# Understanding the effects of TCP-25 and EDTA interaction

A study of the biophysical aspects of a naturally derived peptide formulation with EDTA for topical application


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Thank you,
*Ebba Kant*

# Abstract

Chronic wounds are an emerging healthcare issue and current methods of healing chronic wounds address bacteria but are ineffective against resistant bacteria and the biofilm formation commonly observed in these wounds.

Antimicrobial peptides (AMPs) derived from the endogenous wound healing system have been shown to combat bacterial infections, exhibit immunomodulatory effects, as well as improving wound healing. A 25 amino acid AMP derived from the C-terminal region of thrombin (TCP-25) have been thoroughly investigated by the Xinnate group. This peptide has proven successful antimicrobial and anti-inflammatory activity both *in vitro* and *in vivo* studies conducted on chronic wounds. As a result, the peptide has been formulated into a gel and is currently undergoing a first-in-human clinical safety study.

To improve the current formulation, efforts are made to develop a formulation able to effectively defeat biofilms. Specifically, a formulation incorporating EDTA has been investigated. Preliminary studies have shown the EDTA enhances the bactericidal activity of TCP-25. Biophysical properties studied during this project indicated that EDTA did not impact the peptide structure or LPS binding. More importantly, EDTA addition was found to have a positive impact on the peptide stability at RT, which could be related to increased oligomerization observed during thermal denaturation studies. In summary, the study suggests that the addition of EDTA improves the formulation, thereby highlighting its potential as a promising strategy for developing a new gel containing TCP-25 as active ingredient.

# Populärvetenskaplig sammanfattning

**Kroniska sår är sår som inte lyckas att läka över en lång tidsperiod. De karaktäriseras av kolonisering av bakterier vilka bildar en så kallad biofilm som ger upphov till ökat motstånd mot behandling. De behandlingsmetoder som finns idag mot kroniska sår är inte fulländade, då stor risk för resistens finns samt att de inte kan förstöra biofilmen. Idag är över 40 miljoner människor drabbade av kroniska sår och antalet ser ut att öka framöver, vilket understryker vikten av att hitta välfungerande behandlings-metoder.**

Ett sätt att angripa kroniska sår är att se till naturens egna metoder för sårläkning och bekämpning av bakterier. Inom sårläkning har det nyligen visat sig att så kallade antimikrobiella peptider, AMPs, verkar ha en betydande roll. En av dessa AMPs, vid namn TCP-25 har en roll i att både döda bakterier och minska inflammation vid sår, vilket är intressanta egenskaper för kroniska sår. TCP-25 har studerats i över 20 år av Xinnate AB där både studier på bakterier samt prekliniska studier har gjorts. Dessvärre är TCP-25 inte perfekt i sig själv, då den saknar möjlighet att ta bort biofilm vilket gör det svårt att göra sig av med bakterierna i såret. Dessutom är peptider generellt känsliga och har en låg stabilitet i jämförelse med traditionella läkemedel.

Baserat på bristerna var det intressant att testa en ny formulering med EDTA, vilket är ett ämne som används idag i flertalet olika läkemedelsformuleringar och som nyligen har setts kunna ta bort biofilmer. Innan denna masteruppsats utfördes försök på bakterier där det gick att se att EDTA förstärkte den bakteriedödande effekten ihop med TCP-25.

För att studera den nya formuleringen med TCP-25 och EDTA utfördes det här examensarbetet för att se hur struktur och funktion hos TCP-25 påverkades av att ha EDTA tillsatt. För att undersöka detta gjordes flertalet experiment med olika mätmetoder.

Från projektet sågs indikationer på att EDTA inte påverkar strukturen på TCP-25 eller dess möjlighet att agera som vanligt, vilket är lovande för en framtida användning i läkemedel. Dessutom, under en stabilitetsstudie sågs EDTA förbättra stabiliteten hos TCP-25. Detta kan bero av att TCP-25’s peptider binder ihop sig med varandra och skapar så kallade oligomerer.

Utifrån resultaten behövs vidare studier för att förstå hur interaktionen verkligen ser ut, men det verkar lovande att tillsätta EDTA för att förbättra formuleringen.

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# Abbreviations

| | |
|---|---|
| **AMP** | Antimicrobial Peptide |
| **BN-PAGE** | Blue Native-Poly-Acrylamide Gel Electrophoresis |
| **CBB** | Coomassie Brilliant Blue |
| **CD** | Circular Dichroism Spectroscopy |
| **CN-PAGE** | Clear Native Poly-Acrylamide Gel Electrophoresis |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **HNE** | Human Neutrophil Elastase |
| **IFS** | Intrinsic Fluorescence Spectroscopy |
| **LPS** | Lipopolysaccharide |
| **MBC** | Minimum Bactericidal Concentration |
| **MIC** | Minimum Inhibitory Concentration |
| **RP-HPLC** | Reverse Phase High Pressure Liquid Chromatography |
| **RT** | Room Temperature |
| **TCP** | Thrombin derived C-terminal peptide |
| **WHO** | World Health Organization |

# 1. Introduction

## 1.1 Problem statement and relevance

In healthy individuals, wound healing is an effective and well-functioning process. However, for approximately 40 million patients worldwide, failures in the healing process lead to chronic wounds that have a high impact on patient health and healthcare costs [1]. Chronic wounds are often defined as "wounds that have not proceeded through an orderly and timely reparation to produce anatomic and functional integrity after 3 months" [2]. Colonization of bacteria is very common to chronic wounds [1] where aerobic or facultative pathogens such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) are the most frequent causes of delayed sore healing and infection [3]. Another issue prevalent in chronic wounds is biofilm formation, which affects healing even more negatively than bacteria in planktonic form [4], because this formation provides boosted stability to the bacteria by being able to resist the patient immune response and not be as sensitive to antibiotics [5]. Especially the *P. aeruginosa* species are able to form biofilms in wounds [6].

