University of Twente

Bachelor Assignment

Structure-property correlations of hybrid networks from Poly(trimethylene carbonate-co-ϵ-caprolactone) and human recombinant collagen

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July 11, 2023

Abstract

This study gave the structural-property correlations of the hybrid networks made with P(TMC-co-ϵ-CL) and human recombinant collagen. To make to functionalized copolymer, the oligomer P(TMC-co-ϵ-CL) was polymerized with 1,1,1 methylol propane, resulting in a 3-armed molecule with alcohol groups at each end. Methacrylic anhydride was used for the functionalization. With the use of ^{1}H-NMR the molecular weight turned out to be 21000 g/mol and the degree of functionalization was 99%. To make the functionalized Ecol, the collagen was dissolved in PBS and stirred for several days. Methacrylic anhydride was also used for the functionalization. After adding the MA, the collagen was dialyzed against demi water for 4 more days and freeze-dried afterward for 6 more days. ^{1}H-NMR with acetic acid D4 was used to show the characteristics of functionalization. After both the copolymer and collagen were properly functionalized, they were separately dissolved in DMSO pH2 overnight. 4 different networks (collagen, 3:5, 3:15, and copolymer) were prepared in duplo. Irgacure 2959 was used as a photoinitiator and the samples were eventually photo crosslinked with irradiation of 365nm at $-15°$C under nitrogen for 1 hour. Images of the networks and SEM images were taken after network formation. The networks showed high gel contents between 89.3% and 99.7%, increasing water uptake when more collagen was present in the network and high porosity ranging between 77.1% and 84.1%. Ratios were determined using FTIR, which didn't differ a lot from the intended ratios. Thermal properties were gathered using DSC and TGA, showing similar T_g values and an increase in decomposition temperature up till the 3:15 network. Mechanical properties were gathered with the use of DMA, which provided the E-Modulus, σ , ε_{break}, Toughness, SRS and $Toughness_{srs}$. The toughness and $Toughness_{srs}$ showed an increasing trend and were significantly high for the 3:15 ratios. Overall a lot of structural property correlations were successfully determined in this study.

Deze studie gaf de correlaties tussen structuur en eigenschappen van de hybride netwerken gemaakt met P(TMC-co-ϵ-CL) en menselijk recombinant collageen. Om het gefunctionaliseerde copolymeer te maken, werd het oligomeer P(TMC-co-ϵ-CL) gepolymeriseerd met 1,1,1-methylolpropaan, resulterend in een 3-armig molecuul met alcoholgroepen aan elk uiteinde. Methacrylzuuranhydride werd gebruikt voor de functionalisatie. Met behulp van ^{1}H-NMR bleek het molecuulgewicht 21000 g/mol te zijn en de graad van functionalisatie 99%. Om het gefunctionaliseerde Ecol te maken, werd het collageen opgelost in PBS en enkele dagen geroerd. Methacrylzuuranhydride werd gebruikt voor de functionalisatie. Na het toevoegen van MA werd het collageen 4 dagen lang dialyseerd tegen demi-water en vervolgens 6 dagen lang gevriesdroogd. ^{1}H-NMR met azijnzuur D4 werd gebruikt om de kenmerken van de functionalisatie te tonen. Nadat zowel het copolymeer als het collageen op de juiste wijze waren gefunctionaliseerd, werden ze afzonderlijk opgelost in DMSO pH2 gedurende de nacht. Er werden 4 verschillende netwerken (collageen, 3:5, 3:15 en copolymeer) bereid in duplo. Irgacure 2959 werd gebruikt als fotoinitiator en de samples werden uiteindelijk gephotocrosslinked met bestraling van 365nm bij $-15°$C onder stikstof gedurende 1 uur. Beelden van de netwerken en SEM-beelden werden genomen na de netwerkvorming. De netwerken vertoonden hoge gelgehaltes tussen 89,3% en 99,7%, een toename van wateropname wanneer er meer collageen aanwezig was in het netwerk, en een hoge porositeit variërend tussen 77,1% en 84,1%. Verhoudingen werden bepaald met behulp van FTIR, die niet veel verschilden van de beoogde verhoudingen. Thermische eigenschappen werden verzameld met behulp van DSC en TGA, waarbij vergelijkbare T_g-waarden werden verkregen en een toename van de ontledingstemperatuur tot het 3:15-netwerk. Mechanische eigenschappen werden verkregen met behulp van DMA, waarbij de E-modulus, σ, $\varepsilon_{\text{break}}$, taaiheid,

SRS en $taaiheid_{\text{srs}}$ werden bepaald. De taaiheid en $taaiheid_{\text{srs}}$ vertoonden een stijgende trend en waren significant hoog voor de 3:15-verhoudingen. Over het algemeen werden in deze studie veel correlaties tussen structuur en eigenschappen met succes bepaald.

Contents

1 Introduction

1.1 Relevance

Biomedical engineering plays an important role in advances in new technology and medicine. In the field of tissue engineering, the aim is to create tissues that are appropriate and acceptable for their host. With new advantages in biomedical engineering, more and better tissue can be provided for research. When looking at the field of tissue engineering its demand has increased due to the exponential growth of people that need operations or help in which tissue engineering is needed. Rising chronic diseases, accidents, and trauma injuries are a big factor in the contribution to its growth. People in need of implants are also increasing[1][2]. When looking at the market of tissue engineering, is expected to grow to 31,23 billion USD by 2030, more than doubling its global market size compared with 2021 as seen in Figure 1. With an increase in the need for better and compatible tissue development, further research about biomaterials is required. Especially because biomaterials are used as the main components of many tissue replacement therapies [2]

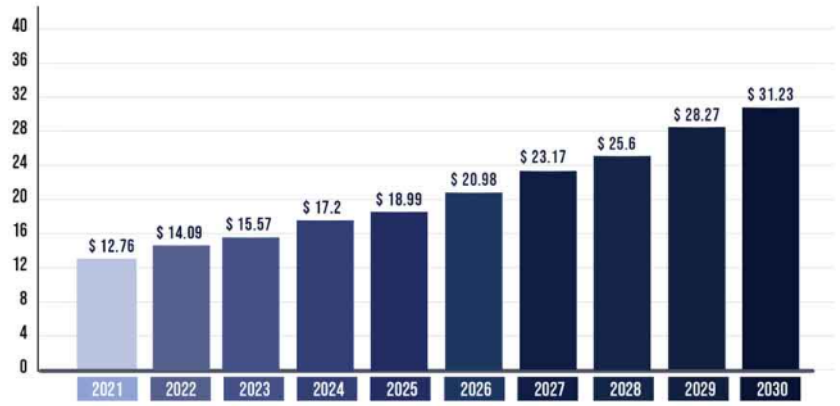

Figure 1: Expected market size of Tissue Engineering from 2021 to 2030 in USD billion dollars [2].

1.2 Biomaterials

In tissue engineering biomaterials play an important role. The biomaterials provide an architectural framework, similar to the extracellular matrix, to encourage cell growth and start tissue regeneration [3]. There are a lot of different biomaterials leading up to many different properties to choose from. Biomaterials and a combination of these are used in several drug delivery devices, cell encapsulation devices, dental composites, tissue adhesives, tissue barriers and to create scaffolds [4]. When biomaterials are used for scaffolds, each scaffold differs in functionality depending on the kinds of biomaterials being used. Synthetic and natural biomaterials are often used to create specific characteristics of scaffolds. Adjusting and mixing these different types of biomaterials opens up a wide range of biodegradation, surface-to-volume ratio, and mechanical properties. Often a mixture is used to create the optimal characteristics to integrate into the host and fulfill its functions, without its environment poorly responding to it. [5].

Biomaterials are derived from polymers, metals, ceramics, composites, and biological materials such as collagen, hyaluronic acid, and chitosan[6]. This thesis focuses on polymers. Polymers are mainly divided into synthetical and natural polymers. Natural polymers are furthermore categorized into polypeptide and protein-based, polysaccharide-based, and polynucleotide based. The main advantages of natural polymers are their bioactivity, biocompatibility, geometry, nontoxic by-products of biodegradation, and structural resemblance to biological extracellular matrices.[5]. The disadvantages are decreased tunability, immunogenic reaction, uncontrollable degradation, and poor mechanical strength. Synthetic polymers are advantageous because of their tunable properties, forms, and established structures. The mechanical strength is also more tunable due to interlinkage and adjustable molecular weight[5].

