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ORIGINAL PAPER

Easy detectable isocyanate in the reaction with gelatin

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Abstract Gelatin reactivity with isocyanate was studied by using the easy detectable 1-naphthyl-isocyanate (NphI). Four different NphI/gelatin feed ratios were investigated with NphI molar amount ranging between 1/10 and 1/1 with respect to the possible reactive groups of gelatin. The reactions were carried out at 45 °C in DMSO as solvent, under nitrogen atmosphere. Modified gelatin samples were characterized by IR, UV–VIS, fluorescence spectroscopies as well as by proton and DOSY NMR. Spectroscopy results allow to evidence the presence of both bonded and unbonded naphthyl derivatives in the gelatin samples. Unbonded species were present particularly at the highest NphI/gelatin feed ratio and their formation was correlated to the increasing competition of the reaction with water since the amount of available reactive groups on gelatin was comparable or smaller than the amount of residuum water in dry gelatin.

Keywords Gelatin · Isocyanate · UV–VIS spectroscopy · Fluorescence spectroscopy

Introduction

Isocyanates can react under mild conditions with all nucleophilic species bearing hydrogen atoms to give carbamate, urea, thioureas, etc. [1]. As results of this high and broad reactivity they have been successfully used as monomers to obtain polyurethane [2, 3] and polyureas [4], as coupling agents to modify polymers [5, 6]

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and surfaces [7] as well as to prepare graft copolymers [8–10]. In particular, isocyanates have been proved to be efficient and versatile reagents for the modification of natural polymers by reaction of the hydroxyl and amine groups present [6, 8–10]. However, natural polymers usually contain the so-called "bonded water" that cannot be removed without damaging the material [11, 12]. Indeed, water competes with the desired reaction as isocyanates react very easily with moisture to give amines and carbon dioxide. The formed amines can react with the remaining isocyanate to give urea derivatives; this side reaction affects the final polymer structure and properties. The only favorable exception is in case of the foaming process [1, 2], where the release of carbon dioxide is exploited to produce empty cavities. In any case, the reaction of isocyanate with water is slower than the reaction with amine [1] to an extent which depends on the specific reaction conditions, namely solvent and catalyst.

In a previous work carried out in our laboratory gelatin was crosslinked by 1,6-diisocyanatohexane and a very high gelling rate was observed without catalyst addition [6]; amide groups present in the gelatin acting most likely as internal catalysts [13, 14]. The complex final structure did not allow the determination to an accurate level of the isocyanate derivative incorporated in the gel.

In this work, we studied then the reactivity of isocyanate toward proteins by using an isocyanate bearing an easily detectable chromophore. Indeed naphthyl-isocyanate can be easily detected by spectroscopy, particularly UV–VIS, fluorescence, as well as, NMR. Indeed, gelatin show low intensity peaks in the aromatic region, due to the scarce occurring of aromatic aminoacids such as phenylalanine [15]. NphI/gelatin feed ratios in the 0.04–0.4 g/g (1/10–1/1 mol/mol) range were investigated so as to explore the reactivity of all the possible reactive groups of the protein macromolecule. The amount of bonded isocyanate was evaluated by the above mentioned spectroscopy methods. The results were discussed in relation to the effective amount and type of reactive groups and considering the main and secondary reaction paths.

Experimental part

Materials

Gelatin type B from bovine skin (Aldrich) with bloom value 250 was dried under reduce pressure at room temperature to constant weight (3 weeks). Water residuum was 6.4% weight by TGA analysis.

Dimethylsulfoxide (J.T.Backer) (DMSO) was dried on Na_2SO_4 overnight, refluxed at 80 °C on CaH_2 during 6 h under nitrogen, distilled under reduced pressure (81–84 °C at 18 mmHg) and kept on molecular sieves under nitrogen until use. Ethyl acetate (Carlo Erba, HPLC grade) was refluxed on K_2CO_3 under nitrogen atmosphere, distilled (73 °C, 1 bar) and kept on molecular sieves under nitrogen until use. 1-Propanol (Carlo Erba, HPLC grade) was dried on Mg activated with I_2 under nitrogen atmosphere and then distilled. Propionic acid (Carlo Erba) was dried

on Na_2SO_4 overnight and then distilled on $KMnO_4$ under nitrogen. NphI (Aldrich) was distilled under reduced pressure before use (67 °C at 0.1 mmHg).

2,2,2-Trifluoroethanol (BioChemika Ultragrade, Fluka) (TFE) was used as purchased from Fluka.

All the other solvents were of analytical grade and were used without further purification.