Today, different kinds of antiseptics and antibiotics are used topically on chronic wounds to decrease the pathogen populations [1]. The current treatments are anti-infective but do not work against excessive inflammation, which is frequently seen with bacterial infection and may cause pain, erythema, fibrosis and delayed healing even if they are no pathogens left [7]. Also, biofilm is today often removed by debridement, but total removal is unlikely [8]. Furthermore, antimicrobial resistance is often seen after treatments, and is one of the ten threats to global health, according to the World Health Organization (WHO) [9]. There is a dire need for drugs that can act on all aspects of chronic wounds and to successfully heal them without resistance risk.

## 1.2 Role of antimicrobial peptides in wound healing process

The wound healing process is complex and involves interaction between biological and physiological pathways to restore the tissue integrity after an injury [10]. In a normal setting, the process consists of four overlapping phases that are well managed; haemostasis, inflammation, proliferation and remodelling [11]. In addition to these phases, emerging evidence suggests that antimicrobial peptides (AMPs) are involved in wound healing [12, 13].

AMPs play a crucial role in wound healing, primarily due to their ability to combat microbial infections. Wounds are susceptible to colonization by various pathogenic microorganisms, which can impede the healing process. AMPs have been shown to be active against a broad spectrum of bacteria, fungi, and even some viruses [14]. AMPs can reduce infection by acting directly on the pathogens, creating a favourable environment for fast wound healing.

Secondly, AMPs also have immunomodulatory roles. They can modulate production of pro- and anti-inflammatory cytokines and activate immune cells such as macrophages, neutrophils and dendritic cells. Moreover, they can stimulate production of other host defence peptides to promote bacterial clearance [15].

Furthermore, AMPs can enhance tissue regeneration by stimulating angiogenesis and promoting wound healing [16]. Numerous AMPs have shown positive results after topical application, such as for example LL-37 that promoted migration, angiogenesis and dermal cell proliferation which are all important for wound restoration [17]. Overall, the research interest in AMPs for therapeutics application has significantly increased, thanks to all properties mentioned. In fact, between 2017-2020 more than 5000 articles were published on the subject [18].

### 3.3 Thrombin as a source of antimicrobial peptides

Thrombin is generated through proteolytic cleavage of prothrombin by coagulation factor X and mediates degradation of fibrinogen and formations of clots during the acute wounding phase [19]. After the clotting process, thrombin undergoes further proteolysis by human neutrophil Elastase (HNE), and releases AMPs of different lengths from the C-terminal region (hence the name TCPs) [20]. The fragments of ≈11kDa have been found to possess the ability to aggregate bacteria as well as their endotoxins, such as lipopolysaccharide (LPS) which in turn enables their removal through immune cell phagocytosis [19]. Shorter TCPs of ≈2 kDa have demonstrated strong activity against bacteria and inflammation [21]. Therefore, TCPs constitute an important class of AMPs that play a crucial role in combating infection and septic shock [20].

## 1.3 Mode of action of TCP-25

One specific TCP of particular interest in this project is TCP-25. TCP-25 is a patented synthetic peptide developed by Xinnate AB with the endogenous amino acid sequence GKYGFYTHVFRLKKWIQKVIDQFGE. The action of TCP-25 is both anti-inflammatory and antibacterial [22]. ***Figure 1*** provides an illustration of the mode of action of TCP-25 and its influence on the LPS signalling pathway. Briefly, TCP-25 can bind competitively to the binding pocket of CD14 which typically interacts with LPS, thus impeding their interaction which would normally cause inflammation. TCP-25 is also able to bind directly to LPS, thereby sequestering it [23].

