INVESTIGATION OF THE REACTION MECHANISM BETWEEN BOVINE COLLAGEN AND A TRIAZINE-BASED COUPLING REAGENT

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Abstract. The triazine-based coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) is a promptly water-soluble white solid commonly used in chemical synthesis, which is proven to act as effective tanning agent. This research work provides an experimental evidence that the tanning ability of DMTMM is associated to an increase of the cross-linking density in the collagen molecule. As a result of the coupling reaction, DMTMM is converted into water-soluble by-products that can be removed by washing.

1 Introduction

The compound 4-(4,6-Dimethoxy-1,3,5-Triazin-2-yl)-4-Methylmorpholinium Chloride (DMTMM) is a versatile triazinic coupling agent[1] used in organic synthesis to promote the formation of carboxamides via direct condensation of carboxylic acids with amines.[2,3] Although several chemical species are available for the same purpose,[4,5] DMTMM is attractive for green chemistry industrial applications because it is highly water-soluble[6,7] and is commercially available in the form of air- and moisture-stable solid powder (melting point 116 – 117 °C).[8] It can be stored at room temperature for up to a month or for several months in the refrigerator without detectable decomposition.[9]
This research work provides experimental evidence to the hypothesis that the powerful tanning effect of DMTMM is due to an increase of amide cross-link density in the collagen molecule. The reaction is investigated here using soluble collagen and DMTMM in aqueous solution; furthermore, the coupling reaction has been modelled by condensing a carboxylic acid and an amine with DMTMM in water.

2 Materials and Methods

DMTMM, acid soluble Calf Skin Type I Collagen and D_2O (99.9 % D), amylamine (≥ 99%) and propionic acid (ACS reagent, ≥ 99.5%) were purchased from Sigma Aldrich. Thermogravimetric analyses were performed with a Q5000 IR instrument with a temperature gradient set at 10 °C/min, in the interval 50 - 700 °C. A Q20 DSC calorimeter was used for differential scanning calorimetry measurements; both were supplied by TA Instruments. The ^{1}H-NMR spectra were recorded at room temperature (298 K) with a Bruker DRX-400 (400 MHz) spectrometer. The Fourier transform infrared spectroscopy of samples was performed with a JASCO FT/IR-4100 Type A spectrometer equipped with a PIKE MIRacle ATR accessory (Ge crystal); spectra were recorded with 128 scans per spectrum at 2 cm^{-1} resolution. MS spectra of all samples were collected with an Agilent Technologies LC-MSD-Trap-SL 10367 system. A 1:1 mixture of H_2O and acetonitrile (ACN), both acidified with 0.1% CH_3COOH, was used as eluent.

An unexpected, massive presence of citric acid was detected in the commercial collagen sample, likely attributable to the extraction procedure from the biological material.[10,11] By means of TGA measurements, it was possible to estimate a content of about 18 % wt. of citric acid. In order to purify the product, a solution was prepared in milliQ water (28.8 mg in 5.76 mL, pH ≈ 4) and subjected to dialysis in a Pur-A-Lyxer Purg10010 Dialysis Kit (Sigma-Aldrich) with a MW cut-off of 1000 Da. The solution was dialysed versus 500 mL of milliQ water, periodically replaced, at room temperature under continuous stirring for 6 days. The solution was eventually freeze-dried in a Lio-5 P Freeze-Drier (CiK Solutions) at T ≈ -50 °C and p ≈ 0.5 mbar, obtaining 20.0 mg of solid collagen (yield 69%). Elimination of citric acid was confirmed by the disappearance of the corresponding signal in the ^{1}H-NMR spectrum of a collagen solution in D_2O (figure 1).