All glassware was flamed under reduce pressure (0.1 mmHg) before use and all reactions were carried out under dry nitrogen atmosphere.

Instruments and characterization methods

IR spectra were recorded with a Perkin Elmer Spectrum One on KBr pellets and with a Spectrum GX equipped with ZnSe horizontal ATR crystal. Both instruments were equipped with Spectrum software version 3.02. ^{1}H-NMR spectra were obtained using a Varian VXR-300 or 200 spectrophotometers in DMSO-d_6 at 60 °C or $CDCl_3$ at room temperature (30 mg/mL), respectively. DOSY experiments were performed on a Varian INOVA600 spectrometer operating at 600 MHz for ^{1}H using a 5 mm broadband inverse probe with z-axis gradient. The sample temperature was maintained at 25 °C. Experiments were carried out by using a stimulated echo sequence with self-compensating gradient scheme, a spectral width of 8,000 Hz and 64 K data points. Typically, a value of 200 ms was used for Δ, 1.0 ms for δ and g was varied in 30 steps (16 transients each) to obtain an approximately 90–95% decrease in the resonance intensity at the largest gradient amplitude. The baseline of all arrayed spectra were corrected prior to data processing. After data acquisition, each FID was apodized with 1.0 Hz line broadening and Fourier transformed. The data were processed with the DOSY macro (involving the determination of the resonance heights of all the signals above a pre-established threshold and the fitting of the decay curve for each resonance to a Gaussian function) to obtain pseudo two-dimensional spectra with NMR chemical shifts along one axis and calculated diffusion coefficients along the other.

UV–VIS spectra were recorded with a Perkin Elmer Lambda 650 spectrometer. Steady-state fluorescence spectra were acquired under isotropic excitation with a PerkinElmer luminescence spectrometer LS55 controlled by FL Winlab software. Solution concentrations were 0.1 g/L for UV–VIS analysis and 0.01 g/L for fluorescence analysis, except otherwise indicated. Gelatin and modified gelatin solutions were prepared by dissolving the dried samples at 50 °C under stirring in TFE. 1,3-di-1-naphthylurea (DNU) was dissolved in DMF at first and then diluted in TFE to final TFE/DMF ratio 100/1 by volume.

The elemental analyses were performed at the Dipartimento di Chimica e Tecnologia Farmaceutiche, Università di Pisa.

Synthesis of propyl 1-naphthylcarbamate

Propyl 1-naphthylcarbamate (PNC) was prepared by following a procedure already reported in literature [16]. Briefly, 1-naphthylisocyante (1.7 mL, 0.012 mol) and 1-propanol (1.8 mL, 0.024 mol) were dissolved in 10 mL of ethyl acetate under

nitrogen atmosphere and refluxed for 24 h. The precipitate was removed by filtration and the product recovered by solvent evaporation. The crude of reaction was purified by recrystallization from acetonitrile and gave a white powder in 72% yield.

It was soluble in several common organic solvents such as chloroform, dichloromethane, acetone, DMSO, DMF, and TFE.

^{1}H-NMR (200 MHz, $CDCl_3$) δ ppm: 7.95–7.40 (m, 7H, Np–H); 6.94 (s, 1H, N–H); 4.17 (t, $J = 6.7$, 2H, C(O)–O–CH_2–CH_2–CH_3); 1.72 (m, 2H, C(O)–O–CH_2–CH_2–CH_3); 0.98 (t, $J = 7.4$, 3H, C(O)–O–CH_2–CH_2–CH_3).

^{13}C-NMR (200 MHz, $CDCl_3$) δ ppm: 10.5 (CH_3–); 22.4 (–CH_2–); 67.2 (O–CH_2–); 119.4 (Np–C2); 120.7 (Np–C8); 125.1 (Np–C4); 125.9, 126.1, 126.3, (Np–C3,6,7); 126.9 (Np–C1); 128.8 (Np–C5); 132.7 (Np–C9); 134.2 (Np–C10); 154. 8 (N–C(O)–O).

FT-IR (KBr) cm^{-1}: 3292; 2966; 1694; 1531; 1502; 1346; 1254; 1233; 1105; 1071; 1009; 789; 771.

Elementary analysis; calculated for C, N, H: 73.34; 6.11; 6.59; found: 73.52; 5.58; 6.68.

Synthesis of 1,3-di-1-naphthylurea

2 g of NphI and 1.78 g of propionic acid were dissolved in 10 mL of ethyl acetate at room temperature under nitrogen atmosphere. A white color precipitate formed immediately and no unreacted isocyanate was detected in the reaction mixture by FT-IR spectroscopy. The organic phase was washed with basic water ($NaHCO_3$). The white solid powder recovered by rotavaporing was insoluble in the most common organic solvents except DMF and DMSO.