Both natural and synthetic polymers have their advantages and disadvantages. Combining different properties of biomaterials can give promising results in the future and might be able to enhance scaffold material[7]. For example, combining the strong mechanical properties of a biomaterial with the biocompatibility of another can be a good option if used in the right circumstances and environment. Due to the wide range of biomaterials, it's difficult to find the optimal mix of biomaterials itself that is the most usable for its host. Combining different types of biomaterials might be able to open new paths for improved scaffolds or other innovative uses.

In tissue engineering biodegradable polymers such as poly(trimethylene carbonate) (PTMC) and the polyesters polycaprolactone (PCL) and poly(d,l-lactide) (PDDLA) have been investigated for biomedical purposes. The polymers differ in characteristics and properties from each other. For example, PTMC is a flexible polymer, which suits soft tissue engineering. PDDLA is used in hard tissues such as bone regeneration due to its high elastic modulus of close to 3GPA ($N/m^2 * 10^9$). PDDLA is one of the few polymers that comes close to the mechanical properties of bone, which is around 3-30 GPA[8]. With the use of copolymerization, copolymers can be created with combined characteristics and properties to adjust to the right field of tissue engineering. After the functionalization of different polymers, photocrosslinking can be used to further enhance the properties by creating a network[9]. Covalent bonds will be formed into polymer chains during photocrosslinking to form a network. For instance when photocrosslinking PTMC, it can lead to improved mechanical properties. The cross-linking of PTMC led to significantly higher stress at break values, while the strain at break values remained very high in earlier studies [10]. Photocrosslinking can also improve the thermal properties and alter the degradation rate of PTMC.

1.3 PTMC, PCL, and ECOL

PTMC is a polymer prepared by ring-opening polymerization of cyclic trimethylene carbonate. It is an amorphous polymer with a low glass transition temperature of -19 °C. When crosslinking PTMC, the resistance to creep will highly increase. PTMC networks can be acquired by gamma-irradiation or by photocrosslinking functionalized macromers[11] based on TMC with the use of UV or visible light. In biomedical engineering, PTMC is widely used and very useful due to its good compatibility *in vitro* and *in vivo*. PTMC-based polymer networks show degradation with enzymatic surface erosion, which doesn't release any acidic degradation byproducts in contrast to PCL and PLA. Due to the degradation in surface erosion, the mass decreases but the mechanical properties of the polymer stay intact. Therefore PTMC is a well-suited biomaterial for tissue engineering and drug delivery and is investigated for vascular tissue engineering, bone defect repair, and drug-loading

implantation. [12][13]. Many characteristics such as the degradation rate can be altered when making a copolymer.

PCL (Polycaprolactone) is an aliphatic polyester that is capable of biodegrading, and it has a low melting point of approximately 60°C. Its glass transition temperature (T_g) is approximately -60°C. It is synthesized in the presence of a catalyst, through ring-opening polymerization of ϵ-caprolactone. The degradation of PCL happens when its ester bonds are broken down through hydrolysis under physiological conditions such as in the human body[14]. Another advantage of PCL is that it is relatively low-cost. These features have made it an area of interest for use as an implantable biomaterial, especially for the development of long-term implantable devices. Additionally, PCL could be used for controlled drug delivery. A drawback to PCL is its poor mechanical properties, which can be compensated by using it as a copolymer for other biomaterials[13].

Both TMC and CL are used to make a copolymer named poly(trimethylene carbonate-co-ϵ-caprolactone). With trimethylol propane, a three-armed polymer with a random sequence was created. The copolymer with its 3-arm formation is visualized in Figure 2. In earlier research about the degradation of this copolymer, a 50:50 molar ratio and a 75:25 molar ratio of PTMC:PCL were assessed. Both ratios showed degradation mainly based on surface erosion. However, the 50:50 molar ratio appeared to have bulk erosion in the later stages of degradation. To minimalize the bulk erosion a 75:25 molar ratio was made in this thesis[4].

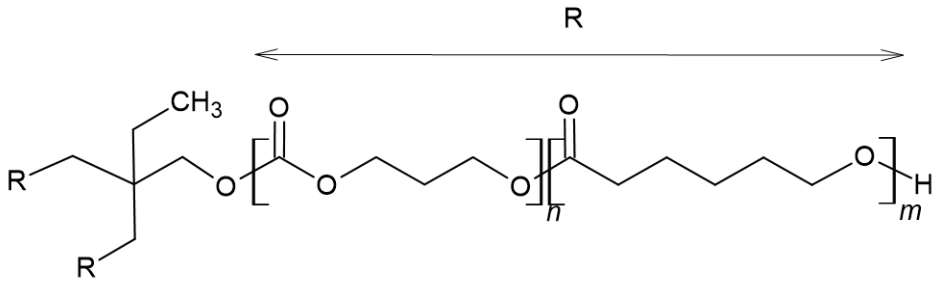

Figure 2: P(TMC-co-ϵ-CL) after formation in a 3 arm[15].

Most collagen used in tissue engineering has been isolated from animal tissue. The collagen that is mainly used is collagen Type 1. Type I collagen is suitable for transplantations and implantations because only a small amount of people develop an immune or allergic response against the collagen. A serologic test can be performed to look at the patient's immunity to prevent this from happening. Animal collagen generally has favorable biocompatibility and cell interaction with its host. Collagen type 1 contains all round good properties and is easily available, resulting in it being the golden standard in tissue engineering up till now [16]. Animal collagen still has some safety and quality disadvantages. It can cause immunogenic reactions and has a risk of infection. Contamination among the type 1 collagen also occurs. Although these disadvantages will not arise often, it is interesting to look further into new types of collagen.[17].

Collagen type 1 is a heterogeneous mixture and due to its amount of contaminations by other collagen and variability of crosslinking that differs with the animal's age and condition, it is difficult to retrace the origin of the material. Recently, it has become more available to obtain human collagen from recombinant DNA-based production systems. The human recombinant collagen is produced using yeast fermentation with the co-expressor human prolyl 4-hydroxylase (P4H). Using the expressor P4H it is shown that the stability and melting point of the collagen is increased. Human recombinant collagen has enhanced safety, performance, biocompatibility, and endurance compared to animal derive collagen but it's still in relatively early development[18].

Collagen-based networks have been made and used for tissue replacement of the liver, skin, blood vessels, and small intestine. A disadvantage of these networks is that they have a limited range of mechanical properties. The networks are in general short of physical strength for further application and are potentially immunogenic. To enhance its physical strength hybrid networks are formed. For further utilization of collagen-based networks with the use of other polymers, networks can be made that match the physical properties of other parts of the body.[19].

1.4 Aim

This thesis uses copolymers to make a three-armed macromer with trimethyl propanol as the initiator. Evonik collagen (natural) was also provided. With the copolymer and ECcol, a polymer mixture is made. With the created polymer mixture, this thesis will provide an answer to the research question:

What are the structural-property correlations of the hybrid networks from poly(TMC-co-ϵ-CL) and human recombinant collagen?

In this thesis, the copolymer and the collagen are functionalized with methacrylic anhydride (MA). The P(TMC-co-ϵ-CL)-tMA was already provided beforehand by preparation with 1,1,1 trimethylol propane to make it three-armed. The Ecol-MA was made during this thesis. With the use of MA, methacrylate groups will be made. The methacrylate groups contain a double bond in which a radical reaction between the functionalized groups is possible allowing for crosslinking.

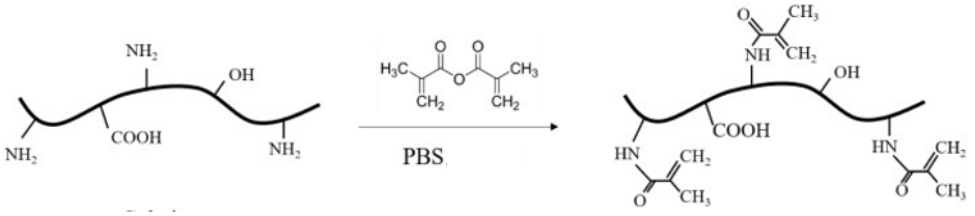

Figure 3: Reaction NH_2 groups collagen with MA to create methacrylate groups[20].

The network and its precursor materials will be characterized to get the results of the physico-chemical properties, thermal properties, and mechanical properties. Examples of techniques being used are FTIR, NMR, Total water content, DMA, SRS, DSC, and TGA. More information about the use of these techniques will be provided in the Materials and Methods chapter.

A polymer mixture of PTMC and ECOL, which is not much different from networks in this study, has been used in other studies and tested to try and create solid networks. The water uptake and gel content were also provided in this earlier study. This thesis aims to determine additional properties of the hybrid networks to obtain a better understanding of the structure-property correlations.

