The kinetics of 1,4-butanediol diglycidyl ether crosslinking of dermal sheep collagen

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Abstract: Dermal sheep collagen was crosslinked with 1,4-butanediol diglycidyl ether (BDDGE) or modified with glycidyl isopropyl ether (PGE). The reduction in amine groups as a function of time was followed to study the overall reaction kinetics of collagen with either BDDGE or PGE. Linearization of the experimental data resulted in a reaction order of 2 with respect to the amine groups in the PGE masking reaction, whereas a reaction order of 2.5 was obtained in the BDDGE crosslinking reaction. The reaction orders were independent of the pH in the range of 8.5–10.5 and the reagent concentration (1–4 wt %). The reaction order with respect to epoxide groups was equal to 1 for both reagents. As expected, the reaction rate was favored by a higher reagent concentration and a higher solution pH. Because the BDDGE crosslinking reaction occurs via two distinct reaction steps, the content of pendant epoxide groups in the collagen matrix was determined by treating the collagen with either *O*-phosphoryl ethanolamine or lysine methyl ester. The increase in either phosphor or primary amine groups was related to the content of pendant groups. Crosslinking at pH 9.0 resulted in a low reaction rate but in a high crosslink efficacy, especially after prolonged reaction times. A maximum concentration of pendant epoxide groups was detected after 50 h. Reaction at pH 10.0 was faster, but a lower crosslinking efficacy was obtained. At pH 10.0, the ratio between pendant epoxide groups and crosslinks was almost equal to 1 during the course of the crosslinking reaction. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 51, 541–548, 2000.

Key words: collagen; crosslinking; epoxy compounds; kinetics; pendant groups

INTRODUCTION

Crosslinking of collagen-based materials can be achieved by bifunctional reagents such as glutaraldehyde,[1–4] hexamethylene diisocyanate,[5,6] dimethyl suberimidate,[7,8] and diglycidyl ethers.[9–11] These compounds, which have aldehyde, isocyanate, bisimido ester, and epoxide functional groups, respectively, react with the amine groups of (hydroxy)lysine residues present in the collagen. Despite the common use of these crosslinking agents for stabilization of the collagen component of biomaterials, few studies on reaction kinetics have been described in the literature. A few studies deal with the kinetics of collagen crosslinking using epoxy compounds in aqueous solutions.[10,11] Also, extensive studies on amine-epoxy systems used in epoxy resins have been carried out.[12–14] Several kinetic models which describe the curing process were proposed and tested experimentally. In general, these nonaqueous systems contain a mixture of an aromatic diepoxy compound in the presence of primary and secondary amines. Sometimes a hydroxyl-containing compound such as water, an alcohol, or an acid is added to influence the curing reactions. The hydroxyl group promotes the interaction of the epoxide group with an amine, by forming a trimolecular transition state.[14] If no catalyst is added, an initial reaction rate dependency proportional to the square of the amine concentration is measured. Addition of a hydroxyl-containing compound decreases the order with respect to the initial amine concentration.

Eloundou et al. reacted 1,4-butanediol diglycidyl ether (BDDGE) with 4,9-dioxane-1,12-dodecanediamine at relatively low temperatures, between 50 and 95°C. They found two reaction orders with respect to the amine groups of 1.20 and 0.45 for the noncatalyzed

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and the hydroxyl group catalyzed reaction, respectively. Moreover, it was deduced that especially at lower temperatures the reaction of a primary amine with an epoxide group is considerably faster than the reaction of a secondary amine group with an epoxide group.[13]

In the present study, the kinetics of the reaction of the amine groups of a collagen matrix using the monofunctional glycidyl isopropyl ether (PGE) or BDDGE was evaluated. A better understanding of the reaction kinetics enables one to optimize the reaction process in the development of bioprosthetic materials.

The overall reaction kinetics including the reaction orders with respect to the concentration of epoxide and amine groups were determined. In addition, the content of pendant epoxide groups as a function of the reaction time was measured to further elucidate the reaction mechanism.

MATERIALS AND METHODS

Dermal sheep collagen (DSC) was obtained from the Zuid-Nederlandse Zeemlederfabriek (Oosterhout, The Netherlands) and was prepared as reported previously.[9] The fibrous collagen network was washed four times with deionized water, twice with acetone, and twice times with deionized water before lyophilization.