^{1}H-NMR (300 MHz, DMSO-d_6) δ ppm: 9.20 (s, 1H, N–H); 8.25 (d, $J = 8.6$, 1H, Np–H(8)); 8.08 (d, $J = 7.2$, 1H, Np–H(5)); 7.96 (d, $J = 7.6$, 1H, Np–H(4)); 7.81–7.39 (m, 4H, Np–H(2,3,6,7)).

^{13}C-NMR (300 MHz, DMSO-d_6) δ ppm: 153.3 (NH–C(O)–O); 134.4 (Np–C10); 133.7 (Np–C9); 128.4 (Np–C5); 126.2 (Np–C1); 125.9–125.7 (Np–C3,6,7); 122.8 (Np–C4); 121.4 (Np–C8); 117.4 (Np–C2).

FT-IR (KBr) cm^{-1}: 3281; 2963; 1632; 1622; 1556; 1396; 1344; 1261; 1208; 1096; 1017.

Elementary analysis; calculated for C, N, H: 80.75; 8.97; 5.16; found: 80.07; 7.63; 5.81.

Modification of gelatin with 1-naphthyl-isocyanate

4.08 g of gelatin was dissolved in 80 mL of DMSO at 50 °C overnight under stirring in a flamed round bottom flask equipped with refrigerator and kept under dry nitrogen atmosphere. The solution was cooled to room temperature and the proper amount of NphI was added (Table 1). The reaction mixture was kept at 25 °C for 24 h under stirring, and an excess of methanol was added. The precipitate was filtered and dried under reduced pressure to constant weight. In the case of reaction F3 water instead of methanol was used. The solid was recovered by filtration,

Table 1 Naphthyl groups to gelatin weight ratio in the reaction feeds and in the modified samples

Reactions	Feed ratio (g/g)	Found (g/g)	
		By UV–VIS	By NMR
F1	0.42	0.22	0.22
F2	0.21	0.13	0.13
F3	0.08	0.08	0.08
F4	0.04	0.05	0.03

washed with methanol in Soxhlet extractor for 16 h and dried under reduced pressure to constant weight.

Results

FT-IR and NMR spectroscopy

Gelatin was modified by reacting with NphI. Four different feed ratios NphI/gelatin were used (Table 1). Isocyanate groups dropped to a non-detectable concentration after reaction time of the order of few minutes in all experiments, as evidenced by the disappearing of the typical stretching band at 2270 cm^{-1} in the FT-IR spectrum of the reaction mixtures.

Infrared spectra of purified reaction products, namely F1–F4 samples, showed small but significant differences with respect to gelatin (Fig. 1): (a) a small shoulder to the strong carbonyl band at 1632 cm^{-1} of amide groups appeared in F1 spectrum corresponding to the carbonyl absorption band of PNC; (b) the amide II band in the modified gelatin samples was a slightly shifted with respect to gelatin, most likely

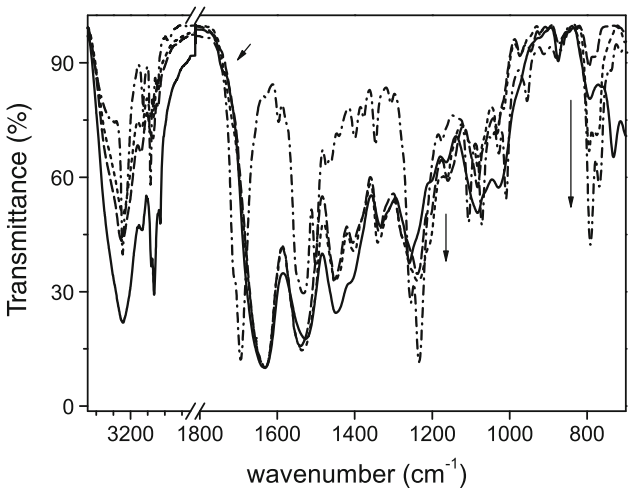


Fig. 1 Comparison among the ATR-IR spectra of gelatin (*solid*), F1 (*dot*), F3 (*dash*), and PNC (*dash dot*). *Arrows* indicate the most important spectrum differences between F1 or F3 and gelatin