2 Materials and Methods

2.1 Materials

The collagen Ecol was provided by Evonik, Germany, PBS Omnipur Calbiochem, Methacrylic Anhydride, HCl, TNBS, $Sn(Oct)_2$, 2-Hydroxy-4' -(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) were purchased from Sigma Aldrich, the Netherlands, the dialysis membrane (12-14 kDa) was purchased from Spectre, the 21000g/mol P(TMC-co-ϵ-CL)MA was made by a ring opening polymerization under argon gas and functionalized by B. van Bochove.

2.2 Synthesis P(TMC-co-ϵ-Cl)-tMA

For the polymerization of the oligomer P(TMC-co-ϵ-CL), 1,1,1 methylol propane was used as an initiator. This resulted in a 3-armed molecule with alcohol groups at each end. The targeted ratio of TMC:CL was 75:25. The intended molecule weight of the created copolymer was 20000g/mol.

To achieve 20000g/mol and a ratio of 75:25, 0.24mol dried and distilled CL, 0.72mol TMC, and 0.005mol TMP were used. 10 drops of $SN(OCT)_2$ were used as a catalyst. The reaction was performed under 130° C for 3 days. The molecular weight (Mn) and degree of functionalization were determined from the ^{1}H-NMR results. Subsequently, the end functionalization was done by adding MA, triethylamine (TEA), and hydroquinone to the copolymer dissolved in 203ml DCM. MA and TEA were added in excess. The copolymer macromer was purified by precipitation with ethanol and dried under a vacuum.

2.3 Synthesis ECOL-MA

To make ECOL-MA the 1,0 gr ECOL was dissolved in 10ml PBS at RT. This solution was stirred overnight and the next day 1ml Methacrylic Anhydride was slowly added while stirring. The solution was shielded from light and stirred for another day. A 12-14 kDA MWCO dialysis bag was used for the dialyzation of the solution against demi water. This process lasted for 4 days, in which the dialysate was frequently replaced, to optimally remove the PBS minerals and unreacted methacrylic anhydride. Lastly, the solution was freeze-dried for 6 days. After freeze-drying, the obtained yield of ECOL-MA was 68,4%.

^{1}H NMR with acetic acid D4 was used to show the functionalization of the collagen (see Appendix 6.3).

2.4 Preperations of Networks

P(TMC-co-ϵ-CL)-tMA and ECOL-MA were separately dissolved in DMSO pH2 (acidified with HCl) overnight. The collagen at RT and the polymer at 70° Celcius. 100% collagen and 100% PTMC networks were made. Furthermore, 37,5:62,5 wt % and 16,7:83,3 wt % ECOL-MA:P(TMC-co-ϵ-CL)-tma networks were made. The networks were made in duplo. For the photoinitiator, Irgacure 2959 was chosen. The amounts used were 6 wt % of collagen and 1 wt % of P(TMC-co-ϵ-CL). The mixtures were mixed for 3 more hours and placed on a plastic petri dish. The samples were stored at $-24°$ Celcius overnight. The photocrosslinking was done with irradiation of 365nm at $-15°$ Celcius under a nitrogen atmosphere for 1 hour. The networks were swollen in DMSO for 1 hour

and demi water was added slowly for 20 more hours overnight to get rid of the unreacted groups and replace the DMSO with demiwater. Afterward, the networks were frozen and freeze-dried.

2.5 Physico-Chemical Properties

2.5.1 SEM

Samples needed to be prepared in order to evaluate them and make SEM images. Samples were frozen with liquid nitrogen and broken. The broken sides were evaluated with the SEM for each sample and the images were made.

2.5.2 Porosity

The porosity was evaluated by weighing 3 samples of each type of network. Subsequently, the volumes of the samples were calculated. With the density of the collagen and copolymer, it is possible to look at the porosity value in the amount of % using equation(1)

$$Porosity(\%) = (1 - \frac{m}{V \cdot p}) \times 100\% \tag{1}$$

Where m is the mass in grams, V is the volume in cm^3, and $\dot{p}$ the density in g/cm^3

2.5.3 Gel Content

The Gel content of the polymer is given by the following equation(2):

$$Gel(\%) = \frac{W_G}{W_T} \times 100\% \tag{2}$$

where Gel(%) is the Gel content in percentage, W_G is the weight of the insoluble fraction (gel) and W_T is the total weight of the polymer sample [21].

W_t is calculated using the equation(3):

$$W_T = W_o \times \frac{m_{polymer}}{m_{polymer} + m_{solvent}} \tag{3}$$

In which W_o is the mass of the networks right after crosslinking containing both polymer and collagen.

2.5.4 Total Water Content

The calculations of the total water content will be made with the wateruptake equation(4):

$$\mathrm{WU} = \frac{m_s - m_{\mathrm{dry}}}{m_{\mathrm{dry}}} \times 100\% \tag{4}$$

m_s is the mass of the polymer network swollen in water and m_{dry} is the mass of the dry polymer network [21]. The networks were swollen in water for 48 hours for the Water Uptake and weighed afterward.

2.5.5 FTIR

Fourier Transform Infrared Spectroscopy (FTIR) was used to determine the collagen:copolymer ratio. When looking at the peaks of the collagen (1640 cm^{-1} 'Amide1) and the peaks of the copolymer (1740 cm^{-1} 'C=O) the ratio can be calculated. The resolution was 4 cm^{-1}, 8 scans were made, and data between 4000 and 400 cm^{-1} was saved.

2.6 Thermal Properties

2.6.1 DSC

For Differential Scanning Calorimetry (DSC), 1 sample per prepared network was used. The weight of the samples was measured individually. The samples were cooled down to $-80°$C and heated to 150° C with a rate of 10°C/min. After this, the samples were cooled and heated again in the second cycle. Results of the second heating cycle were used to get the T_g of the networks.

2.6.2 TGA

For Thermogravimetric Analysis (TGA) 1 sample per prepared network was used. The samples were heated from $30 - 600°$ C with a heating rate of 10°C/min.

2.7 Mechanical Properties

2.7.1 Tensile Strenght

Dynamic Mechanical Analysis (DMA) used small network samples with measured length and thickness that were clamped and slowly pulled apart over time at a rate of 0.5mm/min, resulting in a stress-strain graph that gives the elastic modulus, max stress, elongation at break (%), and the toughness.

2.7.2 Suture retention Strength

DMA was also used for the Suture Retention Strength (SRS). Samples of the same size were used, and a small thread with a diameter of 0.1mm was added to the top of the sample. The force needed to break this thread through the sample was measured. Another stress-strain curve is analyzed to get the SRS and the toughness.

2.8 Statistical Analysis

A one-way analysis of variance (ANOVA) with a Bonferroni posthoc analysis was conducted to assess the variances in the results obtained from the multiple networks. Statistical significance was determined at a significance level of $p<0.05$.

3 Results and Discussion

3.1 Synthesis P(TMC-co-ϵ-CL)

During the polymerization of TMC with CL, the monomer conversion rate turned out to be 99% when looking at the peaks at δ2,08 and δ2,42 ppm and comparing their areas to those of the polymer.

The methyl group of trimethylolpropane at δ0.92 ppm was used as a reference peak to compare the areas of the data from the ^{1}H-NMR with the other peaks created by the TMC at δ2,05 ppm and CL at δ1,38, 1,65, and 2,31 ppm to calculate the number of units available in the molecule and the g/mol of the molecule. This showed a 74:26 ratio for TMC: Cl and a 21000 g/mol molecular weight of the copolymer, which doesn't significantly differ from the intended aim.

The area under the graph is considered and compared to determine the degree of functionalization. The peaks at δ5.57 and δ6.11 ppm were used to calculate the DOF of the copolymer. Comparing those peaks with the peak at δ0.92ppm, showed a degree of functionalization of 99%.

When considering the structure of the copolymer ^{13}C-NMR was used to verify that a random polymer was created instead of a block polymer. The ^{13}C-NMR results showed the presence of a random copolymer due to the double peaks that were present around 173-174 ppm and 155 ppm. Block polymers only show a single peak around these ppms. There also were peaks at around 60 ppm and 65 ppm. See Appendix 6.2. This is characteristic of random TMC/Cl copolymers[22].