Crosslinking

Lyophilized DSC samples (0.50 g) were immersed in 50 mL of a carbonate [0.064*M* sodium hydrogencarbonate ($NaHCO_3$)/0.036*M* sodium carbonate (Na_2CO_3), pH 10.0] buffered solution containing either 0.5, 1, 1.5, 2, 3, 4, or 5 wt % BDDGE (Fluka, Buchs, Switzerland) or 1, 3, 4, or 5 wt % PGE. The reaction was allowed to proceed for definite times at 20°C, followed by extensive washing with deionized water and lyophilization.

The effect of the solution pH on crosslinking or PGE modification was performed following the next procedure. A collagen sample (0.50 g) was immersed in 50 mL of a buffered solution containing 4.0 wt % BDDGE or PGE. The solution was buffered either with 0.025*M* disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$; Merck, Darmstadt, Germany) at pH 8.5 or 9.0 or with 0.064*M* $NaHCO_3$/0.036*M* Na_2CO_3 at pH values of 9.5, 10.0, and 10.5. Reactions were carried out for definite times at 20°C. The samples were washed with deionized water before lyophilization.

Characterization

The amine group content of the collagen samples was determined spectrophotometrically[1,9] after reaction of the primary amine groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) and subsequent hydrolysis of the sample, and is expressed as the number of groups present per mole of collagen (n/mol).

Pendant epoxide groups were determined by the following procedures. Lyophilized DSC samples were crosslinked at pH 9.0 or pH 10.0 as described above for definite times at 20°C. After reaction, the samples were extensively washed before lyophilization. Subsequently, the BDDGE crosslinked samples were either treated with a large excess of lysine methyl ester or with *O*-phosphoryl ethanolamine following the next procedures.

Lysine methyl ester

A sample of crosslinked collagen (0.20 g) was immersed in 20 mL of a 0.1*M* carbonate buffered solution (adjusted with NaOH to pH 10.0) containing 0.5*M* lysine methyl ester dihydrochloride (Sigma Chemical Co., St. Louis, MO). A large excess was taken to promote one-sided reaction, and hence to minimize the content of crosslinking reactions. The reaction was allowed to proceed for 3 days at 20°C. Thereafter, the samples were thoroughly rinsed with water before lyophilization.

The amount of pendant epoxide groups was related to the increase in amine groups before and after lysine methyl ester treatment, as determined by the TNBS assay described above.

O-phosphoryl ethanolamine (O-PEA)

A sample of crosslinked material (0.20 g) was immersed in 20 mL of a 0.05*M* $Na_2B_4O_7 \cdot 10H_2O$ buffer (adjusted with NaOH to pH 9.0) containing 0.5*M* O-PEA (Sigma Chemical Co.). The reaction was allowed to proceed for 3 days at 20°C, followed by extensive washing with water before lyophilization. About 50 mg of O-PEA–treated collagen was hydrolyzed in 4.0 mL 6*M* HCl at 110°C for 20 h. Exactly 0.5 mL of the hydrolyzate was added to 1.0 mL deionized water. Subsequently, 0.8 mL 0.08 wt % hydraziniumsulfate (z.A. Merck, Darmstadt, Germany) and 0.4 mL 5.0 wt % sodium molybdate dihydrate (ACS; Sigma Chemical Co.) were added. The reaction was carried out for at least 3 h at 60°C. The absorbance of the blue solution was measured at 820 nm.[15] The amount of coupled O-PEA was correlated to a calibration curve made of hydrolyzed O-PEA solutions.

Kinetics

Reaction rates were determined by measuring the decrease in amine groups as a function of time. The reactions that were taken into account are presented in Figure 1. Hydrolysis of the epoxide groups is thought not to affect the reaction kinetics because the hydrolysis rate of the reagents is low and <6% of the initial epoxide groups was hydrolyzed after 160 h of reaction at pH 9.0.[9] Furthermore, the reaction rate of epoxide groups with amine groups is supposed to be much slower than the diffusion rate of the reagents to the reaction sites, as shown by Tu et al.[11]. Finally, the reaction