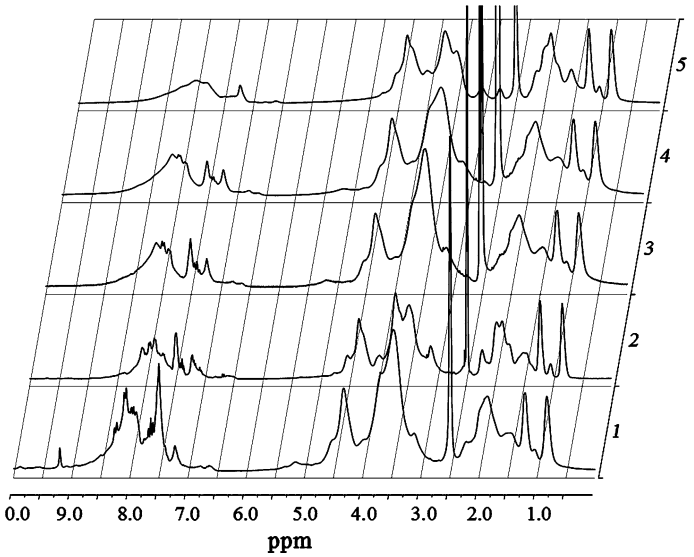


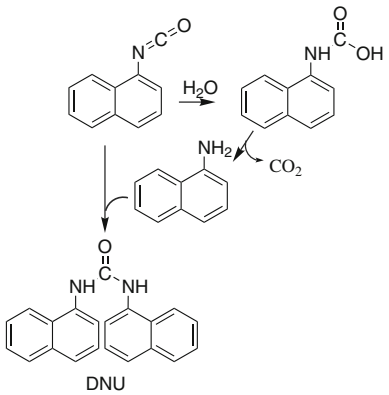
Fig. 2 ^{1}H-NMR spectra of gelatin (no. 5), F1 (no. 1), F2 (no. 2), F3 (no. 3), and F4 (no. 4) in DMSO-d_6 at 60 °C

because the reduced amount of intra and intermolecular hydrogen bonds; (c) some new absorption components are detectable in the modified samples in the C–O–stretching region of PNC in between 1150 and 1300 cm^{-1}: (d) two bands at 794 and 775 cm^{-1} due to the out-of-plane bending of naphthyl C–H are clearly detectable in the modified samples. All together the above mentioned IR features clearly indicate the incorporation of naphthyl group into gelatin by carbamate or urea covalent bonding. Consistently, F1–F4 samples were not soluble in water, where they just swelled. On the other hand, they were soluble in the less polar DMSO and TFE.

^{1}H-NMR spectroscopy analysis of F1–F4 samples showed typical aromatic proton signals in the 7.4–8.3 ppm spectral region (Fig. 2). Gelatin spectrum (spectrum no. 5 in Fig. 2) shows also some signals in the same region due to the natural occurring aromatic protons, particularly phenylalanine. However, the aromatic peak number and intensity are larger in the modified samples than in gelatin, thus confirming the presence of naphthyl groups in F1–F4 samples. In particular, the resonance peak at 7.5 ppm, not present in the gelatin, is detectable in all modified gelatin samples and its intensity increases from sample F4 to F1.

The total integral value of the NMR signals in the aromatic region was exploited to calculate the amount of naphthyl groups in the modified gelatin samples. The integral value of the peak at 0.89 ppm assigned to the protons of CH_3 groups of valine, leucine, and isoleucine [17] was used as internal reference. The corresponding proton amount and the average amino acid weight (AW) for 1000 amino acid residues were 311 and 108.5 g/mol as for a typical aminoacid composition of gelatin [15]. Naphthyl group amounts in the 0.03–0.22 g/g range were calculated (Table 1) under the above-mentioned assumptions.

Scheme 1 Possible formation of the DNU ureic by-product after reaction of NphI with water



^{1}H-NMR spectrum of the most modified F1 sample (no. 1 in Fig. 2) clearly shows a singlet at 9.08 ppm assigned to N–H protons of ureic species by comparison with the spectrum of 1,3-di-1-naphthylurea (DNU). Such ureic groups should be formed by the reaction of NphI with amino functionalities of lysine and arginine residues or by the decarboxylation of isocyanate groups after reaction with water (Scheme 1). Moreover, the spectrum of F1 exhibits aromatic signals better resolved than other peaks of the spectrum. This feature suggests the presence of low molecular weight naphthyl by-products in F1. Similar less intense peaks attributable to low molecular weight species can be identified in F2 sample, also.

The DOSY spectrum of sample F3 (Fig. 3) clearly showed the presence of aromatic species with higher than or comparable to gelatine diffusion coefficient according to presence of both bonded and unbonded aromatic species in F3.