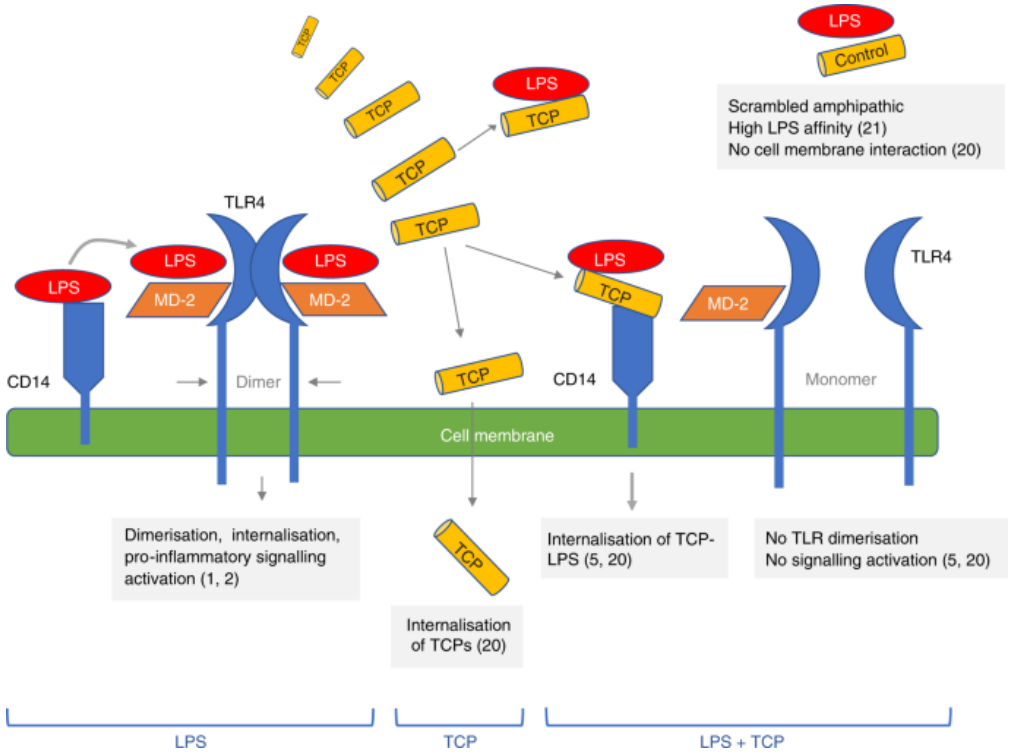



**Figure 1**. Illustration of the effect on LPS on its own and what happens when TCP is present [23].

The effect of TCP-25 has been shown to be pH dependent, as a pH of 5.5 increases the antibacterial effect against *E.coli* by disruption of the membrane but at the same time the binding affinity to human CD14 decreases, suggesting a change in the action mode from being anti-inflammatory at a neutral pH to becoming antibacterial at the acidic pH [24].

In addition to the *in vitro* studies, several *in vivo* studies have also shown promising results. TCP-25 has been shown to protect from *P. aeruginosa* and LPS-induced shock in mice [20]. In a hydrogel formulation of TCP-25, the peptide has been shown to speed up healing of infected wounds in pigs [22]. Currently, TCP-25 is undergoing clinical phase 1 studies to

investigate its safety, tolerability and pharmacokinetics by use of increasing concentrations of the peptide applied topically on epidermal suction blister wounds to healthy volunteers [25].

## 1.4 Preliminary results

As most of AMPs, TCP-25 alone is unable to disrupt biofilms. To enhance the current formulation, efforts are made to develop a formulation able to effectively defeat biofilms, which are often formed in chronic wounds. Specifically, a formulation with EDTA has been proposed. Ethylenediaminetetraacetic acid, EDTA, is commonly used for different applications with pharmaceuticals being one of them. As a drug, EDTA is often used to reduce blood clotting thanks to its ability to complex free calcium ions in the blood. Because of EDTA's ability to bind to ions such as lead ions it can also be used as an antidote for lead poisoning [26]. EDTA has recently been shown to be bacteriostatic because of its ability to reduce biofilms. It can chelate bacterial cell walls and make biofilms more instable by segregating calcium, magnesium, iron and zinc [27]. Apart from its chelating properties, EDTA is often added to pharmaceuticals because of its low toxicity, high stability, compatibility and its ability to increase the action of preservatives and antibacterial agents [28].

TCP-25 and EDTA has been investigated in formulations together to see the effect on bacteria through another master thesis project performed by M.Sc. student Manali Patil. M. Patil tested different combinations of TCP-25 and EDTA in solution and in gel formulation with 25 mM NaOAc at pH 5 to investigate the concentrations needed to inhibit *P. aeruginosa* and *S. aureus*. Two of the experiments performed were Minimum Inhibitory Concentration (MIC) Assay, and Minimum Bactericidal Concentration (MBC) Assay. MIC results can be seen in **Table *1*** and **Table *4*,** MBC Assay results can be seen in ***Table 3*** and **Table *4***.

**Table 1.** MIC table with *S. aureus* and increasing concentrations of TCP-25 and EDTA. Horizontally, increasing concentration of EDTA, vertically increasing concentration of TCP-25.

MIC ASSAY FOR *S. aureus* 29213:

| *S. a* 29213 | 0 mM EDTA | 0.5 mM EDTA | 1 mM EDTA | 2.5 mM EDTA | 5 mM EDTA | 10 mM EDTA |
|---|---|---|---|---|---|---|
| 0 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 1.25 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 2.5 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 5 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 10 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 20 µM TCP | Growth | No growth | No growth | No growth | No growth | No growth |
| 40 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 80 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 160 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |

**Table 2.** MBC table with *S. aureus* and increasing concentrations of TCP-25 and EDTA. Horizontally, increasing concentration of EDTA, vertically increasing concentration of TCP-25.

MBC ASSAY FOR *S. aureus* 29213:

| *S. a* 29213 | 0 mM EDTA | 0.5 mM EDTA | 1 mM EDTA | 2.5 mM EDTA | 5 mM EDTA | 10 mM EDTA |
|---|---|---|---|---|---|---|
| 0 µM TCP | Growth | Growth | Growth | Growth | Growth | Growth |
| 1.25 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 2.5 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 5 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 10 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 20 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 40 µM TCP | Growth | No growth | No growth | No growth | No growth | No growth |
| 80 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 160 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |

**Table 3.** MIC table with *P. aeruginosa* and increasing concentrations of TCP-25 and EDTA. Horizontally, increasing concentration of EDTA, vertically increasing concentration of TCP-25.

MIC ASSAY FOR *P. aeruginosa* PA01:

| *P. a* PA01 | 0 mM EDTA | 0.5 mM EDTA | 1 mM EDTA | 2.5 mM EDTA | 5 mM EDTA | 10 mM EDTA |
|---|---|---|---|---|---|---|
| 0 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 1.25 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 2.5 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 5 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 10 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 20 µM TCP | Growth | No growth | No growth | No growth | No growth | No growth |
| 40 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 80 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 160 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |

**Table 4.** MBC table with *P. aeruginosa* and increasing concentrations of TCP-25 and EDTA. Horizontally, increasing concentration of EDTA, vertically increasing concentration of TCP-25.

