Collagen	Gluteraldehyde	Formaldehyde
0	0.134	0.129	0.130
15	0.131	0.131	0.132
30	0.129	0.100	0.112
60	0.124	0.071	0.069
120	0.122	0.100	1.7234×10^{-2}

hyde-treated collagen shows more difference than native collagen.

DISCUSSION

To probe if aldehyde crosslinking imparts stability to collagen against UV irradiation, various physicochemical studies have been carried out. The viscosity measurements indicate that both native and aldehydes-treated collagen undergo decrease in viscosity on UV irradiation. However, native collagen undergoes more change in viscosity when compared with aldehydes-treated collagen. This shows the stabilizing effect of aldehydes against UV irradiation. It has been shown earlier that aldehydes bring about long range ordering of collagen, which leads to increase in thermal and enzymatic stability of collagen.[21]

It can be seen from the CD studies (Fig. 2) that there are no major alterations in the conformation of aldehydes crosslinked collagen on UV irradiation with increasing concentration of aldehydes. UV irradiation has caused only changes in the amplitude of the spectra and no further modifications are observed. This also indicates that the changes in the CD spectra of collagen in the presence of aldehydes and after UV irradiation is not due to loss of triple helicity because it is known that collagen on complete denaturation undergoes drastic changes, like the disappearance of the positive peak and red shift of the negative peak. It can be seen from Table I that the R_{pn} value for nonradiated aldehydes-treated collagen is less than that of radiated till a concentration ratio of 1 : 10, after which the trend is reversed, whereas for native collagen, the R_{pn} value is more for nonradiated when compared with the radiated. Also there is a slight red shift in the negative peak after irradiation. The effect of duration of UV irradiation on conformation of native and aldehydes crosslinked collagen was studied. It can be seen from Figure 3 that after 15 min of irradiation there is increase in the molar ellipticity of negative peak followed by decrease on further increase in time of irradiation. There is also greater change in helicity observed after 2 h of irradiation (Table II). This shows that prolonged irradiation has a pronounced effect on the conformation of collagen. After 2 h of