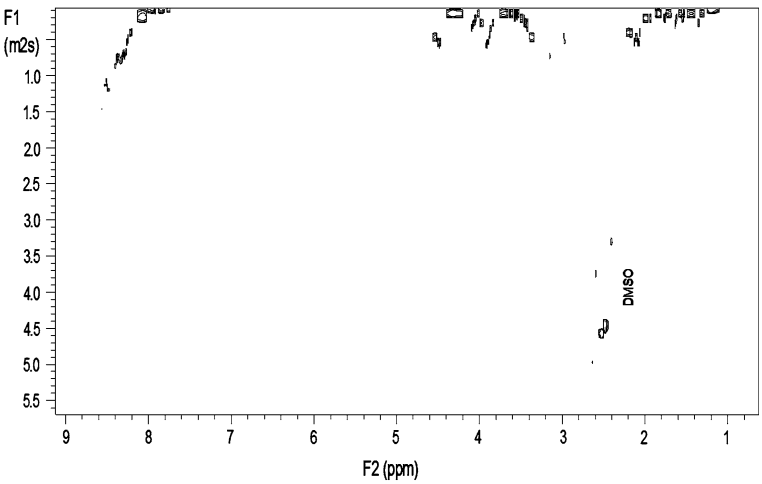


Fig. 3 DOSY spectrum of F3 sample in DMSO-d_6 at 60 °C

UV–VIS spectroscopy

A suitable calibration plot that was obtained with PNC and gelatin mixtures of defined composition allowed the determination of the content of naphthyl groups in F1–F4 by UV–VIS spectroscopy. The obtained values are (Table 1) in good agreement with NMR values.

DNU (Fig. 4), which is the most probable reaction by-product, and PNC (Fig. 4), taken as carbamate model, showed the maximum UV absorptions in TFE at 281 nm ($\varepsilon = 12{,}800$ cm^2/mol) and 279 nm ($\varepsilon = 6{,}100$ cm^2/mol), respectively. DNU adsorption band is slightly red shifted and is flatter than the PNC one. Accordingly, F1 and F3 sample spectra having band maxima flatter should contain a larger amount of ureic derivatives than F2 and F4 (Fig. 5).

Fluorescence spectroscopy

Modified gelatin samples showed emission spectra in TFE solution with maximum at 395 nm, while unmodified gelatin has quite undetectable emission under the same experimental conditions. No differences in the emission band position and shape were observed among F1–F4 samples (Fig. 6), while the emission intensity did not correlate with the total amount of naphthyl groups found by NMR or UV–VIS spectroscopy (Fig. 7). The most intense emission was observed for F2, whereas the sample containing the largest amount of naphthyl is F1.

The analysis of the emission of DNU and PNC clearly indicated that the emission of the former is more intense than that of the latter (Fig. 8). In both cases the emission intensity does not increase linearly with concentration at values larger than

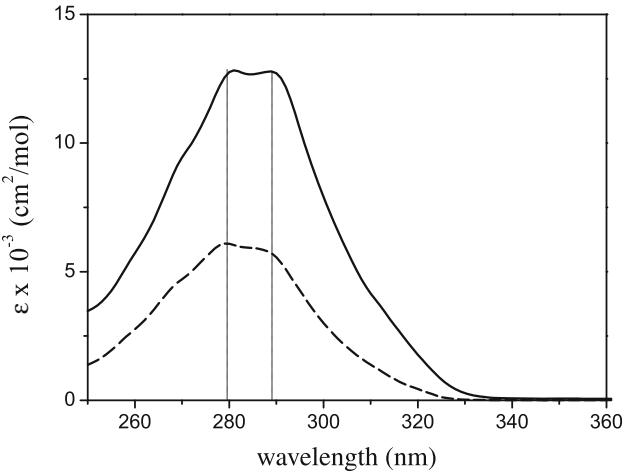


Fig. 4 Comparison between UV–VIS adsorption spectra of PNC (*dash line*) and DNU (*solid line*) in TFE solution (concentrations: PNC: 0.047 mM; DNU: 0.042 mM)

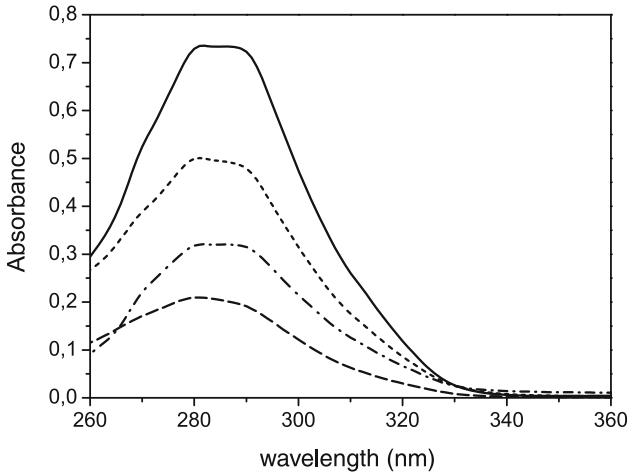


Fig. 5 UV–VIS adsorption spectra of F1 (*solid*), F2 (*dot*), F3 (*dash dot*), and F4 (*dash*) in TFE, 0.01 g/L

