Study of grafting ethylene oxide onto gelatin by one- and hetero two-dimensional ^{13}C n.m.r. spectroscopy

A. Gantar*, A. Šebenik, M. Kavčič and U. Osredkar
The Boris Kidrič Institute of Chemistry, Ljubljana, Yugoslavia

and H. J. Harwood
University of Akron, Akron, Ohio 44325, USA
(Received 14 May 1986; accepted 15 July 1986)

Products of ethylene oxide grafted onto gelatin were prepared and characterized by hetero two-dimensional ^{13}C n.m.r. spectroscopy with the aid of specially prepared model substances. Under the selected reaction conditions grafting took place on the free $-NH_2$ groups of lysine, arginine and asparagine only, while other groups such as –OH, –COOH, –CONH– or the double bonds of the $NH=C<$ groups did not react with ethylene oxide. Three different methods of grafting are possible leading to different molecular weight values for the grafted gelatin. From the ^{13}C n.m.r. spectra it can be seen that main changes in the signals before and after grafting take place in regions corresponding to grafting loci.

(Keywords: grafting; nuclear magnetic resonance; gelatin)

INTRODUCTION

Grafting of different types of vinyl monomers[1-3] and ethylene oxide[4] onto collagen has already been investigated. Due to a number of active groups collagen is very suitable for grafting. It is possible to graft monomers onto the free –OH, –COOH or $-NH_2$ groups of the amino acids and onto the peptide bond of collagen. Each of the free groups has protons, which are not bonded very strongly to nitrogen or oxygen and therefore can be replaced easily by ethylene oxide.

The reactivity of different groups with ethylene oxide is dependent on the charge of the groups, on steric hindrance of the polymeric chain and on the influence of any neighbouring atoms. Ethylene oxide itself is a very reactive cyclic compound which can react not only with collagen but also with water to form high molecular weight poly(ethylene glycol), which may graft to collagen or remain non-grafted.

In this work grafting of ethylene oxide onto gelatin is presented. For characterization of the grafted gelatin we used high resolution one dimensional (1D) and hetero two-dimensional (2DJ) ^{13}C n.m.r. spectroscopy (carbon–proton coupling correlated with carbon). The aim of the investigation was the determination of the mechanisms of the reaction between ethylene oxide and gelatin.

EXPERIMENTAL

Materials

Gelatin was prepared by hot water extraction of limed and bated pig skin. Ethylene oxide (EO) was a product of Hoechst and glycine was a product of Merck.

A 7.7% water solution of gelatin was heated up to 70°C and 20% or 50% of EO calculated on gelatin was introduced into the reaction vessel over 15 min. After the reaction was complete, the quantity of EO which grafted onto the gelatin was determined by weight differences of gelatin (dry matter) before and after grafting. The nitrogen content in dry matter was determined by the Kjeldahl method and the free poly(ethylene glycol) (non-bonded to gelatin) by the extraction of dry matter in chloroform for 24 h.

Model substances to determine the reaction mechanisms were prepared from glycine and ethylene glycol and from glycine and EO in the same way as from gelatin and EO. Glycine was chosen since it is the most frequent amino acid in collagen or gelatin, respectively.

1D ^{13}C n.m.r. spectra were taken on a Varian XL 400 spectrometer (100 MHz for carbon). The spectral window was 20.000 Hz, pulse width was 35 μs (90°) and pulse delay was 2 s. D_2O was used as solvent and all signals were referred to TMS. 2DJ ^{13}C n.m.r. spectra were taken on the same instrument at a spectral window 7000 Hz, pulse width 90°, double precision at normal temperature using a four step programme for incorporation of the high power decoupler. On the second step of accumulation the decoupler was switched off.

RESULTS AND DISCUSSION

There are several possibilities for the reaction between EO and gelatin: reaction at the –OH, –COOH, $-NH_2$, –NH– groups, in the side chains of the protein macromolecules and at the peptide bonds.