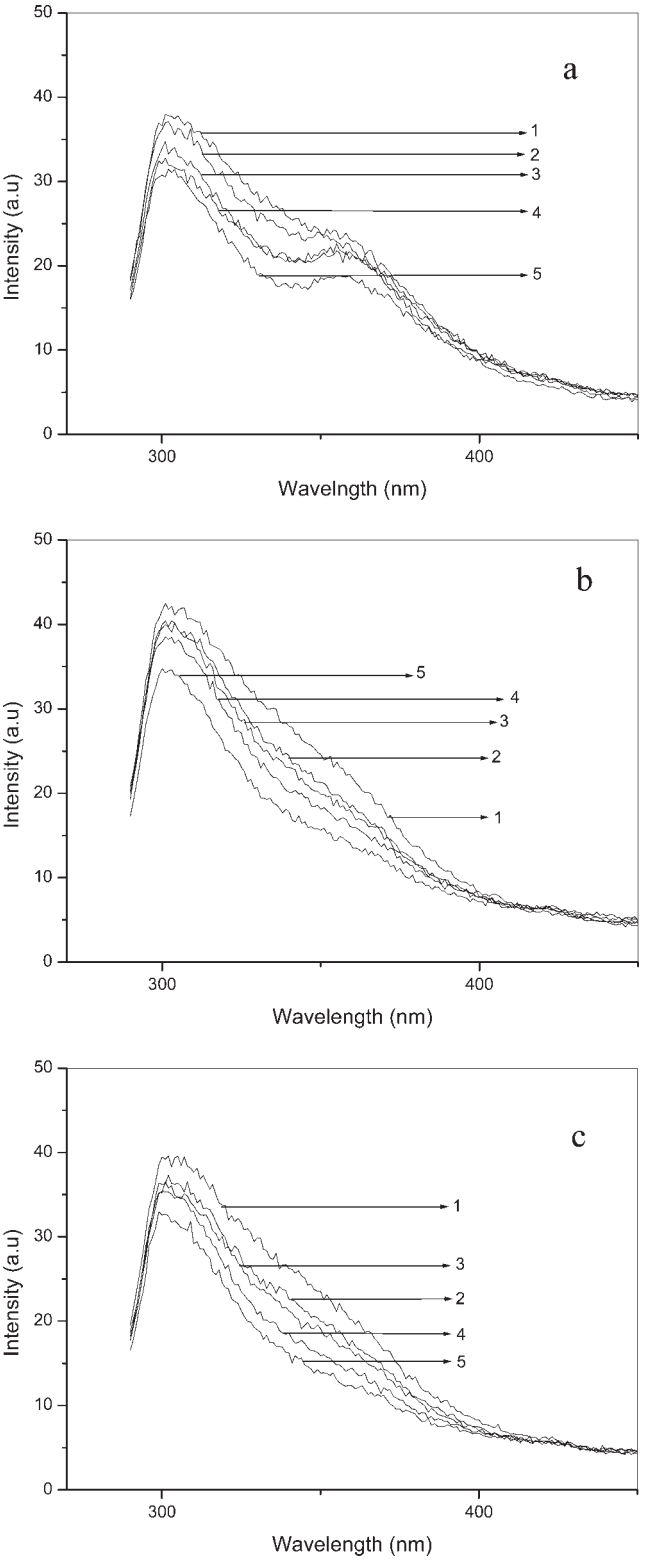

Figure 4 Fluorescence spectra of (a) collagen (0.84 μ*M*), (b) formaldehyde-, and (c) gluteraldehyde-treated collagen solution (1 : 75) on UV irradiation. (1: 0 min; 2: 30 min; 3: 1 h; and 4: 2 h irradiation).

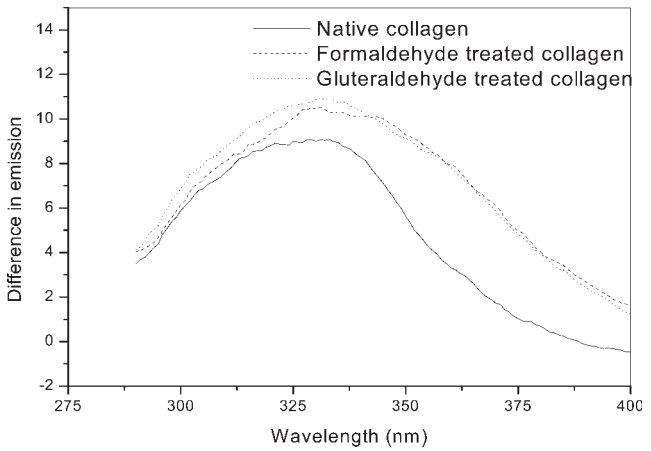

Figure 5 Different emission spectra for native and aldehyde-treated collagen after 2 h irradiation. (1: native; 2: formaldehyde-treated; and 3: gluteraldehyde-treated).

UV irradiation, formaldehyde-treated collagen undergoes denaturation as evidenced from the disappearance of the positive peak and red shift of the negative peak. The same is observed in the case of gluteraldehyde-treated collagen too.

It can be seen from Figure 4(a) that there are two emission peaks for native collagen, and that after treatment with aldehydes the peak centered at around 352 nm disappears [Fig. 4(b,c)]. The emission intensity after irradiation decreases for both native and aldehydes crosslinked collagen. But the change is less in native followed by formaldehyde and gluteraldehyde (Fig. 5). This shows that aldehyde treatment quenches the fluorescent amino acids present in collagen. The increase in absorption after irradiation is attributed to the increase in photoproducts formed due to irradiation of the aromatic amino acids, tyrosine, and phenylalanine. This shows that more UV absorbing centers are formed after irradiation as shown in Figure 6. The difference spectra shown in Figure 7, also confirm that formaldehyde-treated collagen has better stability against UV radiation than native and gluteraldehyde-treated collagen. Native collagen is more susceptible to UV irradiation when compared with formaldehyde- and gluteraldehyde-treated collagen. The crosslinking brought about by aldehydes aids in imparting this stability to collagen.

CONCLUSIONS

This study throws light on the effect of UV irradiation on aldehydes crosslinked collagen with respect to conformational and optical properties. Formaldehyde treatment brings about more stability to collagen against UV irradiation when compared with gluteraldehyde treatment. Duration of irradiation has been found to affect the conformation of collagen more. This study has wider implications, as aldehydes are the widely used crosslinking agents in many biological applications of collagen.