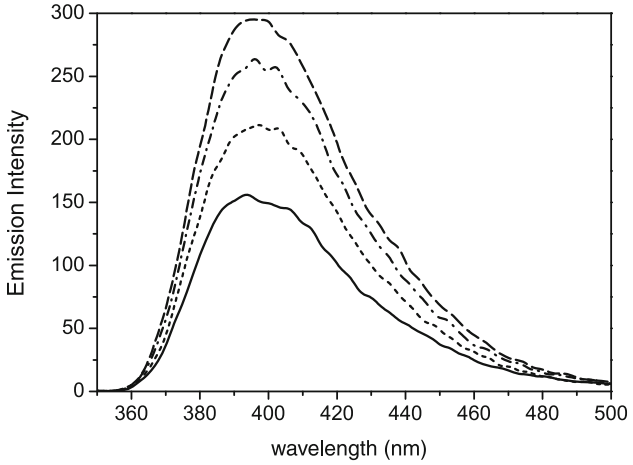


Fig. 6 Emission spectra of F1 (*solid*), F2 (*dash dot*), F3 (*dash*), and F4 (*dot*) samples in 0.01 g/L TFE solutions. Excitation wavelength 290 nm

10^{-5} M most likely because of autoabsorption phenomena [18]. The large difference in the emission intensity between PNC and DNU and the presence of DNU at least in F1, indicated by NMR analysis, can partially explain the lack of linearity of the plot of the emission intensity versus the total naphthyl amount per gram of gelatin (Fig. 7).

The effect on the fluorescence emission of DNU trapped inside gelatin samples was investigated by adding increasing amount of DNU solution to a 0.05 g/L solution of F2 in TFE. The emission intensity of the F2 solution decreased with

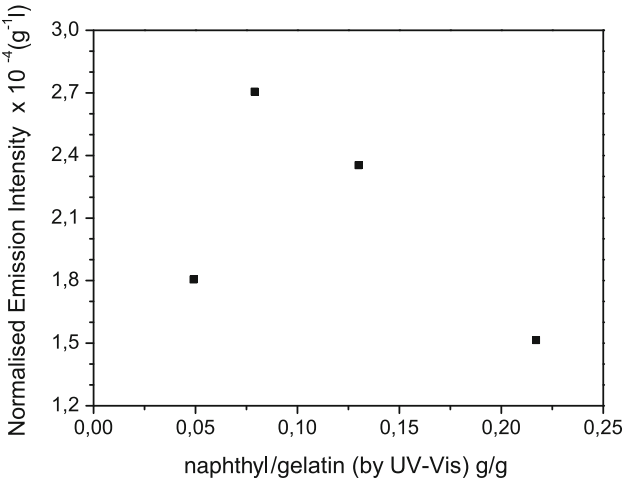


Fig. 7 Emission intensity of F1–F4 solutions in TFE versus the amount of naphthyl found in each samples by UV–VIS spectroscopy. Emission intensities were normalized with respect to the exact solution concentration (~ 0.01 g/L)

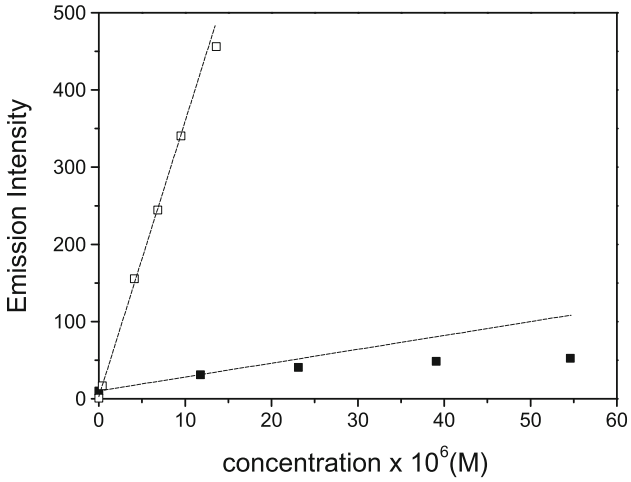


Fig. 8 Emission intensity of PNC (*open symbol*) and DNU (*solid symbol*) in TFE solution in the presence of gelatin 0.05 g/L

increasing the DNU added amount (Fig. 9). Therefore, the lower emission intensity of F1 and F2 with respect to F3 can be attributed to the quenching the emission of naphthyl groups bonded to gelatin by trapped DNU. Indeed energy transfer between two equienergetic states can be very effective as electrons in the excited state of bonded naphthyl groups and DNU should have similar energy [18]. The excited