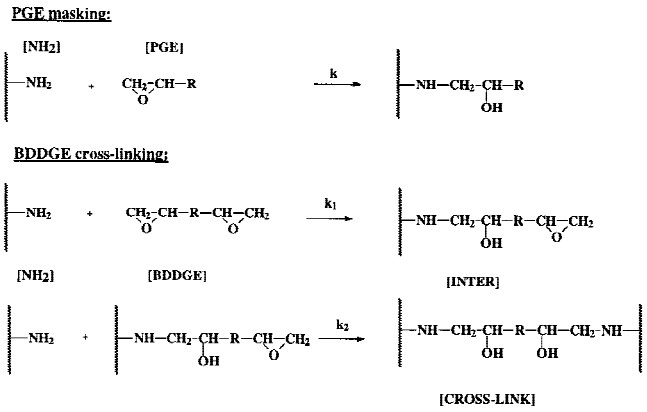

Figure 1. Reactions of amine groups of dermal sheep collagen with PGE or BDDGE.

between secondary amines and the epoxide groups was not taken into account.

The overall kinetics of the crosslinking and masking has been described previously,[11,16] and the reaction orders were determined using the equation:

$$\frac{1}{\left(\frac{[NH_2]_t}{[NH_2]_0}\right)^{(\beta-1)}} = 1 + Kt \quad (1)$$

with

$$K = (\beta - 1)k[\text{Epoxide}]_0^{\alpha}\,[NH_2]_0^{\beta} \quad (2)$$

where [Epoxide] and $[NH_2]$ are the concentrations of the epoxide and the amine groups, t = reaction time, k = overall reaction rate constant, α = reaction order with respect to epoxide groups, and β = reaction order with respect to the amine groups of the collagen material.

The free amine group concentration of the collagen, expressed as the number per 1000 amino acids, was measured as a function of the reaction time; the data were fitted to Equation (1). For a given reaction order of β, a plot of $1/[([NH_2]_t/[NH_2]_0)^{(\beta-1)}]$ against time affords a straight line with a slope K. The value of β was calculated by applying a linear regression on the experimental data.

The order of the reaction gives no direct information about the mechanism of the reaction, although it may give some clues.[16] The crosslinking reaction of collagen with BDDGE most probably takes place via distinct sequential steps, thus forming an intermediate (INTER). The concentration of the intermediate depends on the reaction rates k_1 and k_2 and may also depend on the availability of amine groups in the collagen matrix. Therefore, the concentration of pendant epoxide groups was determined as a function of time to assess the ratio of crosslinks and masked amine groups during the course of the reaction.

RESULTS

Dermal sheep collagen was crosslinked with BDDGE or modified with PGE at pH 10.0. Reaction between the amine groups of the collagen and the epoxide groups of either BDDGE or PGE took place as reflected by a decrease in free amine groups as a function of the reaction time (Fig. 2).

Higher concentrations of both PGE and BDDGE resulted in a faster reduction of amine groups. Despite the almost similar epoxide concentration in the BDDGE and PGE reaction solutions, a faster decrease in amine groups as a function of the reaction time was observed during BDDGE crosslinking. This implies that the second reaction step of the BDDGE crosslinking reaction (k_2 in Fig. 1) contributes considerably to the overall reaction rate. The reaction orders with respect to the amine groups of the collagen and the epoxide groups of the BDDGE and PGE were determined.

PGE reaction

The kinetic data of the PGE reaction (Fig. 2) were fitted to the kinetic model by linear regression. It was found that a β of 2 resulted in the best fit (Fig. 3) for the modification reaction with either a 1 or 4 wt % PGE solution. The pH of the reaction solution was altered to investigate whether the reaction kinetics are applicable for a pH range between 8.5 and 10.5 (Fig. 4). Application of a β of 2 resulted in all cases in a good fit.

