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ScienceDirect
Journal of Photochemistry and Photobiology A: Chemistry 193 (2008) 187–192
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Laser induced foaming and chemical modifications of gelatine films

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Received 17 May 2007; received in revised form 14 June 2007; accepted 18 June 2007
Available online 20 June 2007

Abstract

This paper reports on the investigation of the microfoaming and chemical modifications following single-pulse laser irradiation conducted at 248 nm (KrF excimer laser, 20 ns fwhm) and at 266 and 355 nm (Nd:YAG laser 4th and 3rd harmonic, 6 ns fwhm) of gelatine films. Fluorescence emissions of the films were studied by laser induced fluorescence and spectrofluorimetry and the emission lifetimes were measured by time-correlated single photon counting. The main fluorophores responsible of the observed emissions are due to aromatic amino acids like tyrosine and derived crosslinked products, possibly formed during the laser foaming. It is observed that laser irradiation with a single ns UV pulse induces modifications on the relative intensity of emissions depending on the irradiation wavelength and type of film. It was earlier anticipated that the foaming mechanism can be viewed as a cavitation phenomenon mainly induced by the laser launched tensile wave at the film surface. Spectroscopic investigations confirm that the major accompanying chemical modifications are related with the relative increase of the fluorescence of dityrosine moieties and other products derived of the photoxidation of tyrosine. These modifications are related to the structural disruption induced by foaming and to the increase of temperature in the irradiated region. This approach opens up the possibility to gain information on the molecular structure of the new nanofoamy material, as well as on its microscopic distribution with emerging fluorescence-based confocal systems allowing high spatial resolution. The results shown have important consequences for the laser processing of biopolymers of interest in biomedical applications since the resulting laser foam exhibits properties close to those of the natural extracellular matrix.

Keywords: Gelatine; Biopolymers; Laser processing; Nanostructuring

1. Introduction

Laser-assisted modification of polymers and biopolymers is a rapidly growing field due to the advantages over other chemical and physical methods. Laser processing features high precision capability, low heat deposition onto the substrate and high level of flexibility [1,2]. Recent studies have reported on the fabrication of nanoporous and nanofibrous materials by laser irradiation of polymers and biopolymers [3–9]. Gelatine is a proteinaceous material obtained by thermal denaturation or physical and chemical degradation of collagen, the most widespread protein in the body connective tissues [10,11]. Due to its biodegradability it attracts wide interest in food, in pharmaceutical and in photographic applications. In medicine, this biomaterial is investigated as a scaffold to mimic the supramolecular structure and biological functions of the extracellular matrix (ECM) and for three-dimensional tissue regeneration and the development of artificial organs [12,13]. Gelatine presents some advantages over collagen as its physicochemical properties can be suitably modulated. For instance the thermal and mechanical properties of gelatine, of key importance in the possible applications as a biomaterial, can be enhanced by crosslinking. It is also a much cheaper material than collagen and easier to obtain in concentrated solutions.

In recent works, some of us have reported pulsed laser submicron foam formation in gelatine using single laser pulses of nanosecond (ns) and femtosecond (fs) duration [3–8]. Due to its interest in biomedical applications, it is important to ascertain whether the observed morphological modifications are accompanied by photothermal or photochemical modifications. In the

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doi:10.1016/j.jphotochem.2007.06.024

present paper, we describe the chemical modifications following irradiation of films of gelatine, differing in gel strength (bloom values 75 and 225) and in crosslinking degree, by using spectrofluorimetry and laser induced fluorescence (LIF) techniques. We also report the decay times of the observed emissions by time-correlated single photon counting (TCSPC). From these measurements it is possible to establish that fluorescence from gelatine films finds its origin into the aromatic amino acid tyrosine and from derived crosslinked products like dityrosine. The enhanced contribution to fluorescence of the tyrosine crosslinked products upon nanosecond UV laser irradiation of the films is indicative of chemical modifications which are related with the structural changes induced by foaming on micro and submicroscopic scales.

2. Experimental

Self-standing films of 30 μm thickness were prepared by solvent evaporation at 37 °C using aqueous solutions (6.67 wt.%) of gelatine supplied by Aldrich Chemicals. Type-B gelatine, with two different gel strength values, Bloom 225 and 75 (B225 and B75, respectively), was used. The bloom value characterizes the gel strength resistance and is a measurement of the gelatine quality. It is defined as the value of the force in grams necessary to apply in a standard plunger to deform 4 mm the surface of the gelatine gel [14]. As reported previously [4], the water solution viscosities, determined at 6.67% (w/w), are 5.50 cP (B225) and 3.64 cP (B75) and the molecular weight averages 77.3 kDa (B225) and 62.3 kDa (B75).