3.2 Synthesis Ecol

Ecol was functionalized with methacrylic anhydride. ^{1}H-NMR with acidic acid D4 was used to look at the functionalization. The results showed peaks around δ5.5 and δ5.8 ppm, which indicate that methacrylic anhydride was present in the collagen. Furthermore, the ^{1}H-NMR showed a decrease around δ3 ppm. This shows that the amount of NH^2 groups decreased in the collagen due to the replacement with the methacrylate groups. The full ^{1}H-NMR spectrum is shown in Appendix 6.2. The use of ^{1}H-NMR is not unusual to look at the functionalization. In an earlier study about the characterization of methacrylated type-I collagen, it was also used to determine if the functionalization was successful[23].

A way of looking at the functionalization is by making use of a TNBS-assay. It is commonly used to determine the degree of collagen functionalization [24]. In earlier research with Ecol, the degree of functionalization was also determined with the use of a TNBS-assay[25]. In this study, the assay was also performed. The results of the TNBS-assay for the collagen that was made indicated a negative degree of functionalization. After performing it a second time with the same results we decided that the ^{1}H-NMR results were clear enough to continue with the Ecol-Ma.

3.3 Physico-Chemical Properties

3.3.1 Networks

The copolymer and collagen were dissolved separately because the copolymer needed to be dissolved in DMSO at 70°C. Later they were added together in different ratios. The networks were made in duplo. After photocrosslinking, the networks were successfully formed. A picture of the networks is given in Figure 4.

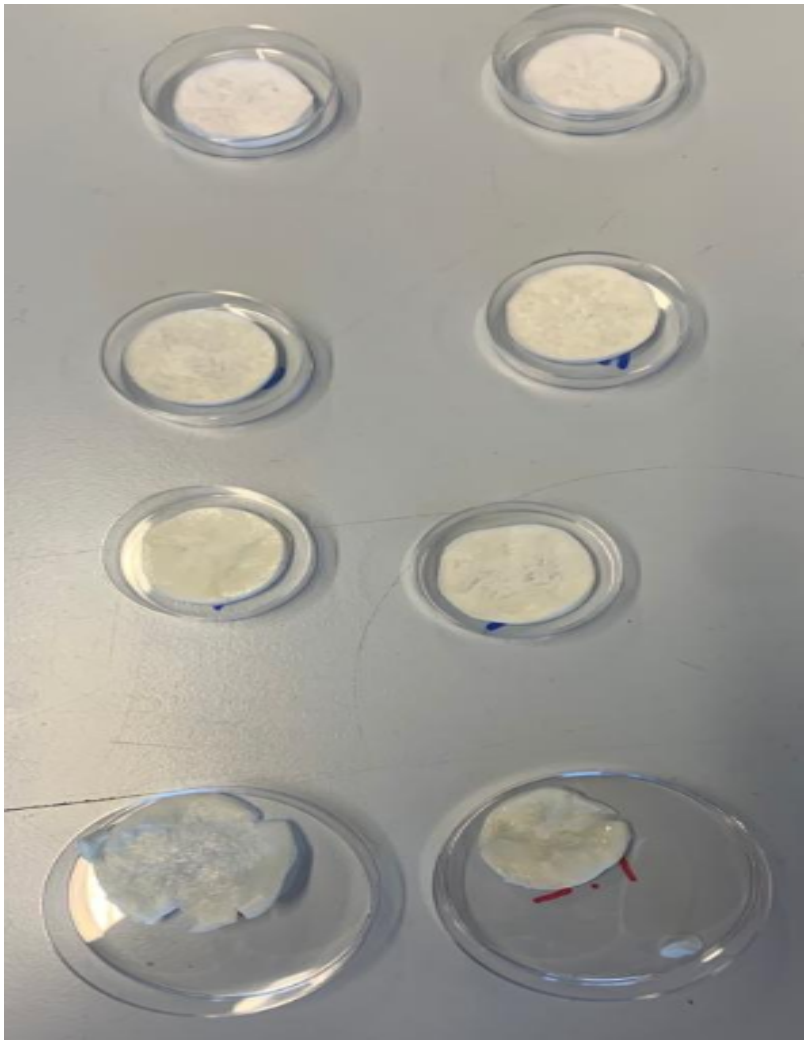

Figure 4: Polymer networks with collagen on the bottom following 3:5 ratio, 3:15 ratio, and lastly the copolymer network on the top.

One of the collagen samples, shown in Figure 4, appeared to be more swollen, as seen in the bottom left of the picture, which caused it to be a little bigger and thinner compared to the other

collagen samples. The samples with more copolymer don't differ a lot from each other based on this image.

SEM pictures were also made to examine the structure of the networks more in detail. These pictures are provided in Figure 5.

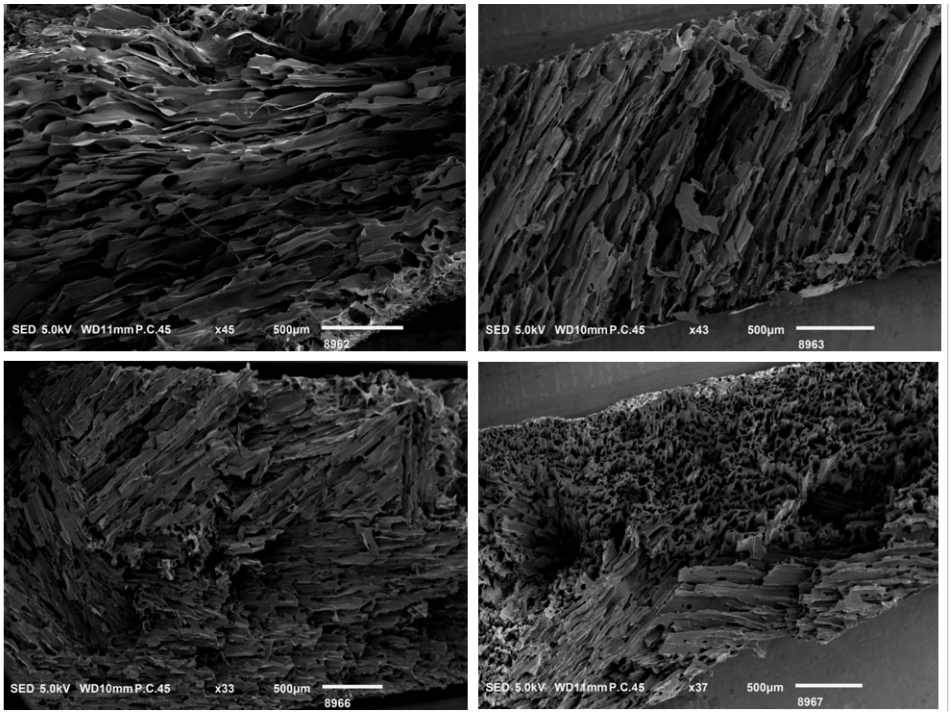

Figure 5: SEM images of the polymer networks with top left collagen, top right 3:5 ratio, bottom left 3:15 ratio, and bottom right the copolymer.

The SEM images, shown in Figure 5, clearly show the samples' size and amount of pores. The differences between the photos are not very large, but there seems to be more structure in the networks as more copolymer is present. The pores also decrease a bit in size. Overall all samples seem porous when looking at these images.

3.3.2 Porosity

With the calculated volume and weight of the samples, the porosity is determined by each different ratio. The results are given in Table 1.

Table 1: Results of the Gel Content, Water Uptake, and Porosity.

	Gel Content (%)	Water Uptake (%)	Porosity (%)
1 (Collagen)	99.7	549.3±11.5	84.1±1.8
2 (3:5)	91.5	239.9±9.8	88.3±1.2
3 (3:15)	90.4	165.6±4.6	79.4±1.8
4 (Copo)	89.3	104.3±15.5	77.1±2.0

When looking at Table 1, the porosity values generally decrease when less collagen is present in the networks, which is not unusual. What stands out is the lower porosity of the collagen sample. The fact that it shows lower porosity compared to the hybrid 3:5 network might come from the loss of its volume after drying. The collagen networks, which appeared to be more swollen compared to the other networks, might have collapsed during freeze drying resulting in a more compact network ultimately decreasing its porosity.

3.3.3 Gel Content

The gel content of the four different ratios of collagen:copolymer was evaluated to assess the degree of crosslinking and network formation. To determine the gel contents, the weights of the samples after crosslinking and freeze-drying were compared. The DMSO content in the networks was taken into account for the calculations. In Table 1 the results of the gel contents are given.

1 (collagen) exhibited an amount of 99,7%. This indicates that a lot of molecules are part of the crosslinked network. Similarly to 1 (collagen), 2(3:5), and 3 (3:15) also showed high gel contents of 91,5% and 90,5% respectively, demonstrating successful network formations. Lastly, 4 (Copolymer) showed a gel content of 89,35%. The high gel contents achieved with these ratios indicate good network formations, which can lead to improved mechanical and structural properties.