BDDGE reaction

The kinetics of the crosslinking reaction of the DSC with bifunctional BDDGE were determined. Following the procedure as described for the masking with PGE and as described by Tu et al.,[11] a β of 2.5 was

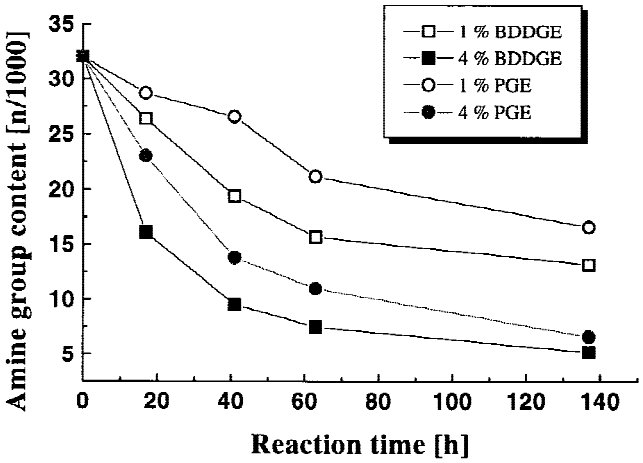

Figure 2. Amine group content as a function of the reaction time during masking with PGE or during crosslinking with BDDGE at two different concentrations (1 and 4 wt %, pH 10.0, 20°C, 1 g collagen in 100 mL reaction solution).

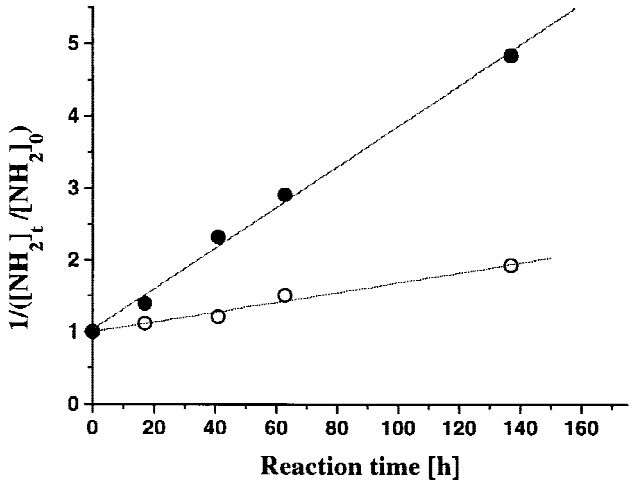

Figure 3. Fit of the kinetic data of the PGE masking reaction with a 1 wt % (○) or 4 wt % (●) solution using second-order reaction kinetics with respect to amine groups of the collagen.

found at different epoxide concentrations and pH of the solution. This value of $\beta = 2.5$ is the same as determined in the crosslinking of bovine internal thoracic arteries with ethylene diglycidyl ether.[11] An increase in the slope of the lines showed that the reaction rate was significantly higher at a higher pH.

The reaction order with respect to the epoxide groups was determined by measuring the K values at different epoxide concentrations. An α of 0.98 was found in case of the PGE masking reaction, whereas an α of 1.08 was obtained for BDDGE crosslinking. These results are similar to those found for the collagen fixation with ethylene diglycidyl ether (Ex-810).[11]

Reaction of collagenous materials with bifunctional reagents introduces more than crosslinks. If the second reaction step (Fig. 1) cannot take place, because no adjacent amine group is available (or already has reacted), pendant epoxide groups will be introduced. It is expected that the extent of this masking of the polypeptide chains will influence the final physical and mechanical properties of the material (vide supra). The concentration of these pendant epoxide groups (INTER in Fig. 1) was determined by reaction with either a large excess of lysine methyl ester or O-PEA. The increase in primary amine or phosphate groups, respectively, was subsequently determined. The concentration of crosslinks is related to the decrease in amine groups and the increase in the free epoxide groups, using: $[\text{Crosslinks}] = ([NH_2]_0 - [NH_2]_t - [\text{INTER}])/2$. The results obtained upon crosslinking of DSC with a 4 wt % solution of BDDGE at pH 9.0 or 10 are presented in Figure 5.

At pH 9.0, a slow decrease in the number of free amine groups was observed. The number of pendant epoxide groups reached a maximum after 50 h; after 150 h, no free epoxide groups were detected. More