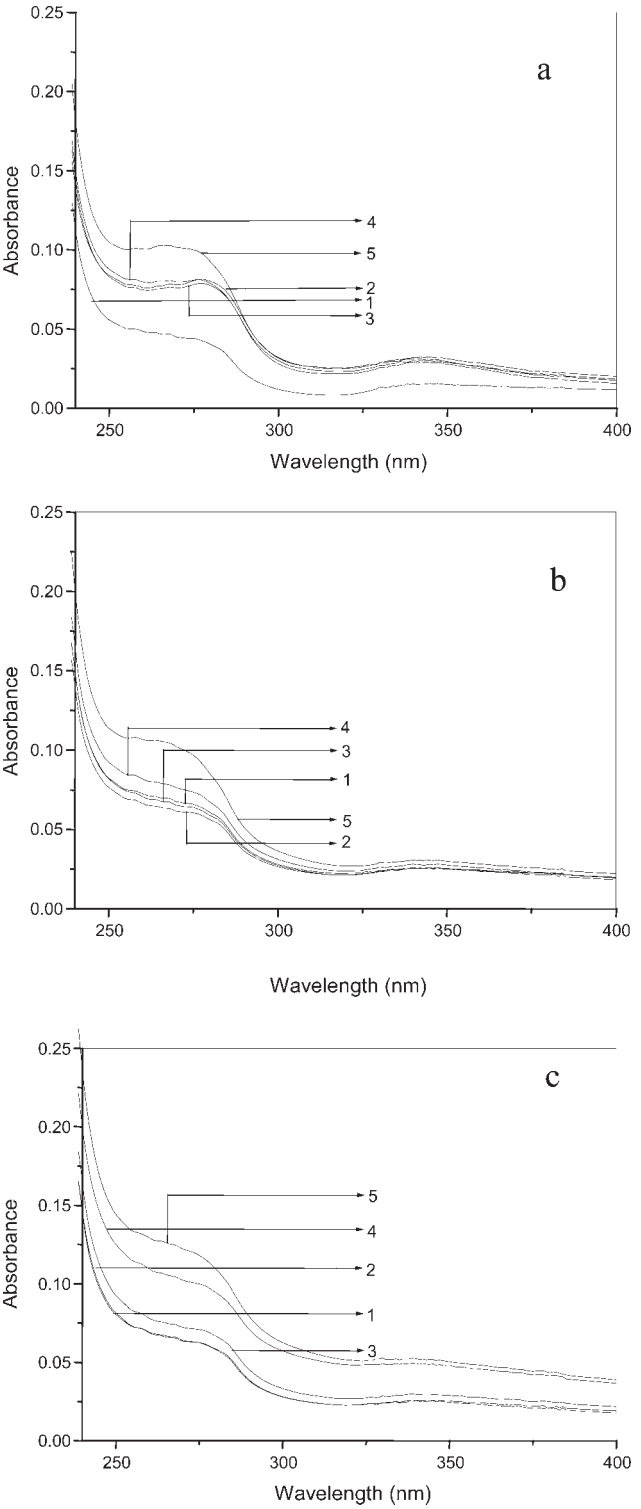

Figure 6 UV absorption spectra for (a) collagen (0.84 μ*M*), (b) formaldehyde-, and (c) gluteraldehyde-treated collagen solution (1 : 75) on UV irradiation. (1: 0 min; 2: 30 min; 3: 1 h; and 4: 2 h).

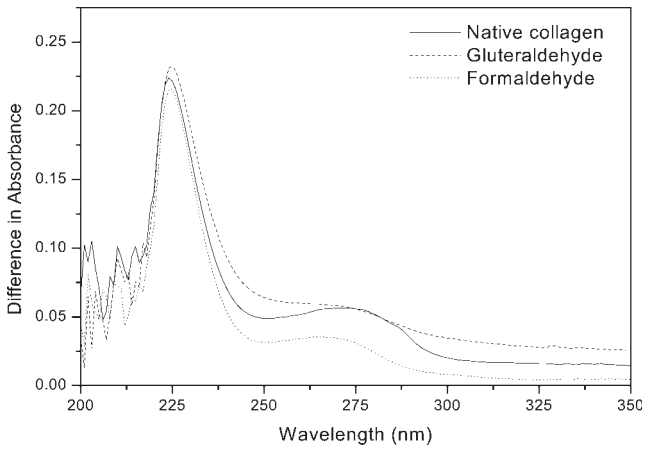

Figure 7 Different absorption spectra for native and aldehyde-treated collagen after 2 h irradiation. (1: native; 2: formaldehyde-treated; and 3: gluteraldehyde-treated).

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