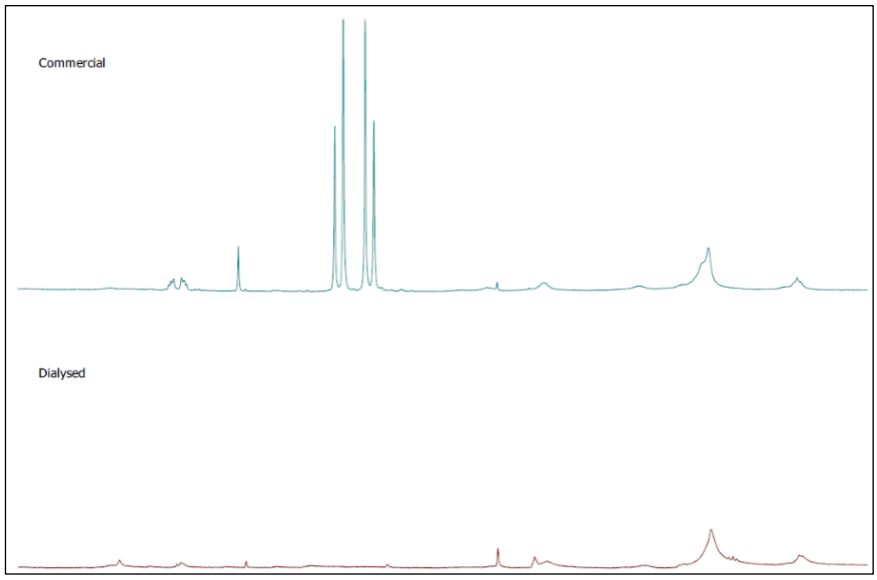

Figure 1. ^{1}H-NMR spectra of commercial and purified collagen.

3 Results

3.1 Cross-linking reaction in water soluble collagen

An aqueous solution of collagen was prepared dissolving 20.0 mg of pristine solid collagen (see Materials and Methods) in 4000 µL of milliQ water and maintaining it under stirring for 8 hours at room temperature. An amount of 22.1 mg of DMTMM was then added while keeping the solution under stirring. Three hours later, the formation of small aggregates was observed, and an additional quantity (22.1 mg) of DMTMM was added to the mixture at this point. After other 2 hours, a whitish gel-like agglomerate appeared within the solution (figure 2). The liquid mixture was subsequently dialysed with the protocol described above in order to remove by-products and residual DMTMM. After freeze-drying, 7.7 mg of solid was recovered. The by-products of the reaction are expected to be DMT-OH (Figure 3) and N-methylmorpholine (NMM), both water-soluble.[4]

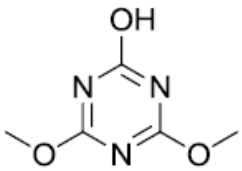

Figure 2. DMT-OH

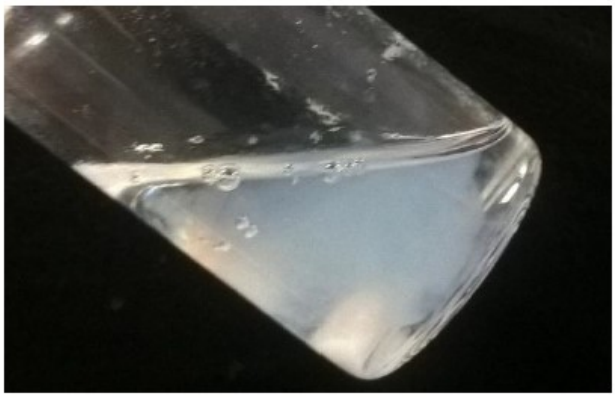

Figure 3. Gel-like agglomerate in the collagen solution after reaction with DMTMM.

3.2 Thermal Gravimetric Analysis (TGA)

The comparative TGA analysis was performed under air flux with 0.340 mg of purified collagen and 0.499 mg of cross-linked product. The results (figure 4) show that the bound water content is 7.9% for the pristine sample and 3.6% for the cross-linked collagen, indicating that, after the reaction with DMTMM, the collagen matrix becomes less hydrophilic. Since the formation of amide bonds takes place at the expense of the highly hydrophilic carboxylic and amino moieties in the collagen side chains, a lower affinity of the substrate for water is expected as one consequence of the crosslinking reaction, in line with the result of TGA measurements.

Another notable outcome of the comparison of TGA curves is that the reaction product starts to decompose at a lower temperature than pristine collagen (see figure 3), indicating that it is slightly less stable. Because of the structural role of water in the collagen molecule,[12,13,14] this may correlate with the lesser content of bound water resulting from TGA measurements.