In *Table 1* experimental and analytical data of grafting EO onto gelatin are given. The quantity of dry matter and the content of nitrogen in the dry matter depend on the quantity of grafted EO. With increasing EO the

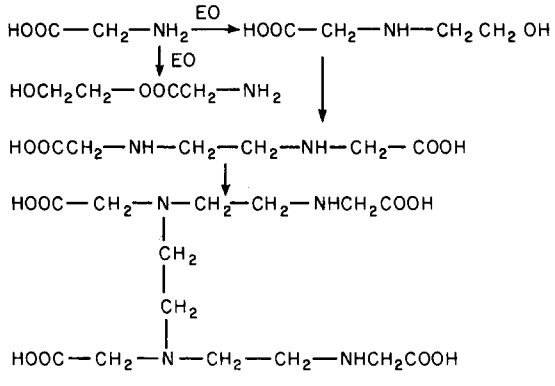
* Part of this work is extracted from the Ph.D. Thesis by A. Gantar, IUV Tannery, Vrhnika.

0032-3861/87/081403-04$03.00

quantity of dry matter increases while the quantity of nitrogen decreases. The quantity of free poly(ethylene glycol) formed during the reaction is very low, which indicates that grafting of EO is the prevailing reaction.

In *Table 2* the results of the analyses of the reaction products between glycine and EO are given. It is evident that EO reacts with glycine, since the concentration of nitrogen and free $-NH_2$ groups decreases with increasing concentration of grafted EO. Also it can be concluded that the reaction between EO and $-NH_2$ groups is the prevailing reaction, since the concentration of free –COOH groups does not alter.

1D ^{13}C n.m.r. spectra of the reaction products between glycine and EO show a significant decrease of $-CH_2-$ groups and an increased number of signals between 45 and 60 ppm which belong to the $-NHCH_2-$ groups, and signals between 60 and 63 ppm, which belong to the $-OCH_2-$ groups. The signals of the –COOH groups are shifted and a signal at 62.5 ppm which belongs to ethylene glycol can be seen. There is no evidence of ethylene glycol reacting with glycine and no evidence of the formation of poly(ethylene glycol), which has signals in the region between 62.5 and 72.0 ppm. From these data, the following reaction scheme for glycine with EO can be postulated:



The 1D ^{13}C n.m.r. spectra (*Figure 1*) of gelatin show a number of signals which are characteristic of different bonded amino acids. The assignments were made on the basis of literature data[5–10] and on the basis of the 2DJ ^{13}C n.m.r. spectra (*Figure 2*). The ordinate of the 2DJ ^{13}C n.m.r. spectrum represents the value of the 1D ^{13}C n.m.r. spectrum, while the abscissa represents the value of the carbon–proton coupling of the carbon atoms. In the 2DJ ^{13}C n.m.r. spectra each –CH< group gives two signals, each $-CH_2-$ group gives three signals and each $-CH_3$ group gives four signals. All of them have the same 163 Hz coupling constant. The intensities of the signals were expressed by the contour of the signals and are in the same ratio as in the non-decoupled spectra: for doublets 1:1, for triplets 1:2:1 and for quartets 1:3:3:1. In the 2DJ ^{13}C n.m.r. spectra of gelatin are four peaks (quartets) at 16 and 21 ppm, which belong to $-CH_3$ groups of leucine and alanine, three peaks (triplet) between 20 and 48 ppm which belong to $-CH_2-$ groups and two peaks (doublet) in the region between 50 and 73 ppm, which belong to the –CH– groups. At 55.5 ppm along the ordinate four peaks can be seen which are split in triplets along the abscissa. These peaks belong to $-CH_2-$ groups of hydroxyproline bonded to different amino acids in gelatin. The signals for the $-CH_2-$ groups of the hydroxyproline ring could be shifted to higher or lower magnetic field due to change of neighbouring –NH– groups or due to interactions with different neighbouring N or O atoms. The signals