It is not uncommon for the gel content to be high in hybrid networks. Gel contents of 90% or higher have been found in earlier research with PTMC and Gelatin hybrid networks [20]. Hybrid networks with PTMC and Hyaluronic Acid have shown gel contents of 82% and higher[26]. This shows that the obtained results are in line with the literature.

3.3.4 Total Water Content

The water uptake of the polymer networks was assessed to evaluate the ability to retain and absorb water in the networks, which are important properties for certain applications in tissue engineering. In Table 1 this data is given. For each ratio, 3 dry and swollen weights were used to calculate the water uptake according to Formula 3.

When looking at Table 1, 1 (collagen) showed a mean water uptake of 549.3% ±11.5%. Collagen has the highest water uptake out of all the samples, indicating excellent hydrophilicity and water absorption in the created network. 2 (3:5) showed a mean water uptake of 239.9%±9.8%. It is expected to have a decrease in water uptake because of the incorporation of the copolymer. This

water uptake still shows that this ratio has retention of hydrophilic properties. 3 (3:15) showed a mean water uptake of 165.6%±4.6%. This is in line with the expectations because more copolymer is present in the network. Lastly, the 4 (Copo) showed a mean water uptake of 104.3 %±15.4%. This is the lowest water uptake from all the ratios used in the experiment.

Looking at the water uptake results indicates that more collagen will lead to more water uptake. Earlier research in hybrid networks containing PTMC and Gelatin also showed decreasing water uptake values when more polymer, in that case PTMC, was present in the network[21]. This is consistent with many other findings because collagen is known for its hydrophilic nature.

3.3.5 Network Composition

The areas of the characteristic peaks shown with FTIR were determined and compared to show the ratios of the created networks. This is semi-quantitative. In Figure 6 the aim and obtained ratios of the polymer networks are presented. The dotted line represents the aimed ratio. The straight lines represent the ratios of the networks made in this thesis.

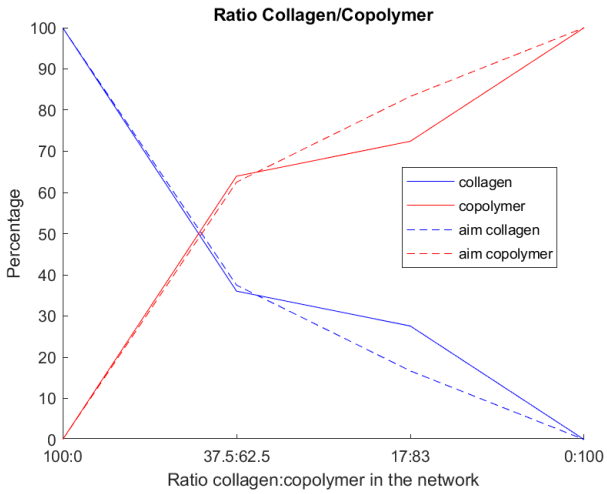

Figure 6: Collagen/Copo ratio that was made

As seen in Figure 6, the obtained ratios of the polymer networks closely match the aimed ratios. The small incomparability present at 3:15 can be a consequence of inconsistencies in the mixing process of the collagen and the polymer. Previous studies with hybrid networks showed similar deviations[26].

3.4 Thermal Properties

The results of the DSC and TGA are shown in Table 2.

Table 2: T_G (°C), $T_{\text{decomposition}}$ (°C), $Weight_{\text{Loss}}$ (%) and $Weight_{\text{Left}}$ (%).

	T_g	$T_{\text{decomposition}}$	$Weight_{\text{loss}}$	$Weight_{\text{left}}$
1 (Collagen)	*	284.8	80.8	19.2
2 (3:5)	-29.8	315.26	92.4	7.6
3 (3:15)	-28.6	318.49	96.7	3.3
4 (Copo)	-29.6	266.70	100	0

*The T_g of the collagen was outside of the DSC its range.

The glass transition temperatures don't significantly differ from each other. This means that adding more collagen or copolymer to the network's overall molecular arrangement at the glass transition region has no significant influence. We can furthermore assume that phase separation is present in the networks looking at these T_g values. The created networks are homogeneous which can be explained by looking at the SEM images and the images of the networks in which no clear phase separation is visible on a molecular level. The decomposition temperatures are also given in the table with an increase when more copolymer is added to the network. However, when only looking at the copolymer this decomposition temperature decreased.

Lastly, the weight loss and residual weight are notated in the table. With this results, Figure 7 was made.

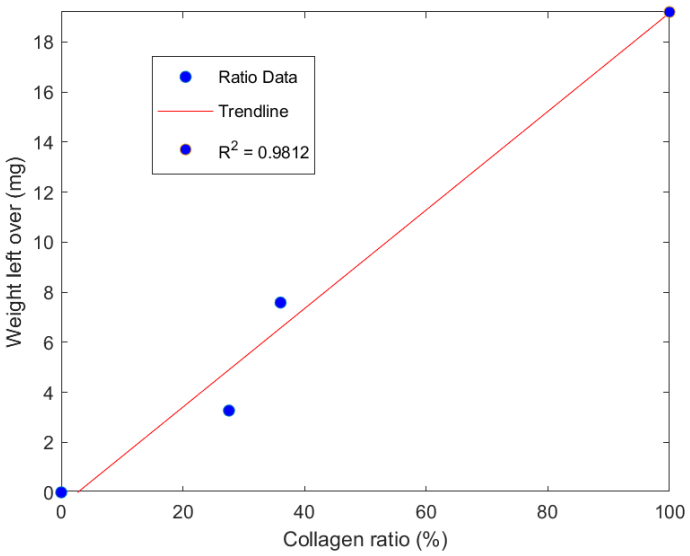

Figure 7: Weight left over after TGA vs Collagen ratio

Figure 7 indicates that the residual weight increases with more collagen in the polymer network. The R^2 was 0.9812 which indicates a high degree of correlation. With the help of this graph, the amount of collagen after degradation can be calculated, and a new ratio of collagen:copolymer can be considered. This is useful to see which component of the network will degrade faster. It gives more insight into how the polymer maintains its composition during degradation.

3.5 Mechanical Properties

3.5.1 Tensile Strenght

Table 3: Average and standard deviation of E-Modulus (MPa), Max Stress (MPa), $\varepsilon_{\text{break}}(\%)$, and Toughness ($N/\text{mm}^2$) with $n = 3$ as standard.

	E-Mod (MPa)	σ (MPa)	$\varepsilon_{break}(\%)$	W (N/mm^2)	SRS (N/m)	W_{srs} (N/mm^2)
1 (Collagen)	1.71 ± 0.42	0.06 ± 0.01	7.1 ± 1.1	0.2 ± 0.1	67.3 ± 22.9^{a}	0.16 ± 0.2^{a}
2 (3:5)	2.99 ± 3.61^{a}	0.10 ± 0.10^{a}	32.5 ± 19.3^{a}	0.9 ± 0.5^{a}	68.3 ± 46.6^{b}	0.60 ± 0.5^{b}
3 (3:15)	2.16 ± 0.41	0.16 ± 0.02	55.4 ± 6.6^{c}	5.5 ± 0.7^{e}	$278.2 \pm 59.2^{a,f}$	$2.93 \pm 1.1^{a,g}$
4 (Copo)	0.063 ± 0.008^{a}	0.11 ± 0.04^{a}	$127.9 \pm 12.5^{a,d}$	$5.9 \pm 2.0^{a,e}$	112.0 ± 15.0	2.75 ± 0.3^{g}

$a : n = 4$
$b : n = 5$
c: p<0.05 compared to 1(collagen) of $\varepsilon_{break}(\%)$
d: p<0.05 compared to all other $\varepsilon_{break}(\%)$ networks
e: p<0.05 compared to 1 & 2 of W (N/mm^2)
f: p<0.05 compared to all oher SRS (N/m) networks
g: p<0.05 compared to 1 & 2 of W_{srs} (N/mm^2)

Table 3 gives the mechanical properties of the polymer networks after making use of the DMA. For the elastic modulus, the E-modulus of collagen is higher compared to that of the copolymer, which is low. The hybrid networks both show a high elastic modulus with 2 (3:5) containing a very large standard deviation.

The maximum stress (σ) shows an increase with more copolymer added to the network. However, the network only containing copolymer has a small decrease comparing it to the 3:15 network.