MBC ASSAY FOR *P. aerguinosa* PAO1:

| *P. a* PA01 | 0 mM EDTA | 0.5 mM EDTA | 1 mM EDTA | 2.5 mM EDTA | 5 mM EDTA | 10 mM EDTA |
|---|---|---|---|---|---|---|
| 0 µM TCP | Growth | Growth | Growth | Growth | Growth | Growth |
| 1.25 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 2.5 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 5 µM TCP | Growth | Growth | Growth | No growth | No growth | No growth |
| 10 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 20 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 40 µM TCP | Growth | Growth | No growth | No growth | No growth | No growth |
| 80 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |
| 160 µM TCP | No growth | No growth | No growth | No growth | No growth | No growth |

From the MIC assay in **Table 1** and **Table 3** it was seen that 2.5 mM EDTA was enough to inhibit growth for both bacterial strains, but not enough to kill them, since growth is seen in the MBC assays in **Table 2** and **Table 4**. It was also noted that a lower amount of EDTA was needed for inhibit growth of *S. aureus* (1 mM) than *P. aeruginosa* (2.5 mM). Based on the data in **Table 1** and **Table 3**, 2.5mM EDTA was chosen for further study.

# 2. Aim

The overall aim of this project was to investigate the effects of EDTA addition on TCP-25 in a solution of 25 mM NaOAc at pH 5. Specifically, we wanted to investigate:

- How biophysical properties of TCP-25, such as secondary structure, thermal stability, and oligomerization, are affected by EDTA
- If EDTA interferes with TCP-25-LPS binding
- How the stability of TCP-25 is affected by EDTA after one month of storage

# 3. Materials and Methods

## 3.1 Methods

### 3.1.1 Peptide stock solution

TCP-25 (MW = 3088.62 Da) was synthetised by AmbioPharm, Inc (North Augusta, SC, USA). The purity and the content (98.4%, and 88.5%, respectively) were confirmed by MALDI-TOF MS (>95%). One mM TCP-25 was dissolved in 25 mM NaOAc at pH 5, following **Equation (1)** and **(2)**:

$$M_p\ (g) = \frac{C\left(\frac{g}{L}\right) * V_f(L)}{Purity\ (\%) * Content\ (\%)} \qquad \textbf{(1)}$$

With $M_p$ = Amount of peptide, C = concentration, $V_f$ = final volume, with the concentration being determined by equation (2)

$$C\left(\frac{g}{L}\right) = MW\left(\frac{g}{mol}\right) * MC\ \left(\frac{mol}{L}\right) \qquad \textbf{(2)}$$

With C = concentration, MW = molecular weight, MC = molar concentration

Stock solution was stored at -20°C.

### 3.1.2 Clear Native-PAGE

TCP-25 (50, 100, 250 and 500 µM) with/without 2.5 mM EDTA in 25 mM NaOAc at pH 5 (20 µl as final volume) was mixed with 5 µl 4×Native PAGE sample buffer. Immediately after, samples were loaded on a NativePAGE™ 4-16% Bis-tris pre-cast 1mm Mini protein gel (Invitrogen, Carlsbad, CA, USA). Electrophoresis was performed at 150 V for 90 minutes in Native PAGE 1×buffer before Western Blot.

### 3.1.3 Blue Native PAGE

Two µg TCP-25, with/without 2.5 mM EDTA in 25 mM NaOAc, were mixed with increasing concentrations of *E. coli* LPS (0–300 µg/ml). Samples (20 µl as final volume) were incubated at 37 °C for 30 min, then mixed with 1 µl of 5% Coomassie Brilliant Blue and 5 µl 4×Native PAGE sample buffer (Invitrogen, Rockford, IL, USA). Samples were loaded on a NativePAGE™ 4-16% Bis-tris pre-cast 1 mm Mini protein gel (Invitrogen, Carlsbad, CA, USA). Electrophoresis was performed at 150 V for 90 minutes in a Native PAGE 1×anode buffer (Invitrogen, Carlsbad, CA, USA) in the external chamber and NativePAGE 1× cathode buffer (Invitrogen, Carlsbad, CA, USA) in the inner chamber.

### 3.1.4 Western Blot

The peptide from the gel was transferred to a PVDF membrane using the Trans-Blot Turbo System (Bio-Rad Laboratories, Hercules, CA, USA) and TCP-25 was then detected using the primary antibody polyclonal rabbit antibody against VFR17 (VFRLKKWIQKVIDQFGE; diluted 1:1000, Innovagen AB, Lund, Sweden) followed by the secondary antibody swine anti-rabbit HRP conjugated antibody (diluted 1:1000, Dako). To visualize the peptide the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was added to the membrane and incubated for 2 minutes, followed detection using the ChemiDoc MP Imager (Bio-Rad Laboratories, Hercules, CA, USA).

### 3.1.5 Circular Dichroism Spectroscopy

The secondary structure of TCP-25 alone or with EDTA was evaluated by circular dichroism (CD). In the first set of experiments, TCP-25 at different concentrations (10-100 µM), with and without 2.5 mM EDTA was analysed. Measurements were performed on the JASCO-810 spectropolarimeter (Jasco, Tokyo, Japan) with a Jasco CDF-426S Peltier that was set on 20°C. For samples with 10 µM TCP-25, 0.2 cm cuvettes were used, and for samples with 50 µM and 100 µM TCP-25 0.1 cm cuvettes were used (Hellma, GmbH & Co, KG, Müllheim, Germany). Spectra was recorded

between 260 – 190 nm with 1 nm data pitch, a continuous scanning mode and with a scanning speed of 20 nm/min. The response time was 8 seconds and the final spectra was the result of 3 accumulations. Spectra were corrected for the background of the buffer alone or with EDTA, and values were converted to give the mean residue ellipticity, $\theta$ (mdeg cm$^2$ / dmol). $\theta$ was calculated based on **Equation (3)**, taken from [29].

$$\theta_{MRW} = \frac{A_{mdeg}}{10*L*M} \quad \textbf{(3)}$$

Where $A_{mdeg}$ is the raw values of absorbance from the measurement, L is the length of the cuvette in cm, M is the concentration of the peptide in mg/ml

**Equation (3)** was then modified to only be $\theta$, following the **Equation (4).**

$$\theta = \frac{A_{mdeg}* MRW}{10*L*c} \quad \textbf{(4)}$$

Where MRW was determined from **equation (5).**

$$MRW = MW / N_{aa} \approx 124 \quad \textbf{(5)}$$

Where, MW is the molecular weight of the peptide (Da) and $N_{aa}$ is the number of amino acids in the sequence.