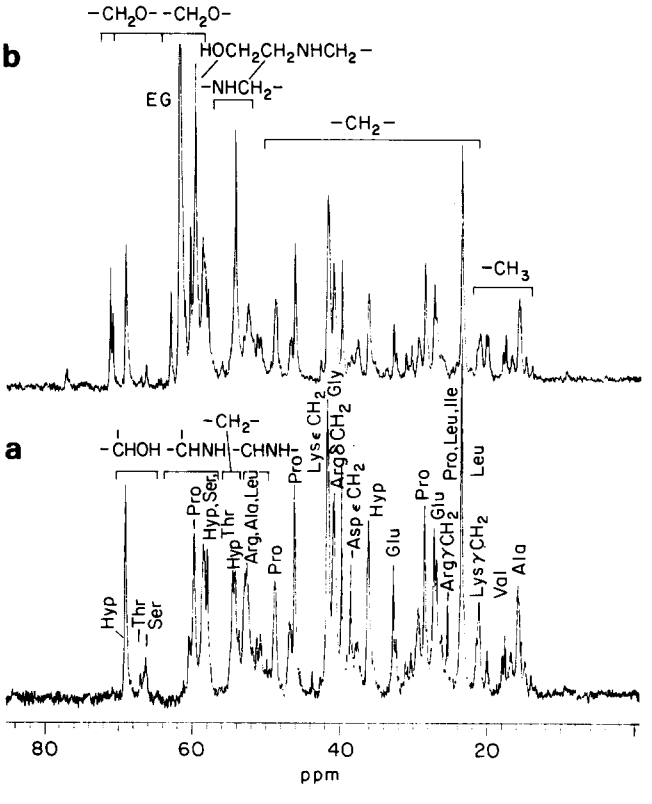


Figure 1 1D ^{13}C n.m.r. spectra of gelatin: (a) before reaction with ethylene oxide, (b) after reaction with ethylene oxide

Table 1 Quantities of dry matter, and bonded EO, nitrogen and free poly(ethylene glycol) in dry matter, respectively, in the reaction products between EO and gelatin

EO/gelatin (%)	Dry matter (%)	Grafted EO (%)	Nitrogen (%)	Poly(ethylene glycol) (%)
0	7.7	0	17.1	0
20	8.9	15.6	13.6	0
50	9.8	28.6	11.7	3.0

Table 2 Quantities of dry matter, and bonded EO, nitrogen, free –COOH and $-NH_2$ groups in dry matter, respectively, in the reaction products between glycine and EO

EO/glycine (%)	Dry matter (%)	Grafted EO (%)	Nitrogen (%)	–COOH (ml 1N NaOH/g)	$-NH_2$ (ml 1N NaOH/g)
0	10.0	0	18.4	4.2	12.5
30	12.0	20.0	15.4	4.3	8.3
50	14.1	41.0	12.1	4.4	5.6
54	15.5	55.0	11.1	4.2	4.5
117	16.3	63.0	10.5	4.0	3.8

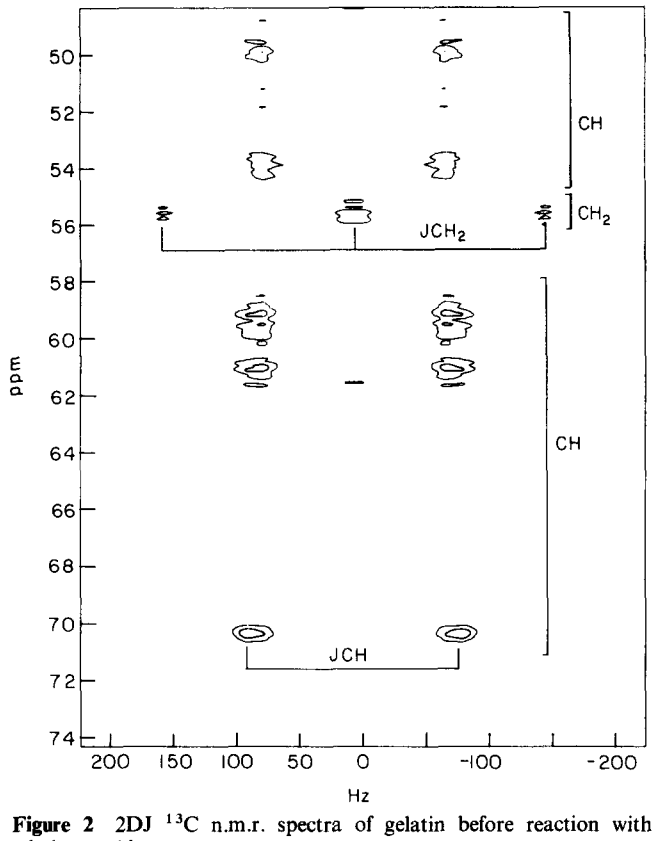


Figure 2 2DJ ^{13}C n.m.r. spectra of gelatin before reaction with ethylene oxide

between 65 and 70 ppm belong to –CHOH groups.