The elongation at break (ε_{break}) shows an increase in % when more copolymer is present in the network indicating that the 100% copolymer network could withstand more elongation before it fractured. The ε_{break} of the copolymer is also significantly higher compared to the other values. The 3:15 of ε_{break} is significantly higher compared to the value of the collagen.

Furthermore, when looking at Table 3, the incorporation of more copolymer increases its toughness. This indicates that the presence of copolymer influences the energy needed, also known as energy absorption, before fracturing. The increase in toughness is a very important mechanical property for further use and implementation in specific applications of tissue engineering. The 3:15 ratio shows lots of comparison with the 100% collagen when looking at the toughness and

seems promising in this regard. Both the 3:15 and the copolymer networks of the toughness showed significantly higher values compared to the collagen and 3:5 networks.

3.5.2 Suture Retention Strenght

Table 3 also shows the SRS results and the $SRS_{thoughness}$. The SRS values show an increase with more copolymer present in the networks. The 3:15 network results show higher SRS values compared to the 100% copolymer networks, indicating that the small amount of collagen has a positive effect on the resistance to rupture. This is important in further implementation in tissue engineering. The 3:15 value of SRS (N/m) shows a significantly higher value compared to the other networks of SRS.

When looking at the $SRS_{thoughness}$ almost the same trend is visible as seen in the toughness. It increases but for the 100% collagen, it suddenly drops. This indicates that the small ratio of collagen has a positive effect on the energy absorbance, in the specific area, before it fractures, making the material stronger. Both the 3:15 and the copolymer networks of the $SRS_{toughness}$ showed significantly higher values compared to the collagen and 3:5 networks.

4 Conclusion

In this study, the networks were successfully prepared and characterized. For the functionalization of the random three-armed copolymer, the degree of functionalization turned out to be 99%. The molecular weight of the copolymer was 21000 g/mol with a ratio of 74:26 PTMC/Cl. For the collagen ^{1}H-NMR with acidic acid, D4 was used to look at the functionalization. With methacrylate peaks present in the spectrum at around 5.5 ppm it was concluded that the collagen was functionalized correctly.

The network ratios obtained after photocrosslinking were pure collagen, 3:5 ratio, 3:15 ratio, and pure copolymer. The morphology of the networks was assessed with the SEM, which revealed their interconnected and porous nature. Networks appeared to be more structured when more copolymer was present. This was in line with the results of the porosity, which were 84.1% for 100% collagen, 88.3% for a 3:5 ratio, 79.4% for a 3:15 ratio, and 77.1% for 100% copolymer.

Furthermore, the gel contents of the created networks showed a high degree of functionalization across all networks with values ranging from 89.3% up to 99.7%. This shows that most molecules are part of the network and crosslinked properly. Water uptake was performed to look at the network's availability to absorb and retain water and its hydrophilic nature. The water uptake for collagen was 549.3% followed by 3:5 with 239.9%, 3:15 165.6%, and copolymer with 104.3% showing the decrease in hydrophilicity when more copolymer was present in the network. With the use of FTIR, the composition of the networks was assessed and discussed as shown in Figure 6. This concluded that the ratios of the networks that were made did not deviate much from the intended ratios.

Thermal properties were assessed with the DSC and TGA analysis. T_g values didn't differ and showed together with the SEM images that there was no phase separation present in the networks. The $T_{decomposition}$ temperatures increased with more collagen present in the network from 284.8 to 318.49 but dropped for the 100% copolymer network. With the TGA results, figure 7 was made showing the weight on the y-axis against the collagen % on the x-axis. A trendline was used and with this figure, the degradation can be looked at in more detail

Lastly, the mechanical properties were assessed using DMA. This provided valuable information about the stress-strain behavior of the networks. There was no clear trend visible in the Elastic modulus of the networks. The elongation at break and maximum Stress showed an increase with more copolymer present in the network. The toughness, which is an important characteristic for further application in tissue engineering showed 0.2±0.1 for collagen, 0.9±0.5 for a 3:5 ratio, 5.5±0.7 for a 3:15 ratio, and 5.9±2.0 for the copolymer, which clearly shows an increasing trend. For the $SRS_{toughness}$ and SRS values, this trend is also visible except it decreases for the copolymer network. The $SRS_{toughness}$ for collagen is 0.16±0.2 followed by 0.60±0.5 for 3:5, 2.93±1.1 for 3:15, and 2.75±0.3 for the copolymer. Almost the same trend is possible in the SRS values, which show 67.3±22.9 for collagen, 68.3±46.6 for 3:5, 278.2±59.2 for 3:15, and 112.0±15.0 for copolymer indicating that forming a hybrid network has a positive impact on the amount of energy needed to fracture the networks.

In conclusion, this thesis solved a lot of structural-property correlations of the hybrid networks that were made with the copolymer and the human recombinant collagen answering the initial research question. It also contributes to the knowledge of hybrid networks and their potential to be integrated into tissue engineering. Further research can improve and fine-tune these results to explore these characteristics in more specific tissue engineering applications.

5 Recommendation

This research showed the functionalization of human recombinant collagen and the functionalization of the copolymer P(TMC-ϵ-co-Cl). With the correctly functionalized biomaterials networks were made and assessed. A lot of characteristics and properties were found using these networks. The structural-property correlations that were addressed in this thesis are obtained from dry networks. Follow-up research with swollen networks would be interesting. The difference in mechanical properties can be tested and evaluated. Cell culture, in which these networks play a role, could also be explored in the future. Simultaneously more specific applications in the field of tissue engineering can also be considered in further research. The TNBS-assay showed negative results [25]. Since the TNBS-assay was used in previously made networks with human recombinant collagen, it could be interesting to look at the suitability of the TNBS-assy with networks with human recombinant collagen.

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6 Appendix

6.1 ^{1}H-NMR PTMC/PCL

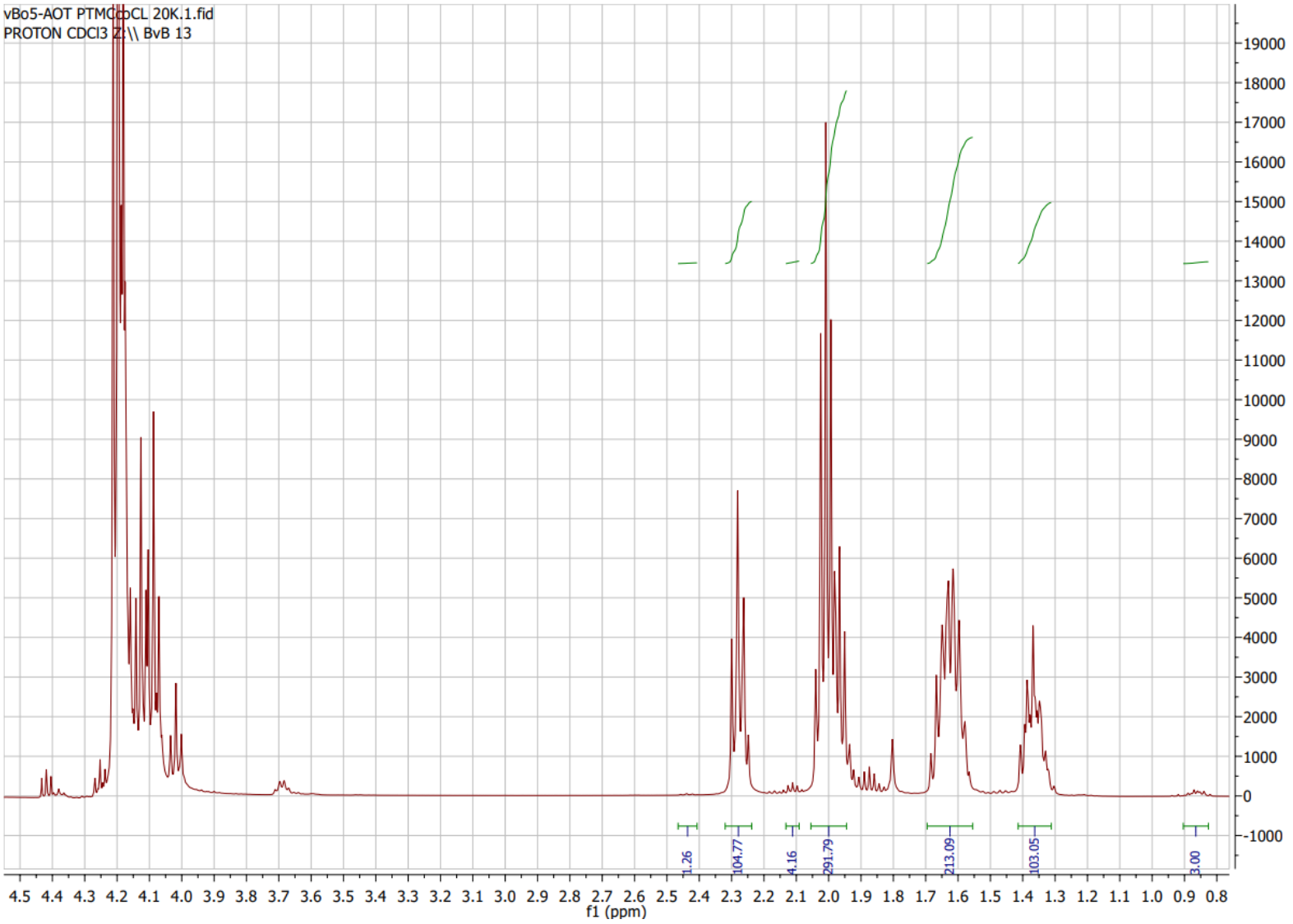

Figure 8: H-NMR results (0.8-4.5ppm)