The alpha helical content from the measurements was calculated using the following **Equation (6)** [30].

$$FH = \frac{\theta_{exp} - \theta_\lambda^u}{\theta_\lambda^n - \theta_\lambda^u} \quad \textbf{(6)}$$

Where $\theta_{exp}$ is the θ at 222nm, $\theta_\lambda^u$ is 3900, $\theta_\lambda^n$ is -36000 according to Morsiette et al [30]. 222nm was chosen because that is the second negative band of an alpha helical structure.

In the second set of experiments, we investigated the ability of TCP-25 to bind *E.coli* LPS in the presence of EDTA. TCP-25 was diluted in 25 mM NaOAc at pH 5 with/without 2.5 mM EDTA at 10 µM as final concentration. Then, 10 µg/ml of LPS were added to the peptide. The secondary structure was evaluated as described above. For all measurements, values below 200nm were regarded as non reliable because of high prevalence of scattering.

### 3.1.6 Intrinsic Fluorescence Spectroscopy

Exposure of tryptophan residues in the structure of TCP-25 structure to the environment was evaluated by intrinsic fluorescence spectroscopy (IFS). Measurements were performed using the Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan) with an FMO-427s monochromator. Spectra were recorded between 300 – 450 nm with excitation at 280nm. The data pitch was 1nm and response 0.25 s, with a bandwidth of 2 nm and accumulation of 3 measurements. A 3×3mm cuvette (Hellma, GmbH & Co, KG, Müllheim, Germany) was used.

For the first set of experiments 10 and 100 µM TCP-25 in 25 mM NaOAc with and without 2.5 mM EDTA was used (Two hundred µl final volume). Peptide was exposed to thermal denaturation by increasing temperature from 20–100°C according to the following pattern: +2°C between 20 and 30°C, +5°C between 30 and 100°C. Sensitivity was adjusted according to values at 20°C, to be in a range between 0.6-0.8 A.U. After reaching 100°C, the sample was cooled to 20°C and measurements were taken again to see if denaturation was reversible. Raw data was normalized based on the values of the 20°C measurement to give the fluorescence increase and were plotted against the wavelength in nm.

In another set of experiments, structural changes upon LPS addition were investigated. Two hundred µl of 10 µM TCP-25 were mixed with increasing concentrations of *E. coli* LPS (for details of sample preparation see **Table 5**). LPS was prepared by vortexing the stock solution of 1 mg/ml for 15 minutes.

***Table 5.*** Summary of sample preparation

| Conc of LPS in peptide solution (ug/ml) | LPS solution |
|---|---|
| 1,0 | 0.1mg/ml LPS |
| 2,0 | |
| 2,9 | |
| 3,8 | |
| 4,8 | |
| 14,2 | 1 mg/ml LPS |
| 23,4 | |
| 32,4 | |
| 41,3 | |
| 50,0 | |

Two different solutions of LPS were used and added cumulatively to the peptide solution. Concentrations of LPS shown in **Table 5** determined following the **Equation (7):**

$$Conc\ LPS \frac{=(V_{LPS\ sol} * C_{LPS\ sol})}{(V_{peptide\ sol} + V_{LPS\ sol})} \qquad \textbf{(7)}$$

Where the $V_{peptide\ sol}$ was the peptide solution, which was always set to 200 µL.

### 3.1.7 RP-HPLC

The stability of TCP-25 in 25 mM NaOAc at pH 5 without and with 2.5 mM EDTA was evaluated by reverse-phase high pressure liquid chromatography (RP-HPLC). Hundred µM TCP-25 freshly diluted from stock solutions (1 mM stored at –20°C), after 1 month of storage at room temperature and at +4°C, were analysed. Phenomenex Kinetex C18-column (150x2.1 mm, 4.6 µm, 100Å pore size, Torrance, CA, USA) and the Agilent 1260 Infinity System were used. The column was equilibrated in a combination of 90% buffer A containing 0.05% Trifluoroacetic acid (TFA) in MilliQ and 10% buffer B containing 0.05% TFA in acetonitrile. Ten µl of each sample were loaded on the column. The elution profile of the peptide was monitored during the gradient of 35% B at 10 min, 45% at 20 min) and the 215 nm spectrum was recorded. The flow rate of the column was 1 ml/min and all runs were performed at 50°C.

### 3.1.8 Statistical analysis

All experiments were performed in triplicate except for the HPLC measurements, which were conducted in duplicate. Graphs were done in PRISM Software version 9 or in Excel Spreadsheets.

# 4. Results

## 4.1 Effects on secondary structure of TCP-25

Circular dichroism (CD) was used to evaluate whether the TCP-25 structure was affected by addition of EDTA. TCP-25 was dissolved at different concentrations (10, 50, 100 µM) in 25 mM NaOAc at pH 5 with or without 2.5 mM EDTA. As seen in ***Figure 2,*** the structure of TCP-25 predominantly remained in random-coil conformation and between being with and without EDTA the difference is neglectable, apart from 100 µM with EDTA where there is a slight decrease for the sample with EDTA. The average and standard deviation of triplicates for each condition are shown in ***Appendix A***.