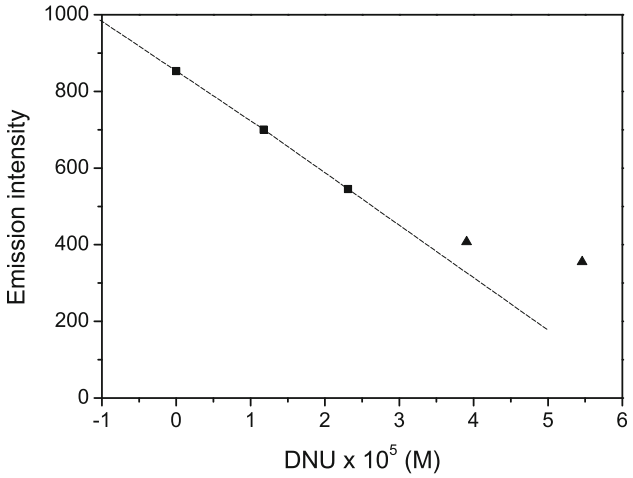


Fig. 9 Emission intensity of a F2 solution (0.046 g/L in TFE) versus the amount of added DNU

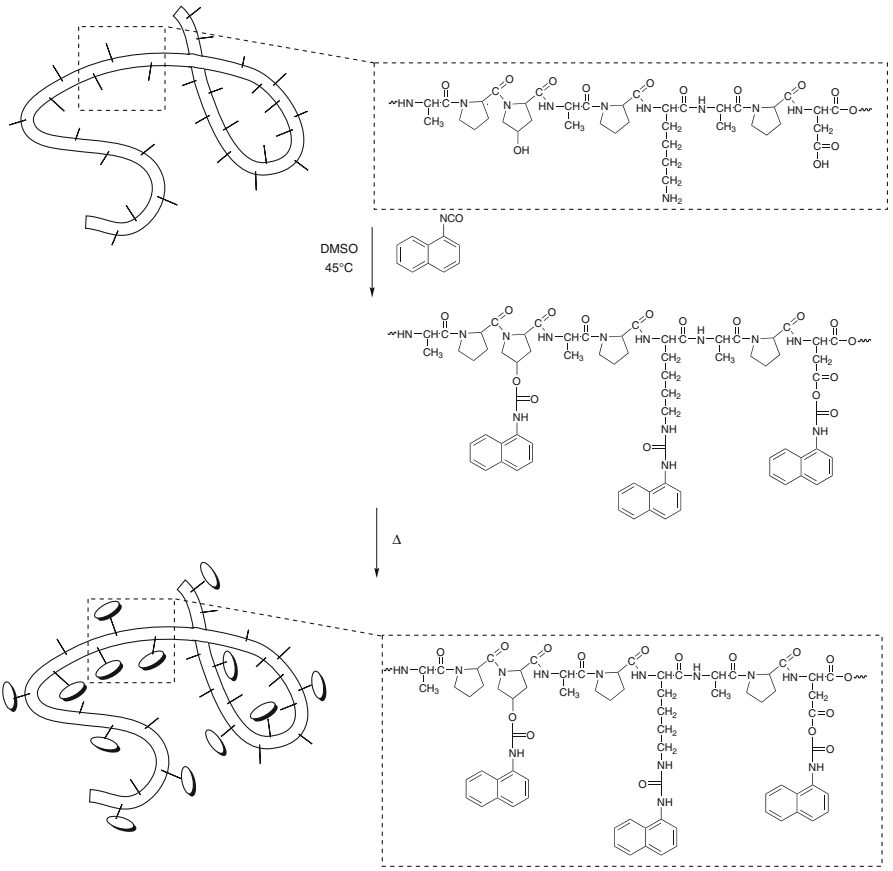
DNU* while moves far away from bonded naphthyl groups can decays to its ground state by light emission which has an intensity lower than bonded carbamates (see Fig. 8).