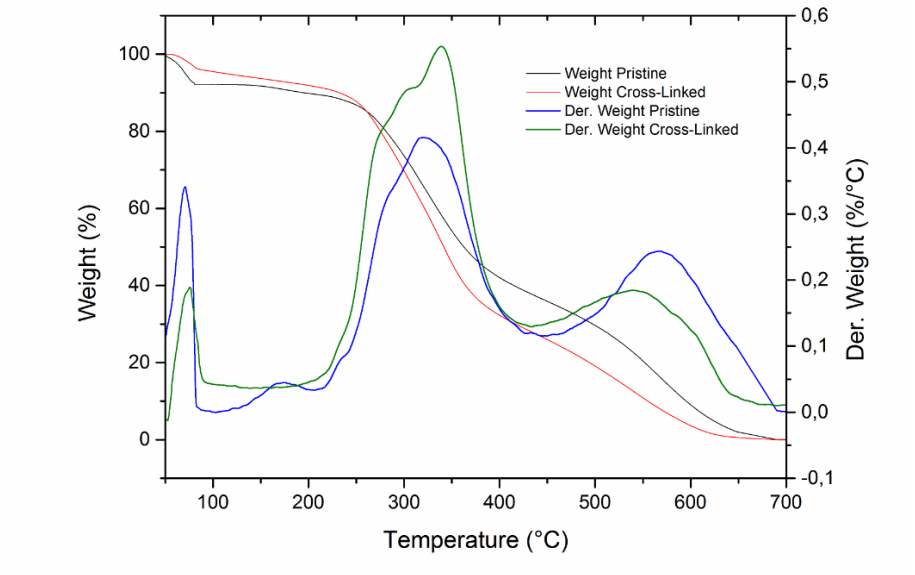

Figure 4. TGA and D-TGA curves of pristine (black and blue) and cross-linked (red and green) collagen.

3.3 Fourier transform infrared spectroscopy

Infrared spectroscopy is thoroughly exploited in the investigation of protein structure.[15] Specifically, resonances related to the peptide bonds are classified as Amide I, Amide II, Amide III, Amide A, etc. based on their position in the spectrum, and they are influenced by the structural features of the protein. For the collagen molecule, the Amide I band falls at around 1650 cm^{-1}, the Amide II at 1550 cm^{-1}, the Amide III at 1245 cm^{-1},[16] while overtones and Fermi resonances (Amide A and B) are found at 3293 cm^{-1} and 3097 cm^{-1}, respectively.[17]

FT-IR spectra of both freeze-dried pristine and cross-linked collagen are shown in figure 5. The similarity between band frequencies for the two samples indicates that the transformations undergone by collagen do not affect its chemical composition, although some structural modifications occurred. The most notable differences regard Amide A and Amide II bands. The Amide A band is independent on the backbone conformation but is very sensitive to the strength of hydrogen bonds (note that it is superimposed to the O-H stretching band of collagen-bound water). For quantitative comparison, the spectra were normalized using the intensity of the C=O band (Amide I), assuming that the amidation reaction has no effect on it. The decrease of the Amide A band in the cross-linked collagen indicates the disappearance of amino groups; since the cross-linking degree is correlated to the ratio A_I/A_A, the increase of this ratio from 2.90 to 3.73 in the final product confirms that DMTMM promotes cross-linking in collagen and suggests that the reaction product contains less bound water. The decrease of both Amide II and the carboxylate symmetric stretching band at around 1401 cm^{-1} in the reaction product indicates the reduction of amino and carboxyl groups, consistent with an increase of cross-linked collagen.

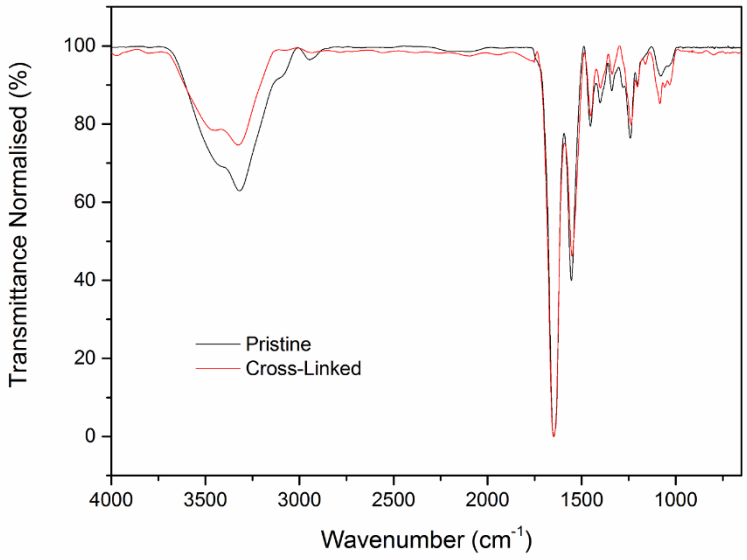

Figure 5. Superimposed FT-IR spectra of collagen before (black line) and after (red line) reaction with DMTMM.

The last observation regards the difference between the frequencies of Amide I and II, which denotes the presence of denatured collagen for $\Delta\nu > 100$ cm^{-1}.[18] In this case, the values are 94 cm^{-1} for the original sample and 95 cm^{-1} for the reaction product, indicating that no denaturation occurred after reaction of collagen with DMTMM.