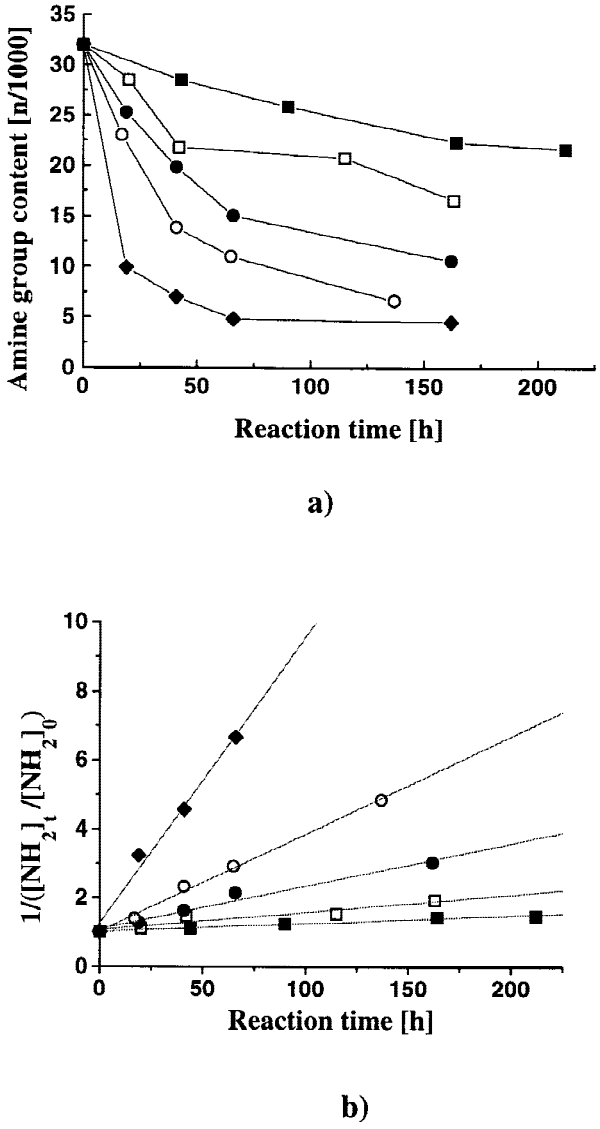

Figure 4. Content of amine groups as a function of reaction time at different pH values (a). Fit of the kinetic data for second-order reaction of the PGE masking reaction of dermal sheep collagen at different pH values (b). ■ = pH 8.5; □ = pH 9.0; ● = pH 9.5; ○ = pH 10.0; ♦ = pH 10.5. Reaction conditions: 4 wt % PGE, 20°C, 1 g collagen in 100 mL reaction solution.

important, a continuous increase in crosslink density was detected during the course of the reaction. A faster decrease in amine groups was found at pH 10, and both the concentration of pendant epoxide groups and crosslinks increased gradually with time.

The concentration of BDDGE and deprotonated amine groups present in the collagen material had an effect on the rate of reaction, degree of crosslinking, and crosslink efficacy. The contents of amine and pendant epoxide groups as well as the amount of crosslinks formed were determined as a function of the initial BDDGE concentration after 24 h reaction time (Fig. 6). An increase in epoxide concentration re-

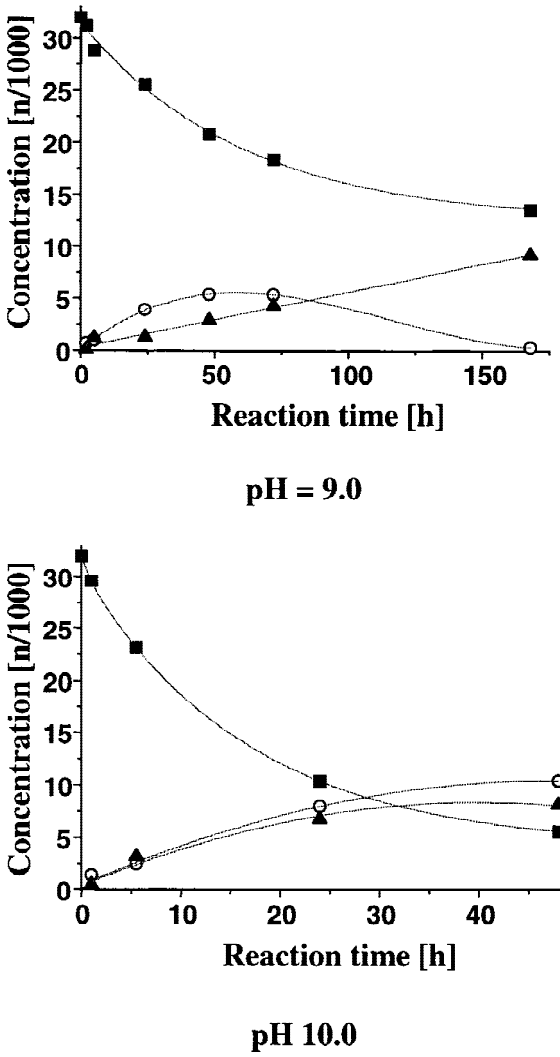

Figure 5. Concentrations of free amines (■), intermediates (○), and crosslinks (▲) as a function of the reaction time during crosslinking of dermal sheep collagen at pH 9.0 and 10.0 (4.0 wt % BDDGE, 20°C, 100 mL/g collagen).