Crosslinked B225 gelatine films were prepared by the procedure described earlier [4,6,7,14,15] by immersing the films in a 4% aqueous solution of formaldehyde. The crosslinking degree was characterized by their weight swelling ratio q [16]. A longer treatment with formaldehyde gives a higher degree of crosslinking and subsequently a lower swelling ratio. Highly crosslinked B225 gelatine films with $q = 4$ (B225-c) were prepared for the experiments herein. The moisture content of the gelatine films determined by thermogravimetric analysis (TGA) was 4% in all the tested materials. UV–VIS spectra of films were measured using a Hewlett Packard 8452A, Diode Array Spectrophotometer.

Single-pulse laser irradiation of the films was carried out at 248 nm (KrF excimer laser, 20 ns fwhm) and at 266 and 355 nm (Q-switched Nd:YAG laser 4th and 3rd harmonic, 6 ns fwhm). The pulses were focused with a 10 cm focal length lens (for experiments at the three wavelengths). The gelatine samples were placed at the focal plane of the beams and were mounted on an *XY* positioner (*Z* being the propagation direction of the beam). The morphology of the irradiated areas was inspected using an environmental scanning electron microscope (ESEM, Philips XL30). On the other hand, LIF measurements of the irradiated areas were carried out using laser excitation at 266 and 355 nm and a 0.30 m spectrograph with a 300 lines/mm grating (TMc300 Bentham)-intensified charged coupled detector (2151 Andor Technologies) system. Spectrofluorimetric analysis of the films was performed using a Spectrofluorolog, 1680, 0.22 m double spectrometer. The TCSPC system, data collection and data fitting procedures are described elsewhere [17].

3. Results and discussion

3.1. Spectrofluorimetric and laser induced fluorescence measurements

UV ns pulse laser irradiation of the films (at 248 and 266 nm) leads to the development of micrometer size bubbles and formation of a foamy layer. This is illustrated in Fig. 1 which shows ESEM graphs of a B225 gelatine film irradiated at 266 and 355 nm at fluences above the corresponding threshold for modification of the films (determined as 0.2 J/cm^2 as 266 nm and 1.2 J/cm^2 at 355 nm). The typical thickness of the foaming layer is about 5 μm. At longer wavelengths, 355 nm, melting and resolidification with larger size structures become clear. Such behaviours were also observed for the two other gelatine types B75 and B225-c.

The UV–VIS absorption spectra of the gelatine films are shown in Fig. 2. Also shown in Fig. 2 is the absorbance of a solution of tyrosine in water (10^{-3} mol/l). Tyrosine (Fig. 3), an aromatic amino acid and minor residue of gela-

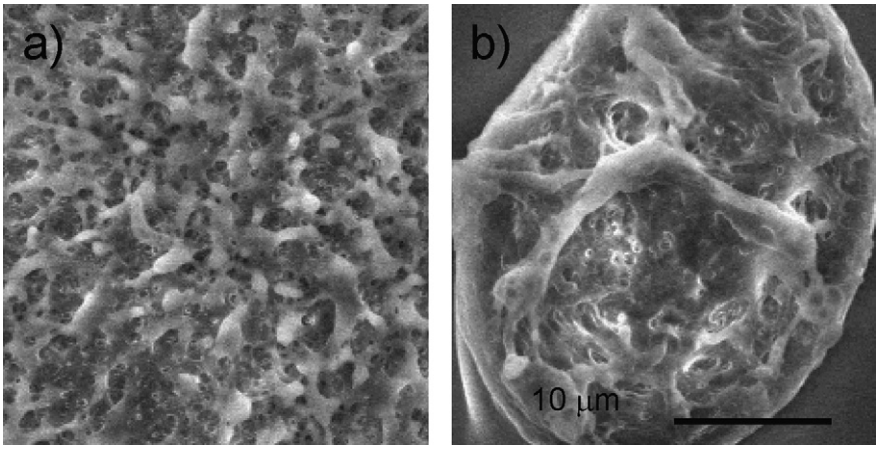

Fig. 1. ESEM graphs of B225 gelatine irradiated above the modification threshold with a single laser pulse at: (a) 266 nm, 0.25 J/cm^2; (b) 355 nm, 3 J/cm^2.