The 1D ^{13}C n.m.r. spectra of gelatin after the reaction with EO are changed for a number of signals. The changes are due to new signals of EO bonded to gelatin and due to shifting of the C atoms of gelatin because of grafting. The differences between the two spectra can be seen in *Figure 1*. Most differences are in the region of 52–72 ppm.

Comparing the 2DJ ^{13}C n.m.r. spectra of gelatin before and after grafting (*Figures 2* and *3*) it can be seen that all new signals belong to $-CH_2-$ groups (triplets). The signals of –CHOH groups belong to the non-grafted gelatin and the signal at 55.5 to $-CH_2-$ groups of hydroxyproline. These signals changed neither in position nor in intensity.

Comparing the 1D and 2DJ ^{13}C n.m.r. spectra in the region between 52 and 72 ppm it can be seen that signals in the 1D ^{13}C n.m.r. are partly or completely overlapped, while in the 2DJ ^{13}C n.m.r. spectra they are well separated.

The new signals in the 1D and 2DJ ^{13}C n.m.r. spectra of gelatine after grafting were assigned on the basis of literature data[11–13] as in the case of grafted glycine and on the basis of model substances. The strong new signal at 62.4 ppm belongs to unbonded ethylene glycol and the signal at 54.5 ppm to $-CH_2-NH-CH_2-CH_2-$ groups which could be formed from amino acids with free $-CH_2-NH_2$ or $-CONH_2$ and $-C(=NH)-NH_2$ groups. Such acids are lysine, arginine and asparagine which, together, amount to 15% of total (*Table 3*). The new signal at 59.3 ppm belongs to the CH_2OH carbon of the $-NH-CH_2-CH_2-OH$ group, which is grafted onto lysine, arginine and asparagine. Due to the grafting of EO onto $-NH_2$ groups the positions of $\varepsilon-CH_2-$ groups of lysine at 40.8 ppm, and of γ and $\delta-CH_2-$ groups of arginine at 25.3 and 40.5 ppm changed.

Since the signal of the carbon atom at 157.5 ppm after grafting did not change either position or intensity, it can be concluded that the NH=C– group in the end group