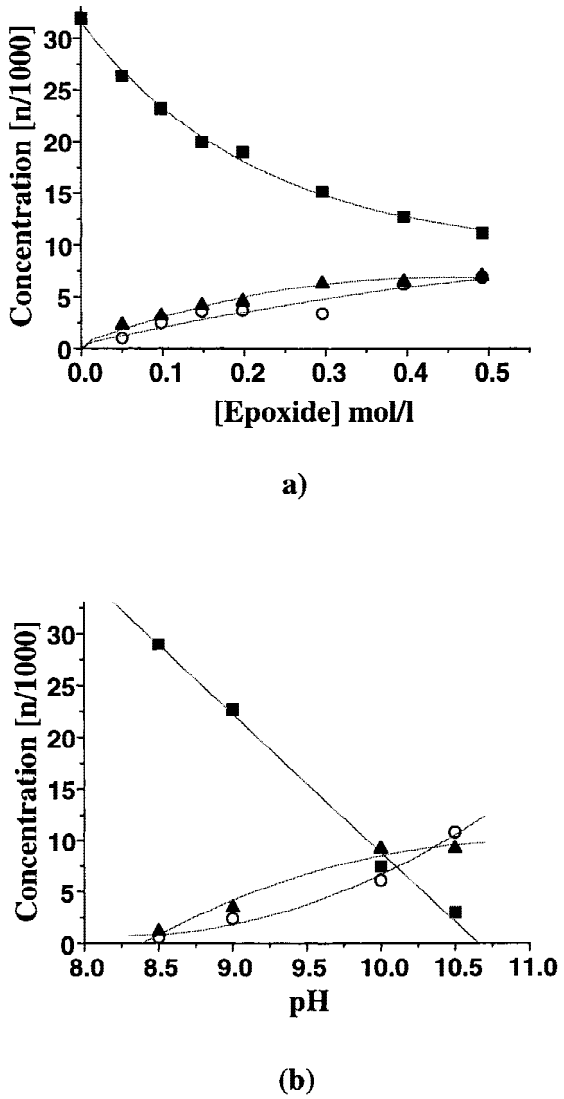

Figure 6. Concentration of amine groups (■), intermediates (○), and crosslinks (▲), respectively, as a function of the initial epoxide (a) concentration (pH 10.0, 24 h, 20°C, 1 g collagen/100 mL reaction solution) or as function of the pH (b) (4.0 wt % BDDGE, pH 8.5, 9.0, 10.0, 10.5, 24 h, 20°C, 1 g collagen/100 mL reaction solution).

sulted in a lower content of amine groups. The crosslink efficacy was only slightly affected, as indicated by a constant ratio of pendant epoxide groups and crosslinks. Increasing the amount of unprotonated amine groups by performing the reaction at a higher pH revealed a faster decrease of amine groups after 24 h of crosslinking. A linear relationship between the concentration of amine groups left in the matrix and the solution pH was found. As a result of the increased rate of the reaction, the amount of pendant epoxide groups introduced increased at higher pH values and approached or even became higher than the amount of crosslinks formed.