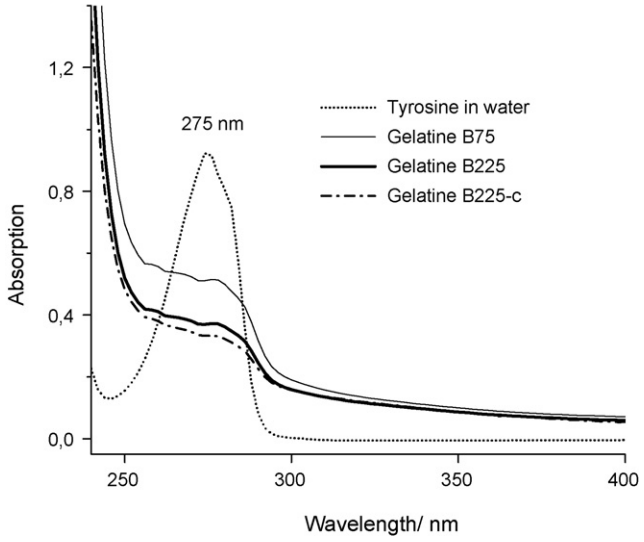

Fig. 2. Absorption spectra (resolution 2 nm) of 30 μm thick gelatine films and of a solution of tyrosine in water (10^{-3} mol/l). The reference is air for the films and water for the solution of tyrosine.

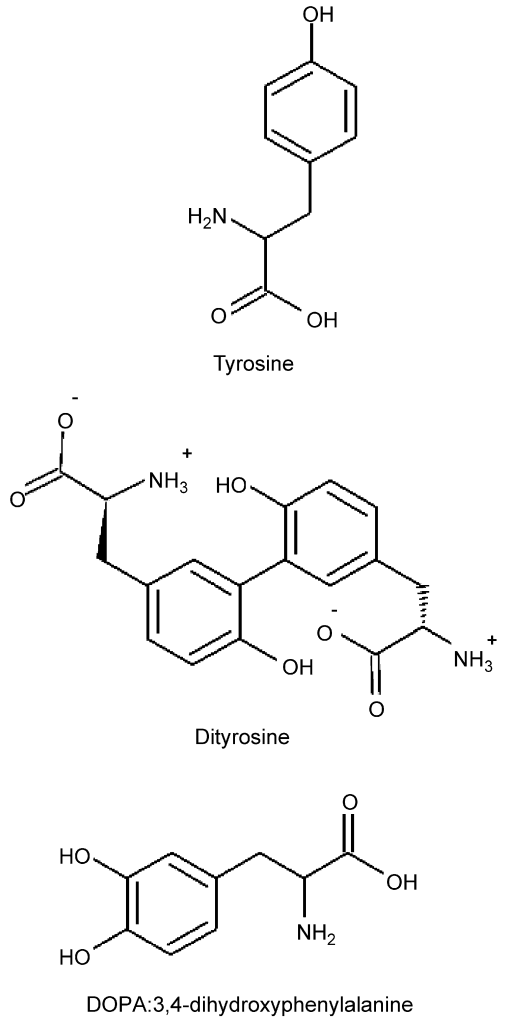

Fig. 3. Chemical structures of tyrosine, dityrosine and DOPA.