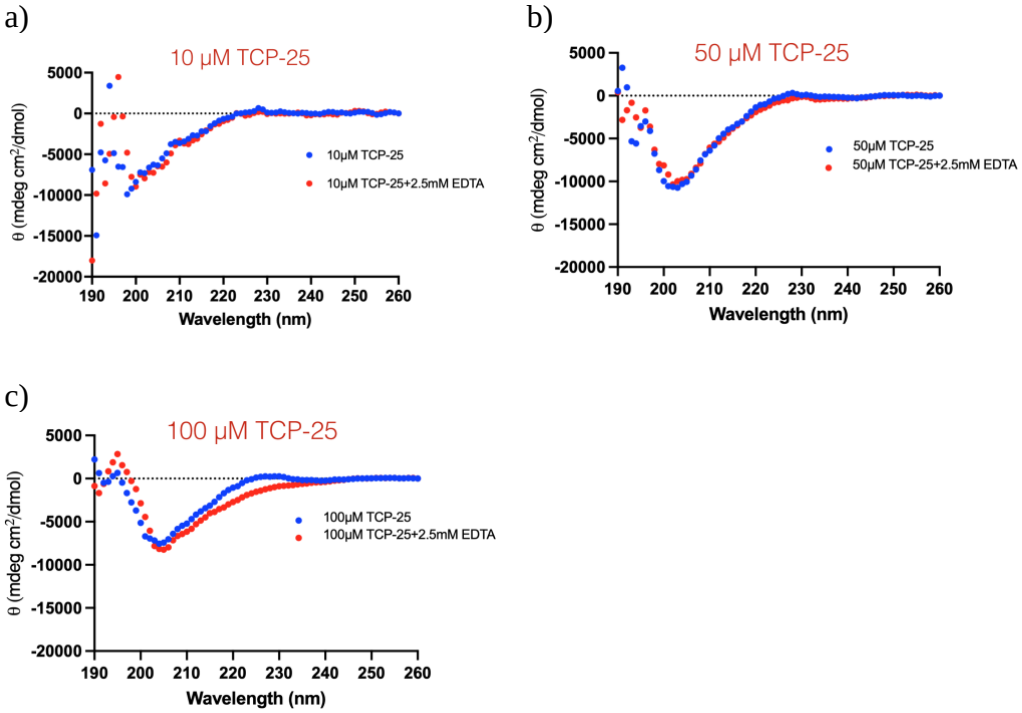
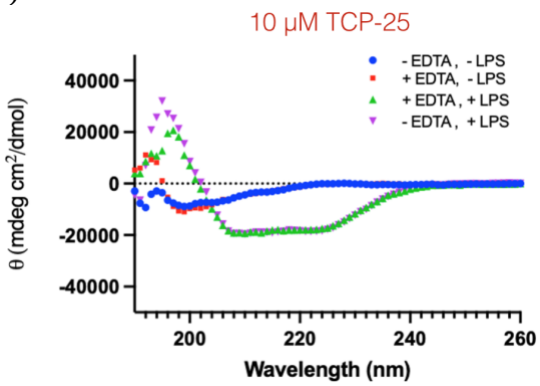



**Figure 2.** Structural analysis of TCP-25. **(a)** Representative far-UV CD spectra of TCP-25 in concentrations 10 µM in 25 mM NaOAc at pH 5 with or without 2.5 mM EDTA. (**b)** Representative far-UV CD spectra of TCP-25 in concentrations 50 µM in 25 mM NaOAc at pH 5 with or without 2.5 mM EDTA. (**c)** Representative far-UV CD spectra of TCP-25 in concentrations 100 µM in 25 mM NaOAc at pH 5 with or without 2.5 mM EDTA. All spectra were acquired at 25°C. Measurements performed in triplicates (n=3).

### 4.2 LPS binding

One of the main mechanisms of action of TCP-25 is LPS binding [23], which occurs primarily through electrostatic interaction. Since EDTA is negatively charged at pH 5 [35], it was of interest to understand if its addition to the formulation was affecting the LPS–TCP-25 binding. Since it is known that TCP-25 adopts α-helical structure when bound to LPS [23], we decided to use CD to test whether the secondary structure in the presence of LPS was affected by the addition of EDTA or not. TCP-25 was diluted to 10 µM in 25 mM NaOAc at pH 5 with or without EDTA and with or without 10 µg/ml LPS. The spectra for all samples are shown in ***Figure 3a.*** As expected, TCP-25 alone in the presence of EDTA was showing no structure, while in the presence of LPS it was adopting a perfect alpha helical conformation. This result was confirmed by calculating the alpha helical content in the structure (***Figure 3****Error! Reference source not found.****b***). A clear increase in alpha helical content (from below 20% to almost 60%) was observed when LPS was added to the samples, which was consistent with previous studies [23]. This result was confirmed by calculating the alpha helical content in the structure (***Figure 3****Error! Reference source not found.****b***). A clear increase in alpha helical content (from below 20% to almost 60%) was observed when LPS was added to the samples, which was consistent with previous studies [23].

a)




b)

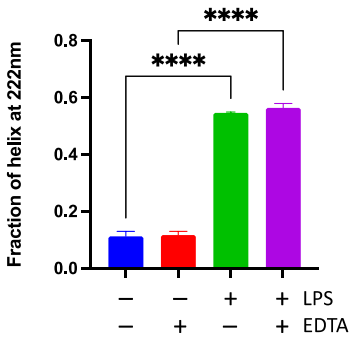



**Figure 3.** Understanding LPS binding. (**a**) Representative image of CD measurement of 10 µM TCP-25 in 25 mM NaOAc at pH 5 with and without 2.5 mM EDTA and 10 µg/ml LPS. (**b**) $\alpha$ – helical content, expressed as fraction of helix ± SD calculated from the CD spectra obtained at 222nm. Two-way ANOVA analysis with Tukey's multiple comparison test showing significant increase of helical content for samples containing LPS, p = <0.0001 . The measurements were performed in triplicates (n=3).