DISCUSSION

Crosslinking of tissues rich in collagen is a well-known method in the preparation of bioprosthetic materials such as wound dressing, vascular grafts, tendons, and aortic heart valves. Glutaraldehyde has been the most widely applied and most extensively studied crosslinking agent. During the past decade, other methods were developed[17–19] to crosslink collagen-based tissues. A relatively new group of crosslinking reagents comprises the poly glycidyl ethers, and several of these multifunctional reagents appeared to be successful in crosslinking of collagen-based materials.[20–23] Despite the large amount of research in this area, the kinetics of crosslinking of collagen materials with epoxy compounds have been described only briefly.[10,11] Tu et al. crosslinked bovine internal thoracic arteries with the bifunctional ethylene diglycidylether and with the trifunctional glycerol polyglyc-

idyl ether. Crosslinking was carried out at 37°C under basic conditions (pH 8.5, 9.5, or 10.5) using three different epoxy compound concentrations of 0.5, 1.0, and 4.0 wt %. They found for all reaction conditions that the decrease in amine groups was first order in the epoxy compound and 2.5th order in amine groups of the collagen-based tissue.[11]

Recently, the crosslinking of DSC with (BDDGE) was described. Under alkaline conditions, this reagent will react with the amine groups of (hydroxy)lysine residues. By controlling the reaction conditions, the crosslink density and efficacy can be altered.[9] Elaboration of the reaction kinetics makes it possible to optimize the crosslinking process and thus design the material properties. To distinguish the two reaction steps in the crosslinking with the bisepoxy compound BDDGE, the kinetics of the masking with the monoepoxy compound PGE were determined. It is assumed that the kinetics for the PGE reaction and the first step in the crosslinking are approximately the same. Figure 1 summarizes the reactions which were expected to lead to a crosslink or masking of an amine group. Because the experimental data showed (Fig. 2) that the reaction with either BDDGE or PGE was slow and not complete within 24 h and that no linear relationship was found for the decrease in amine groups as a function of time, it was concluded that the reaction process is not diffusion controlled.

The reaction order with respect to the amine groups of the collagen was determined by application of Equation (1) to the kinetic data. The value of β, the reaction order with respect to the amine groups, was determined by linear regression of the experimental data. Masking of the amine groups with PGE resulted in a β of 2, which was independent of the solution pH in the range 8.5–10.5 and the PGE concentration (1–4 wt %). On the contrary, a β of 2.5 was found when the collagen was crosslinked with the bifunctional BDDGE, similar to that determined for the collagen fixation with Ex-810.[11] Analogously to the PGE reaction, the value of β was not affected by the BDDGE concentration or pH. This higher value of β suggests that the second reaction (k_2), which resulted in the formation of a crosslink, had a considerable effect on the overall reaction kinetics.

In addition, after the first step, the second epoxide group of the BDDGE molecule could react only with the amine groups which are closely located to the pendant group owing to spatial limitations. Therefore, the effective amine concentration for the second epoxide group was considerably lower than for an incoming BDDGE molecule. However, when a pendant epoxide group was already close to an adjacent amine group, the possibility of this reaction was higher than the reaction of an amine group with the epoxide reagent. Because a faster decrease in amine groups was observed in the BDDGE crosslinking reaction compared to the corresponding PGE masking reaction, the second step appeared to have a higher reaction rate constant than the first step.

As shown in Figure 4, a faster decrease in amine groups was found when the reaction was carried out at a higher pH. The effective concentration of deprotonated amine groups depended on the pH according to the Henderson–Hesselbach equation

$$\mathrm{pH} = pK_a + \log \frac{[\mathrm{NH_2}]}{[\mathrm{NH_3^+}]}$$

or

$$\frac{[\mathrm{NH_2}]}{[\mathrm{NH_3^+}]} = \frac{[H^+]/K_a}{1 + [H^+]/K_a} \tag{3}$$

with pK_a (lysine in collagen[23]) = 10.0 and $[H^+] = 10^{-\mathrm{pH}}$.

A higher effective concentration of amine groups increased the value of K and consequently the overall reaction rate. A linear plot was obtained when the logarithm of K was plotted as a function of the solution pH. The gradient of this line was 1.8 for both PGE and BDDGE (data not shown), which shows that the effect of the pH on the reaction rate was similar for both reactions.

The reaction order with respect to the epoxide groups (α) was equal to 1 for both the PGE and BDDGE reactions. This value, which was also determined by Tu et al.,[11] shows that the same reaction kinetics are valid for the reaction of an incoming BDDGE or PGE molecule with an amine group of the collagen.

The overall reaction rate equation does not give information about the mechanism of the crosslinking. A pendant epoxide group is introduced in the collagen matrix after the first step in the BDDGE crosslinking. Determining the content of these groups as a function of the reaction time will provide detailed information on the course of the crosslinking. DSC crosslinked with BDDGE for different times at pH 9.0 or 10.0 was treated with either an excess of lysine methylester or with O-PEA to quench the pendant epoxide groups. Figure 7 presents the reactions which occurred during lysine methylester or O-PEA treatments.