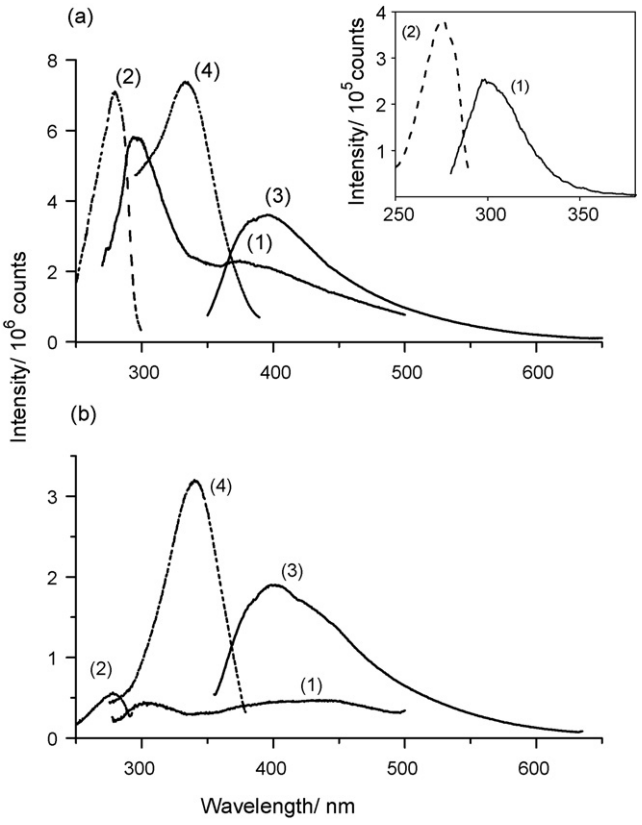

Fig. 4. Spectra of virgin, (a) and irradiated; (b) B225 gelatine films. Continuous lines correspond to emission spectra obtained upon excitation at λ_{exc} = 260 nm (1) and λ_{exc} = 335 nm (3). Dotted lines correspond to excitation spectra obtained by collecting the emission at λ_{em} = 310 nm (2) and λ_{em} = 400 nm (4). The inset shows the spectra of a solution of tyrosine in water (10^{-5} mol/l).

tine (∼1–1.5 wt.%), [18] exhibits an absorption band at 275 nm [4,15,19]. This chromophore significantly contributes to the absorbance of the gelatine films in the corresponding region.

Fig. 4 shows the emission and excitation spectra of B225 gelatine films in virgin areas and in areas irradiated with a single-pulse at 248 nm at a fluence of 1.8 J/cm^2. The films prepared with gelatines with different gel strength (B75) or crosslinked with formaldehyde showed similar emission and excitation spectra. The inset in Fig. 4 shows the emission and excitation spectra collected from an aqueous solution (10^{-5} mol/l) of tyrosine. Upon excitation at 260 nm the fluorescence maximum of tyrosine points at 300 nm. The excitation spectrum monitored at 310 nm displays a maximum at 275 nm [19]. The emission spectra (λ_{exc} = 260 nm) of the three types of gelatine films studied consist of a band centred at 300 nm (similarly to the solution of tyrosine) and a broad emission in the 380–440 nm region. The shorter wavelength band reveals the presence of tyrosine. Further, confirmation of the contribution of tyrosine to the fluorescence of the film is underlined by the band at 275 nm in the excitation spectra (λ_{em} = 310 nm). The longer wavelength and broad band emission indicates the contribution of other fluorophores. In fact, this emission band is used as a reference for dityrosine (Fig. 3), a dimeric species of tyrosine, which absorbs below 350 nm [20]. The contribution of other aromatic amino acids in gelatine like phenylalanine (∼2 wt.%), [18] usu-

ally excited in the 270–290 nm range, can be disregarded due to their low fluorescence quantum yield [15]. No significant modifications were observed in the emission and excitation spectra of gelatine.

Upon excitation of the films at 335 nm, the fluorescence spectrum is mainly pictured by a broad band with maximum at *ca.* 400 nm. While dityrosine mostly accounts for the 400 nm emission, other residues or aging products of gelatine can also contribute to the fluorescence. This is the case of pentosidine crosslinks reported to absorb at 335 nm and to fluoresce in the region above 400 nm [15,19,21]. The excitation spectrum recorded with $\lambda_{em} = 400$ nm shows maximum intensity at *ca.* 335 nm, indicating that pentosidine is also responsible for the 400 nm broad band-like emission.

Upon irradiation, the relative intensity of the two emission bands resulting from excitation at 260 nm, changes dramatically in all gelatines studied; the longer wavelength broad emission in the 380–440 nm region increases in intensity with respect to the band assigned to tyrosine. Together with this effect, a bathochromic shift of the absorption band from 335 to 340 nm is observed (as evidenced in the excitation spectra monitored at $\lambda_{em} = 400$ nm). These effects are observed in all gelatine films studied, irrespective of gel strength or crosslinking.

Further insight in the fluorescent properties of the films and their changes upon laser irradiation was obtained by LIF measurements. LIF spectra recorded on virgin and irradiated areas of the films upon excitation at 266 nm (Fig. 5 a) consist of two broad bands centred at 315 and 420 nm in agreement with data reported in Fig. 4. These bands are present in the spectra of all gelatine types studied and, as discussed above, are assigned to tyrosine (short wavelength) and its dimeric dityrosine species (long wavelength). Again as observed by spectrofluorimetry, upon irradiation with UV ns laser pulses, the relative intensity of the two bands $I_{\text{long }\lambda}/I_{\text{short }\lambda}$ increases (see Fig. 5 a).