Discussion and final remarks

Gelatin was modified by side chain binding of hydrophobic florescent groups by reaction with NphI (Scheme 2). Four different feed ratios NphI/gelatin (Table 1) were selected so as the naphthyl amount ranged between 1/10 and 1/1 by mole with respect to the possible reactive groups of gelatin. These were assumed to be the amine of lysine and arginine, the carboxylic acid of glutamic and aspartic acids, and the hydroxyls of hydroxyproline and serine. Their total amount was 2.48 mmol/g as determined by acid/basic titration [6]. Dimethylsulfoxide was used as reaction solvent as it dissolves gelatin and does not react with isocyanates under the mild conditions adopted.

The above-mentioned conditions allow modulating the modification degree of gelatin at least up to 0.5 mmol/g as indicated by UV–VIS and ^{1}H-NMR (Fig. 10). Actually, spectroscopy indicates a maximum grafting degree of 1.3 mmol/g but at the same time that at least a part of such groups are not covalently bonded to gelatin. DOSY NMR spectrum and fluorescence data clearly evidenced the presence of unbonded naphthyl groups trapped into the modified samples, most likely due to the low solubility of the ureic by-product and hydrogen bonds with gelatin.

The naphthyl incorporation efficiency, assumed as sum of naphthyl groups bonded and trapped into each gelatin sample per amount of fed naphthyl-isocyanate, decreased with the feed ratio (Fig. 11). This behavior can be due to the increasing



Scheme 2 Possible reactions of naphthyl-isocyanate with gelatin residues

amount of not removable incorporated but not grafted naphthyl derivatives. The isocyanate reaction with amine and hydroxyl groups follows a second order kinetic, as in first approximation [13] it can be considered of first order with respect to both the isocyanate and the nucleophyle. The amount of reactive groups per gram of gelatin is almost 0.45 mmol –NH groups, 1.25 mmol –OH and 0.78 mmol –COOH [6], while, the residue water after drying is 3.5 mmol/g. Thus, the probability of reaction between isocyanate and water increases correspondingly. In conclusion, if high grafting yield (>80%) and negligible presence of ureic by-product are desired, the isocyanate/gelatin feed ratio should be <0.5 mmol/g. This means that only 50% of the total reactive groups of gelatin can be reasonably modified.

Moreover, the DOSY NMR but also by simple fluorescence analysis method have allowed to demonstrate the presence of unbonded by-products which have to be considered in order to identify the effective grafting of naphthyl groups to the gelatin macromolecule.

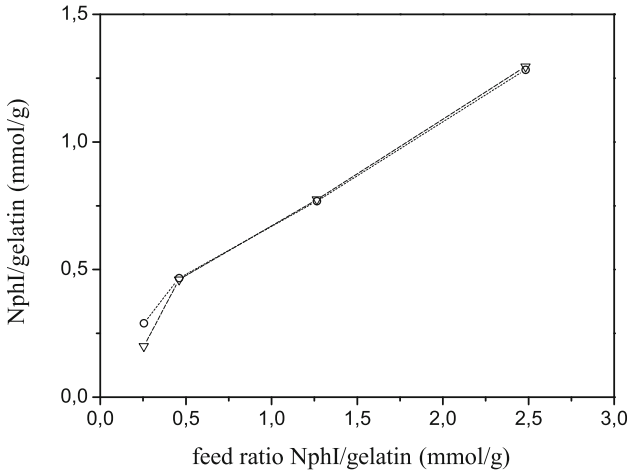


Fig. 10 Amount of naphthyl groups incorporated into gelatin as found by UV–VIS (*circle symbols*) and NMR (*triangle*) spectroscopy versus the corresponding feed ratio

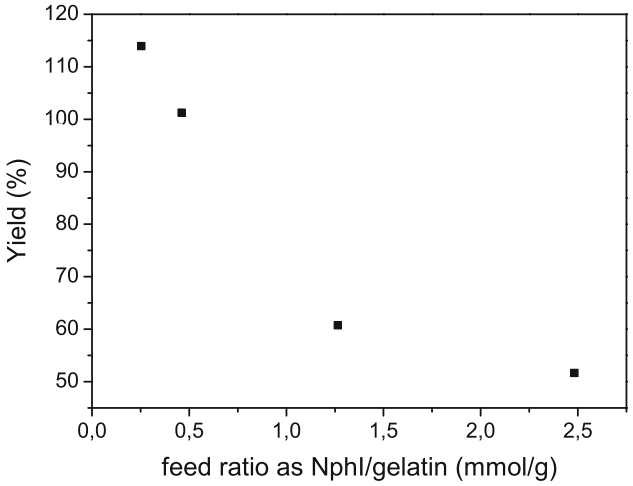


Fig. 11 Yield percentage of naphthyl groups incorporated into gelatin as found by UV–VIS spectroscopy versus the corresponding feed ratio

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