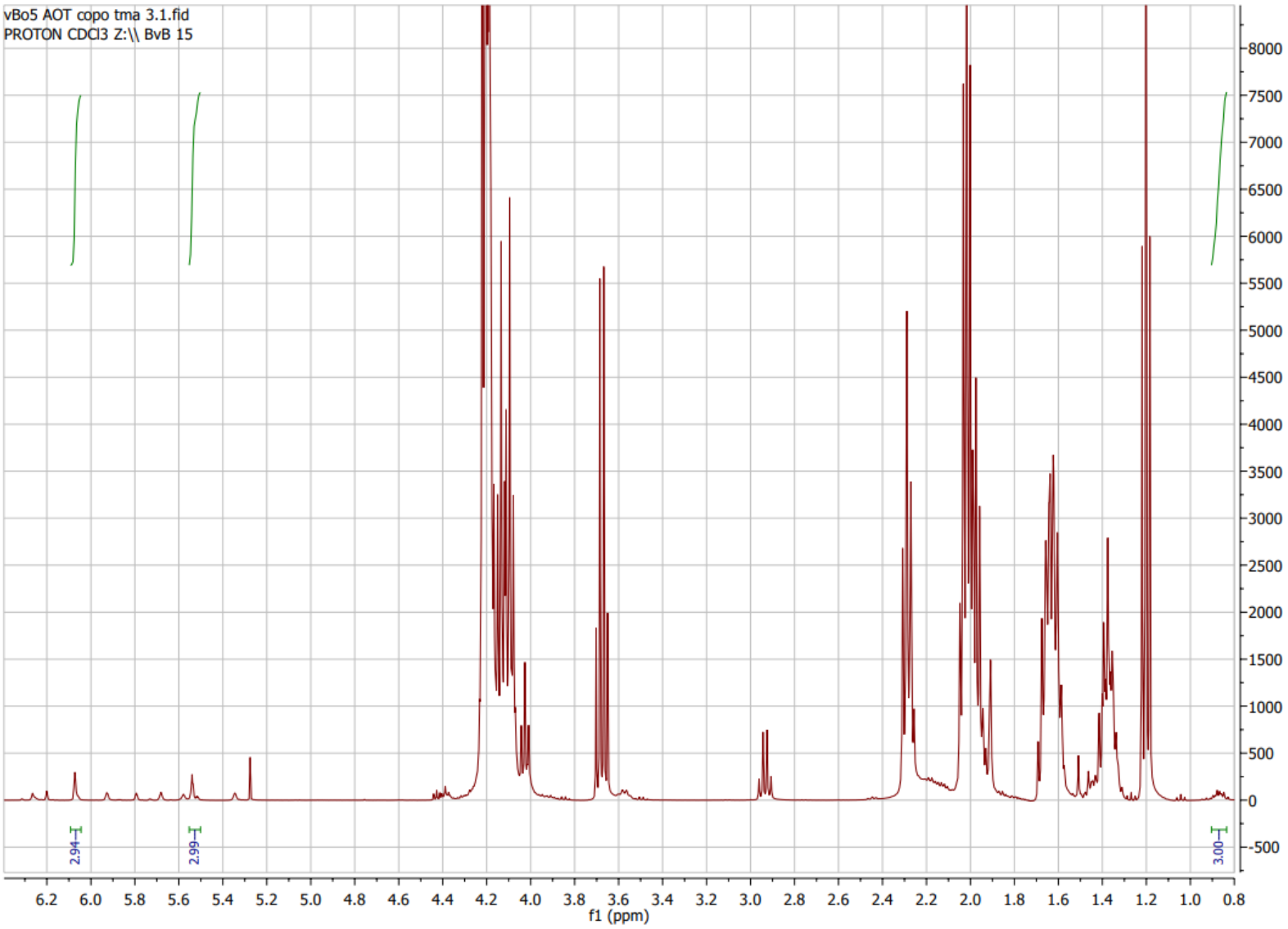

Figure 9: H-NMR results (0.8-6.4ppm)

6.2 ^{13}C-NMR PTMC/PCL

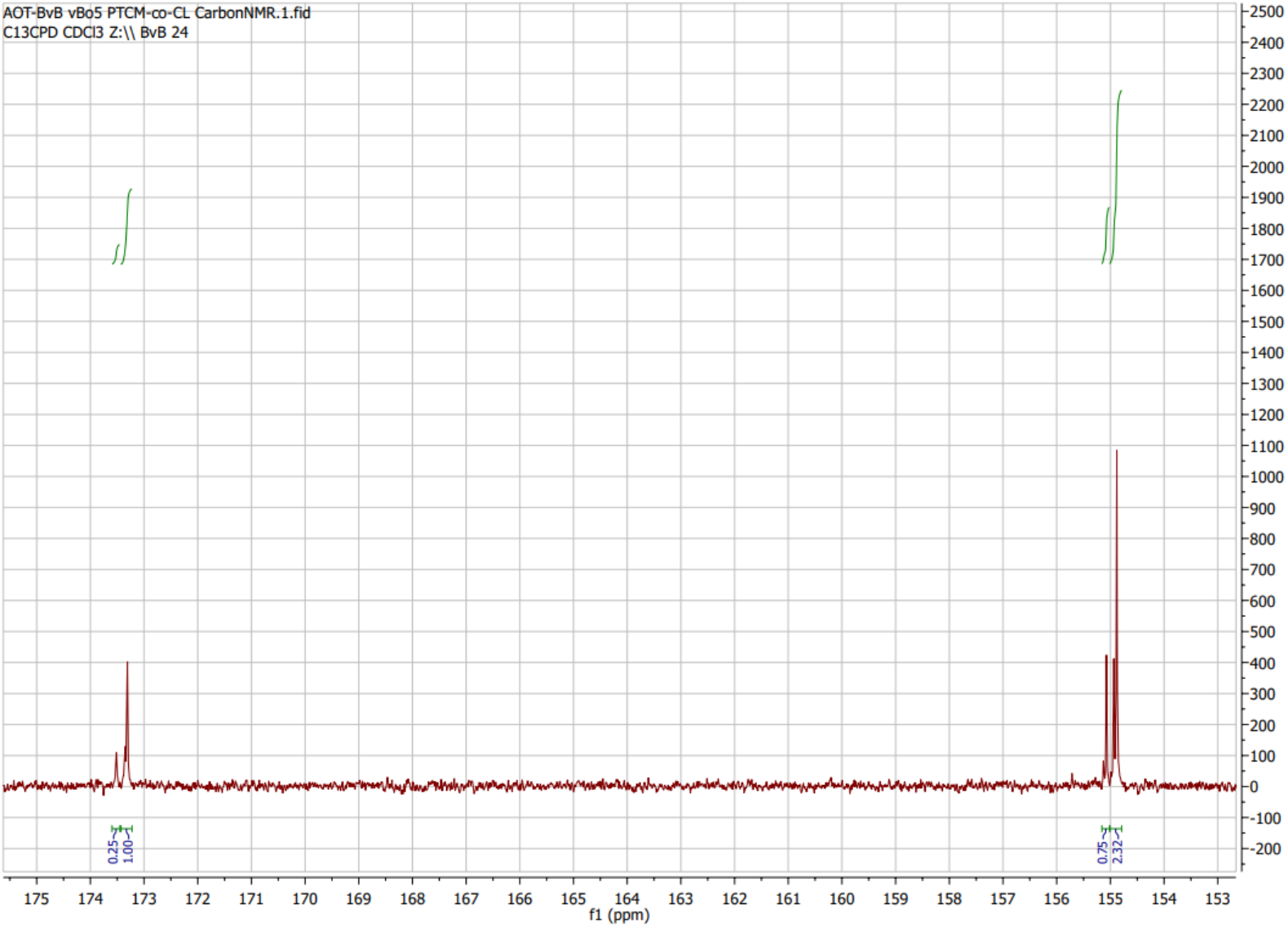

Figure 10: C-NMR results (153-175ppm)