LIF spectra with excitation at 355 nm (Fig. 5 b) also reveal the changes induced by laser irradiation. They show a general increase of the fluorescence band located in the 375–550 nm region with emission maxima at 410 and 440 nm. Similar effects have been reported by Wisniewski et al. [9] on a spectroscopic study of a KrF laser treated thin collagen film, where the 410 and 440 nm bands were assigned to the photooxidation products of tyrosine: dityrosine and 3,4-dihydroxyphenylalanine (DOPA, Fig. 3), respectively, with related products.

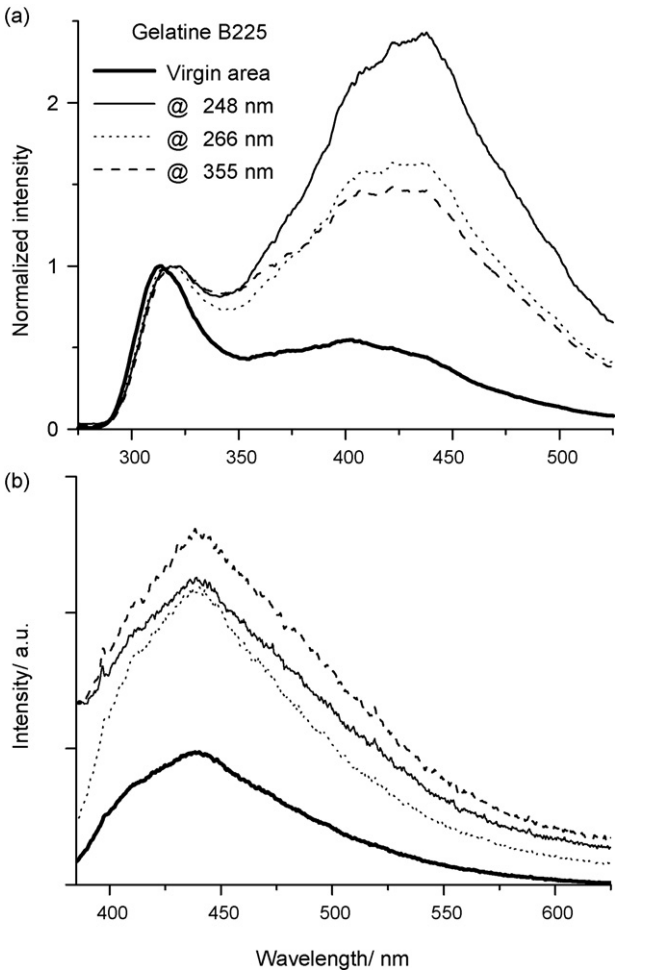

Fig. 5. LIF spectra (resolution 10 nm) of a B225 gelatine film upon different irradiation wavelengths: (a) $\lambda_{exc} = 266$ nm, intensities are normalized in reference to the 315 nm band (of tyrosine); (b) $\lambda_{exc} = 335$ nm. Each spectrum is obtained from the accumulation of 100 individual ones and by detecting the signal with a 500 ns gate at zero delay with respect to the arrival of the illuminating pulse to the surface. Spectra were recorded with 0.1 mJ/pulse.

3.2. Fluorescence lifetime measurements

The fluorescence intensity decays were measured using TCSPC [17]. Table 1 summarizes the results obtained on virgin films excited at 270 nm and on irradiated films excited at 270 and 340 nm, respectively (decays registered at 335 and 400 m). It was not possible to record the lifetimes of virgin films upon excitation at 340 nm due to the too high contribution of scattered light. Also in Table 1 the measured lifetimes of a solution of tyrosine in water are listed. The decay observed in the tyrosine solution is monoexponential with a lifetime of 3.37 ns, in good correspondence with literature data [22]. Fluorescence decays of the short wavelength band of gelatine films ($\lambda_{exc} = 270$ nm) were fitted with a biexponential function (with lifetimes of 1 and 3.5 ns). These values are also in good agreement with fluorescence lifetimes of tyrosine measured in the emission region above 330 nm [19] allowing to ascertain that tyrosine is the main fluorophore in this region. UV laser irradiation of the films does not change substantially these decays. The fluorescence decays of the irradiated films upon excitation at 340 nm (collecting the emission at 400 nm) are substantially different. While the decays are still biexponential, the calculated lifetimes are longer *ca.* 1.7–2 ns and 7.1–7.7 ns. Harms et al. [20] have examined the lifetimes and rotational correlation times of dityrosine emission by TCSPC ($\lambda_{exc} = 287$–300 nm). Fluorescence intensity decays of dityrosine are generally characterized by two decay components with similar lifetimes as those measured here. These data give further support to the assignment of the long wavelength band to the dityrosine group. Small differences between the various types of gelatine reported in Table 1 are probably related with modifications in the physical condition of the films according with their gel strength and purity and degree of crosslinking.