The interaction between TCP-25 and LPS was further confirmed by CN-PAGE. Two µg of TCP-25 with and without 2.5 mM EDTA in 25 mM NaOAc at pH 5 were mixed with increasing concentrations of LPS (0,50,100,300 µg/ml) and after a short incubation evaluated by CN-PAGE followed by Western blot. ***Figure 4*** displays a representative image of the result.

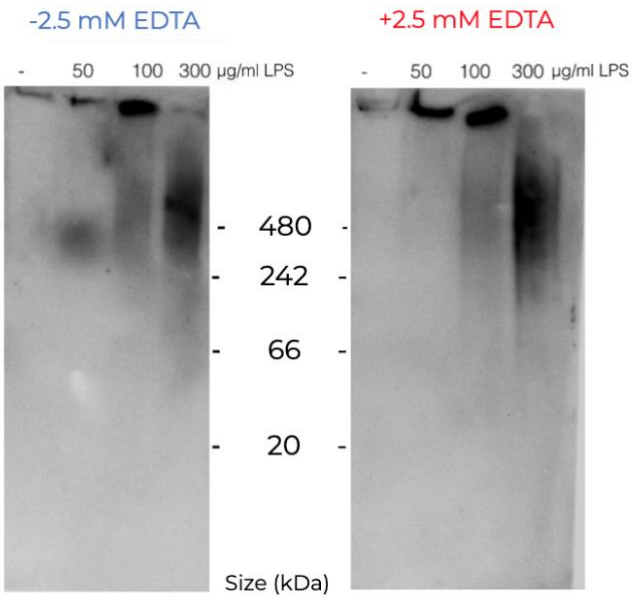
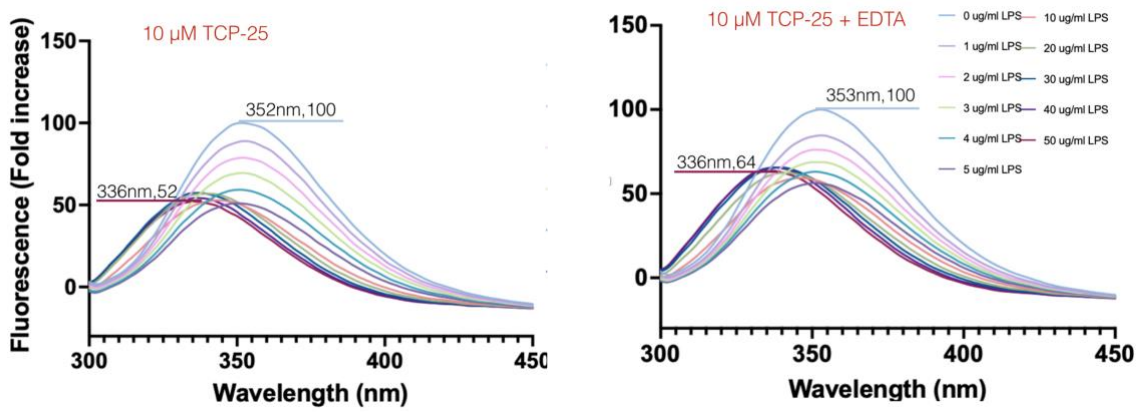



**Figure 4.** Representative image of CN-PAGE measurement of 2 µg TCP-25 in 25 mM NaOAc pH 5 with or without 2.5 mM EDTA and increasing concentration of LPS (50-300 µg/ml) as well as 5 µl 4×Native PAGE sample buffer. All measurements performed in triplicates (n=3).

Under the conditions used, TCP-25 alone cannot enter the gel, but if binding occurs the peptide becomes negatively charged and can migrate towards the positive pole. As seen in the ***Figure 4***, LPS binds to the peptide in both conditions, i.e., with and without EDTA. There is also no obvious difference between the gels, suggesting that EDTA does not influence the TCP-25 binding to LPS.

To further investigate the ability of LPS to interact with TCP-25 when EDTA is added, intrinsic fluorescence measurements were also employed. 10 µM TCP in 25 mM NaOAc at pH 5 with or without EDTA were measured with increasing concentration of LPS from 0-50 µg/ml. ***Figure 5a*** shows representative spectra of the measurements.

a)




b)