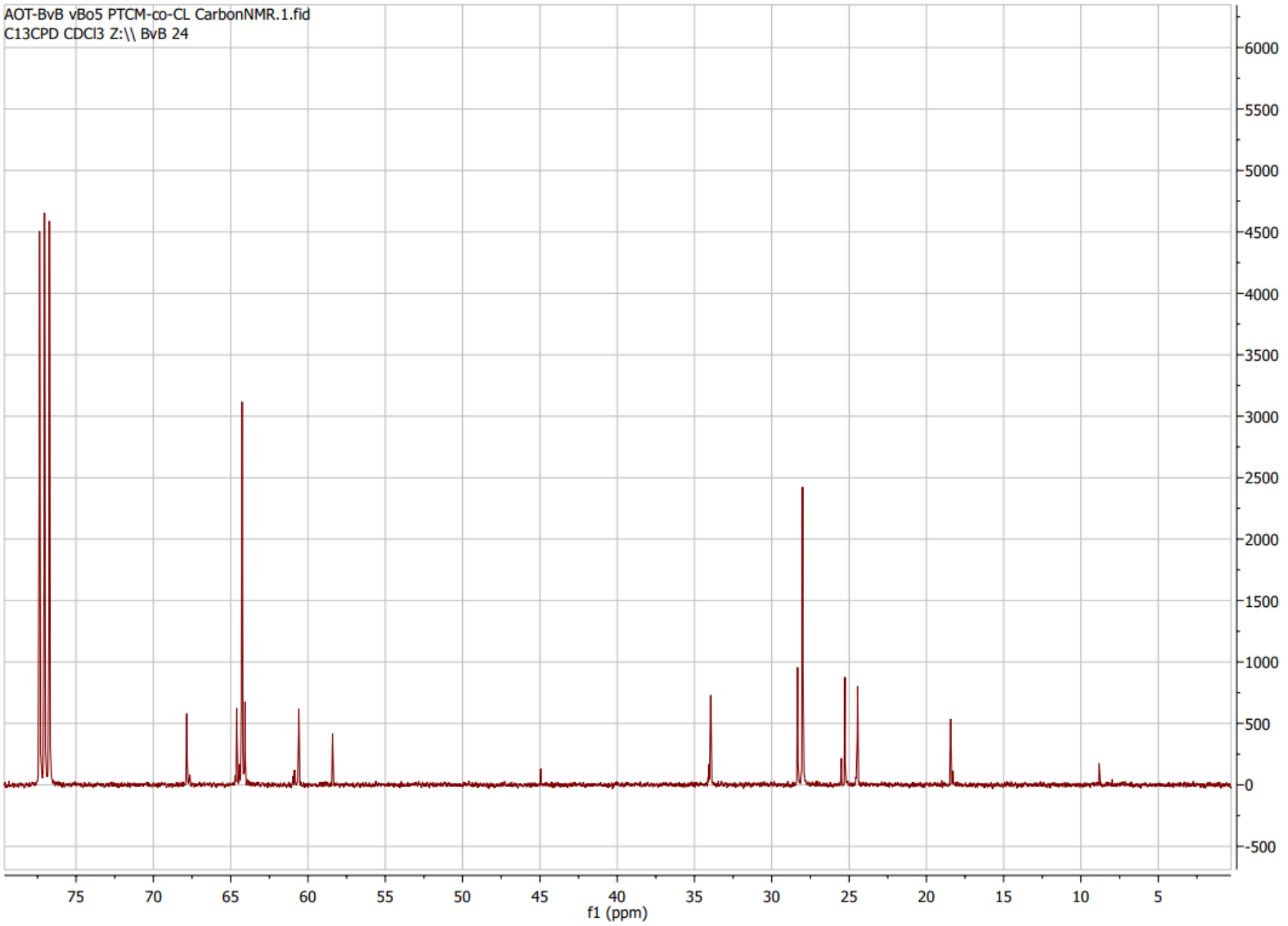

Figure 11: C-NMR results (0-80 ppm)

6.3 H NMR Acetic Acid D4 overlay

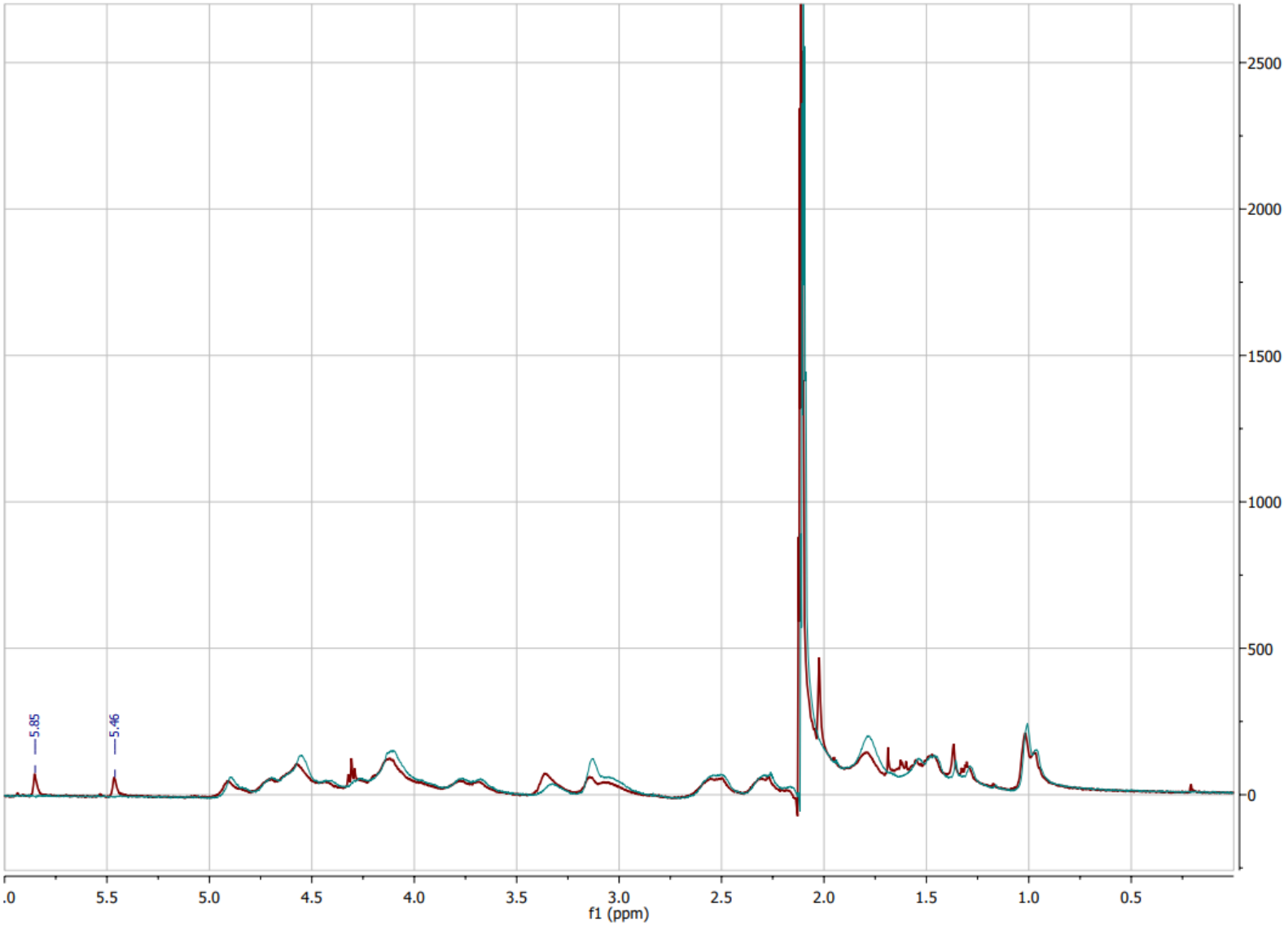

Figure 12: H-NMR results Acetic Acid D4