Table 1
Fluorescence lifetimes of virgin and irradiated (-irr) gelatine films (at 248 nm) as measured by TCSPC

	λ_{ex} (nm)	λ_{em} (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2
Tyrosine in water (10^{-4} mol/l)	270	310	–	–	1	3.37 ± 0.02	1.03
B225	270	335	0.93	0.36 ± 0.04	0.07	3.36 ± 0.07	1.16
B225-irr	270	340	0.93	0.39 ± 0.03	0.07	2.96 ± 0.08	1.02
B75	270	335	0.66	1.0 ± 0.1	0.34	3.79 ± 0.08	1.17
B75-irr	270	340	0.89	0.67 ± 0.04	0.11	3.25 ± 0.09	0.97
B225-c	270	335	0.61	1.1 ± 0.3	0.39	3.7 ± 0.2	1.09
B225-4-irr	270	340	0.91	0.44 ± 0.05	0.09	3.1 ± 0.1	1.03
B225-irr	340	400	0.78	1.81 ± 0.09	0.22	7.5 ± 0.1	0.96
B75-irr	340	400	0.79	1.7 ± 0.1	0.21	7.1 ± 0.1	1.06
B225-c-irr	340	400	0.74	2.0 ± 0.1	0.26	7.7 ± 0.1	1.12

Decays are fitted to one or to the sum of two exponentials: $\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$.

4. Conclusions

Laser irradiation with UV ns pulses induces dramatic structural changes in the irradiated gelatine films in the form of a foaming layer with nanometric size pores and bubbles. As revealed by fluorescence measurements taken on virgin and irradiated films, the major accompanying chemical modifications are related with the relative increase of the fluorescence of dityrosine moieties and other products derived of the photoxidation of tyrosine. These modifications are related to the structural disruption induced by foaming and to the increase of temperature in the irradiated region.

Lazare et al. [3,4,6] have discussed the mechanism of foaming induced by ns UV irradiation of collagen, gelatine and other biopolymers. Foaming requires a sudden and dense bubble nucleation and growth, which are made possible by the transient acoustic wave induced by the laser pulse. The tensile component of the laser induced photoacoustic transient wave is thought to be responsible for most of the bubble nucleation. The high water content of the films reduces the tensile strength of the substrate facilitating bubble nucleation and growth together with the eventual expansion of the material in a microfoam layer. The mobility of the tyrosine residues increases as a result of swelling or volume expansion induced by laser, leading to a higher collision probability and an enhanced formation yield of tyrosine dimers formed by two tyrosine units when they are in close proximity [7,23–25].

On the other hand, the maximum surface temperature obtained at the end of the laser pulse could be roughly estimated-based on the assumption that the absorbed laser energy is converted entirely into heat and that the heat diffusion is much slower than the laser pulse duration, $\Delta T = \alpha F/\rho C$, with α being the optical absorption coefficient at the laser wavelength, F the laser fluence, ρ the density and C the heat capacity. For irradiation at 0.25 J/cm^2, 248 nm ($\alpha \approx 300\,\text{cm}^{-1}$, ρ and C of 1.0 g/cm^3 and 2.25 J/g °C, respectively [4]) the increase of temperature for gelatine is about 30 °C. This gentle heating could be moderated by the presence of water, as part of the laser pulse energy is consumed as latent heat of evaporation and contributes to suppression of the increment of temperature in the irradiated region [26]. Studies on thermal degradation of gelatine [27] have shown modifications in the relative contribution to fluorescence of the tyrosine and its related crosslinked chromophores, in similarity with the observations presented herein. Therefore, it can be concluded that the observed chemical changes have their origin in the structural modifications induced by foaming and to the increase of temperature in the irradiated region. More work is in progress to quantify the relative contribution of these two factors.

Acknowledgements

Funding from MEC (Projects CTQ2006-15583-C02-01/PPQ and MAT2006-05979) is gratefully acknowledged. M.O. thanks CSIC for I3P contract and S.G. thanks the EU for a Marie Curie contract (MESTCT-2004-513915).

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