3.4 Amide condensation in a homogeneous aqueous system

The water-based amidation that leads to increasing the cross-linking density in collagen has been further investigated by reacting the two water-soluble substrates propanoic acid (pKa = 4.9) and amylamine (n-pentylamine, pKa = 10.2) in presence of DMTMM. To this purpose, 100 µL of amylamine and 64.4 µL of propionic acid were dissolved in 400 µL of water and thoroughly mixed. An appropriate amount of HCl 0.1 M was added to the mixture until the pH was lowered from the initial value (about 9) to 6.5, at which value both amino and carboxylic functional groups are electrically charged; in these conditions, the most effective nucleophile in solution is the deprotonated acid.

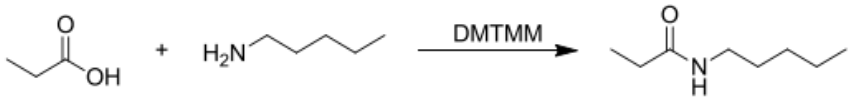

Figure 6. Amide coupling reaction between propionic acid and amylamine.

An amount of 0.2348 g of DMTMM was subsequently added to the mixture while keeping it under stirring. Within a few minutes a white precipitate appeared, and the pH spontaneously decreased to about 4. A small aliquot of the suspension was dissolved in ACN/H_2O 1:1 and, after properly dilution, it was characterised by ESI-MS technique. The resulting mass spectrum (figure 7) demonstrates that N-pentylpropanamide is formed, as expected.

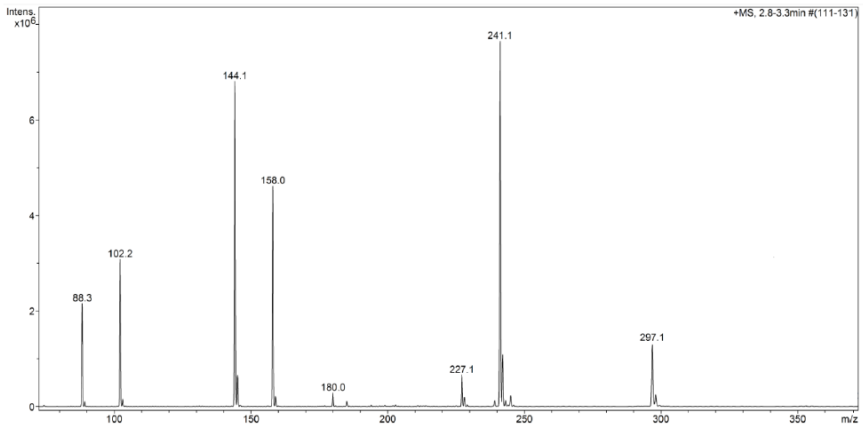

Figure 7. ESI-MS spectrum of the reaction mixture between propionic acid and amylamine.

The assignment of the signals in the spectrum are as follows: 88.3 m/z [amylamine-H+], 102.2 m/z [NMM-H+], 144.1 m/z [N-pentylpropanamide-H+], 158.0 m/z [DMTOH-H+], 180.0 m/z [DMTOHNa+], 227.1 m/z [DMTM-H+], 241.1 m/z [DMTMM+] and 297.1 m/z [(DMT)2O-H+]. Propionic acid was not detected. Thus, ESI-MS confirms that the coupling reaction was successful both for the presence of the amide and of all the expected by-products of DMTMM (NMM and DMT-OH) signals. The mass of the demethylated DMTMM is also present, indicating that de-methylation represents a spontaneous decay route for DMTMM in aqueous environment. Finally, the mass at 297.1 m/z, which could be assigned to bis(4,6-dimethoxy-1,3,5-triazin-2-yl) ether, $(DMT)_2O$, confirms the occurrence of a solvolysis side reaction. A plausible reaction pathway that leads to the formation of the triazine ether is the nucleophilic attack of the negative charged oxygen of DMT-O^- towards the partially positively charged carbonyl carbon of the keto form of DMT-OH (figure 8).

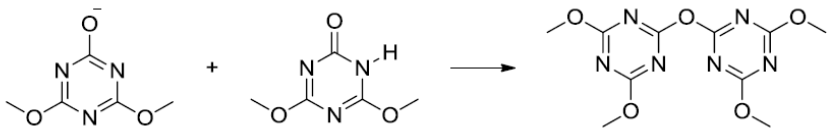

Figure 8. Proposed mechanism for the formation of $(DMT)_2O$.

4 Conclusion

This research work contributes to elucidating the tanning mechanism of DMTMM in aqueous medium. It was demonstrated by means of various techniques (TGA and DSC, but especially with FT-IR) that the cross-linking density in soluble collagen increases after reaction with DMTMM.
The mediating role of DMTMM in water-based amide synthesis was further confirmed with a simple model reaction where reagents (propionic acid and amylamine) are both water-soluble. It was also shown that DMTMM in water undergoes demethylation via solvolysis.

References

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