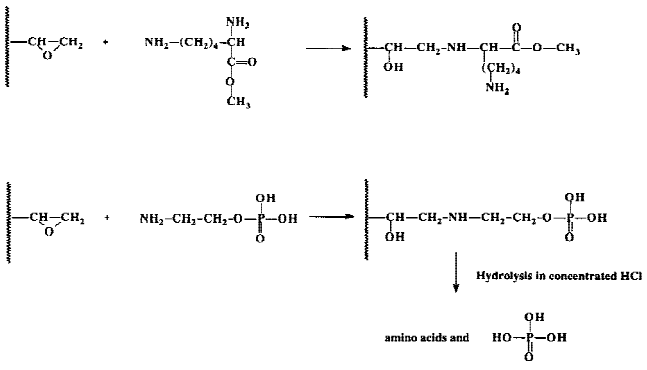

Figure 7. Postreactions of free epoxide groups with lysine methyl ester or *O*-phosphoryl ethanolamine.

Treatment of BDDGE crosslinked collagen with lysine methyl ester did not result in additional crosslinking, as indicated by similar values of the shrinkage temperature before and after treatment (data not shown). This implies that only one amine group of lysine methylester reacted with a pendant epoxide group. The content of epoxide groups can thus be related to the increase in primary amine groups. Moreover, both the lysine methyl ester and O-PEA method afforded the same results. The values obtained are regarded a good indication of the concentration of pendant epoxide groups in the material.

Crosslinking at pH 10.0 showed a faster decline in amine groups (Fig. 5) compared to the reaction at pH 9.0. During crosslinking, competition takes place between the reaction of the epoxide group of an incoming BDDGE molecule with an amine group (k_1) and the reaction of a pendant epoxide group with the same amine group (k_2). A higher pH, and thus a higher effective concentration of deprotonated amine groups will increase the possibility of both reactions. Because of the higher concentration of free BDDGE molecules in the solution, the effect of a higher pH will be more pronounced for the first reaction (k_1). Hence, the formation of crosslinks will be suppressed, which leads to a lower crosslink efficacy. Crosslinking at pH 9.0 showed a rapid increase in pendant epoxide groups in the first stage of the reaction while the amount of crosslinks was only slightly elevated. Therefore, in the initial stage of reaction mainly masking reactions occurred which were responsible for the decline in amine groups. It seems that, because of the low concentration of deprotonated amine groups, the possibility of the reaction of the second epoxide group with an adjacent amine group was low, and the first reaction dominated in the initial stage. After approximately 50 h, a maximum was observed in the amount of pendant epoxide groups. After this, the formation of crosslinks dominated the overall reaction. The increase of the crosslink density was supported by an increase in the shrinkage temperature from 59 to 71°C between 50 and 168 h of crosslinking.[9]

Crosslinking at pH 10.0 resulted in both masking and crosslinking reactions. A maximum in pendant epoxide groups was observed after 48 h. At this time, the content of amine groups was reduced to a level of 4 (n/1000), which seems to be the lower limit of accessible amine groups.[9] As a result, no further crosslinks could form and a relative large amount of pendant epoxide groups remained in the matrix. The lower value of Ts = 62°C of a material crosslinked at pH 10.0 compared to that at 9.0 (Ts = 71°C) is in accordance with this high degree of masking and somewhat lower degree of crosslinking.

Selecting a reaction time of 24 h, the extent of crosslinking and masking was determined to be a function of the BDDGE concentration at pH 10.0 (Fig. 6) and of pH. The data showed that although the number of free amine groups reacted increased, the ratio of pendant groups and crosslinks formed was the same. Therefore, high pH values (>9.0) do not seem efficient even though the crosslinking time becomes shorter. This is confirmed by the data presented in Figure 6(b). After 24 h and at a BDDGE concentration of 4 wt %, an increase in pH revealed fast crosslinking but also a high degree of masking. It is concluded that optimal crosslinking conditions were obtained at a pH of 9.0 despite the long reaction times of 7 days, as depicted in Figure 5.

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