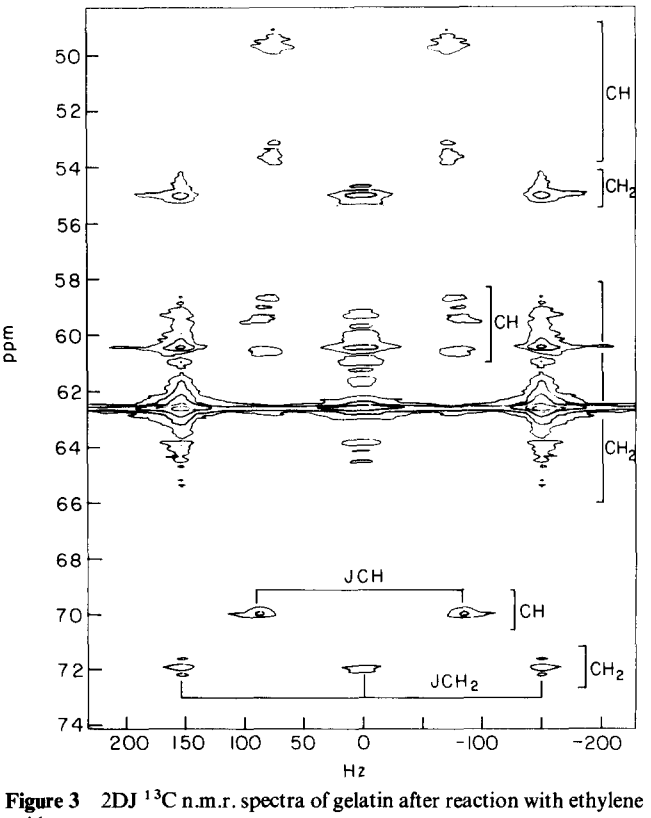


Figure 3 2DJ ^{13}C n.m.r. spectra of gelatin after reaction with ethylene oxide

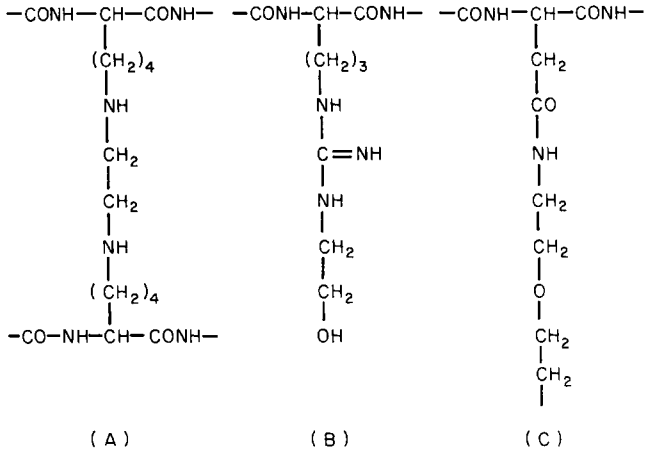
Table 3 Chemical shifts of different groups in gelatin after grafting with EO

		Chemical shifts (ppm)	
		before grafting	after grafting
$HOCH_2CH_2OH$			62.4
1 2 3 $-CH_2NHCH_2CH_2OH$			
(Lysine)	1	40.8	42.8
	2	–	54.5
	3	–	59.3
NH 1 ‖2 3 4 $-CH_2CNHCH_2CH_2OH$	1	40.5	41.0
Arginine	2	157.5	157.5
	3	–	54.5
	4	–	59.3
O 1 ‖2 3 4 $-CH_2CNHCH_2CH_2OH$	1	38.3	37.5
Asparagine	2	174.5	174.5
	3	–	54.5
	4	–	59.3
1 2 3 4 $-NHCH_2CH_2O(CH_2CH_2)_xO-$	1	–	54.0–56.0
	2	–	60.0–63.0
	3	–	70.0–72.0
	4	–	70.0–72.0

NH=C(NH$_2$)–NH– of arginine did not react with EO.

Five new signals between 60 and 73 ppm belong to –CH_2O– carbons of the –$NHCH_2$–CH_2O– groups grafted onto gelatin. The two signals at 70 and 71 ppm belong to poly(ethylene glycol) with different molecular weights and the signals at 60 and 63 ppm to the other –CH_2– group of the poly(ethylene glycol) chain grafted onto gelatin. The signals of HOCH– groups of hydroxyproline, threonine and serine at 66 and 69 ppm did not change position or intensity after grafting. It can be concluded that EO was grafted onto gelatin on the free –NH_2 groups only.

On the basis of all these data the following reaction products could be assumed when EO is grafted onto gelatin:



All amino acids with free –NH_2 groups could react in the three ways. This means that EO could be bonded to –NH_2 groups which are located on the side chains of the amino acids (cases B and C) or when the steric circumstances are favourable, with two basic amino acids forming a bridge between two protein chains (case A). In case (A) an essential increase of molecular weight can be expected.

CONCLUSION

Under the selected reaction conditions grafting of ethylene oxide onto gelatin took place on free –NH_2 groups of the side chains of the basic amino acids (lysine, arginine, asparagine) only. Ethylene oxide did not graft onto the –COOH, –CH, –OH groups or onto the peptide groups of gelatin.

Hetero two-dimensional ^{13}C n.m.r. spectroscopy is thus an excellent method for characterization of the complex reaction products between gelatin and ethylene oxide.

ACKNOWLEDGEMENT

The 1D and 2DJ ^{13}C n.m.r. spectra were recorded at the University of Akron, Akron, USA, the staff of which we gratefully acknowledge.

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