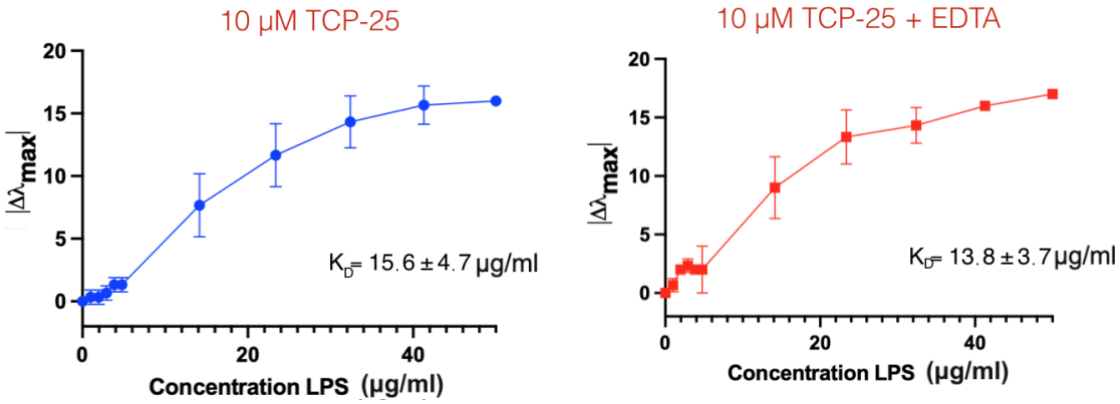



**Figure 5.** Intrinsic Fluorescence measurements with LPS. (a) 10 µM TCP-25 in 25 mM NaOAc buffer pH 5 with or without 2.5 mM EDTA with increasing concentration of LPS (0-50 µg/ml). Measurements performed in triplicates (n=3). (b) $K_D$ value of LPS with 10 µM TCP with or without EDTA in 25 mM NaOAc solution pH 5. Plotted with $|\Delta\lambda_{max}|$ against concentration of LPS.

The values presented on the lines in ***Figure 5a*** show the starting and ending values of $\lambda_{max}$, and its normalized fluorescence with increasing LPS concentration. The difference between the sample containing EDTA and the one without it is negligible, indicating that the interaction between LPS and TCP-25 is unaffected by the addition of EDTA. The binding affinity ($K_D$) between LPS and TCP-25 was calculated by plotting the $|\Delta\lambda_{max}|$ in function of increasing concentrations of LPS (***Figure 5b***). As expected, $K_D$ with and without EDTA are very similar.

### 4.3 Stability effects by EDTA addition

To understand if EDTA addition was positively affecting the stability of TCP-25 at 4°C and RT conditions after one month, RP-HPLC was employed to analyze the peptide content. Previously it was shown that low pH decreases the stability of the peptide [32]. TCP-25 (100 µM) was diluted directly from the stock[1] in 25 mM NaOAc at pH 5 with or without 2.5 mM EDTA. Immediately after, the sample was loaded on the column. The rest of the sample was aliquoted and stored at room temperature (RT) or at +4°C for a month before next round of measurements. The results are shown in ***Figure 6***.

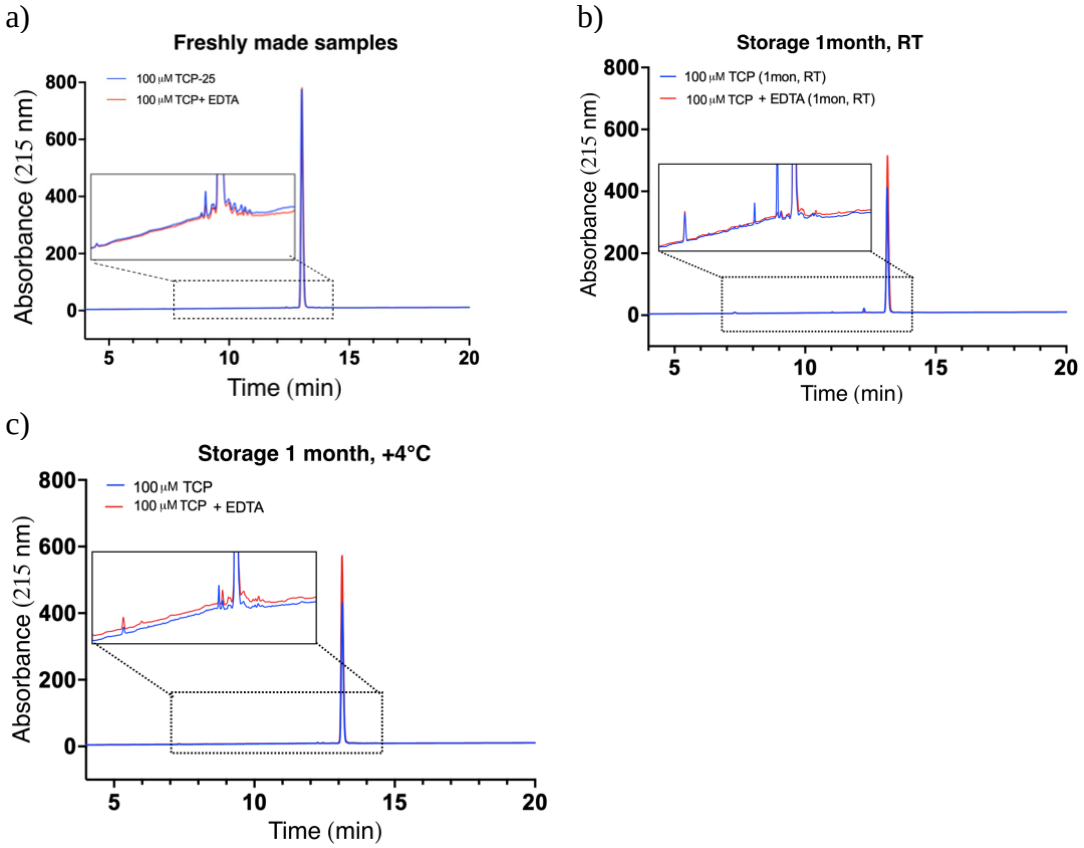



**Figure 6.** TCP-25 stability investigation. **a**) Measurement of fresh[1]sample of 100 µM TCP-25 with and without 2.5 mM EDTA in 25 mM NaOAc at pH 5 directly after being made (**b**) Shows 100 µM TCP-25 with and without 2.5 mM EDTA in 25 mM NaOAc pH 5 that has been stored 1 month at room temperature. (**c**) Shows 100 µM TCP-25 with and without 2.5 mM EDTA in 25 mM NaOAc pH 5 that has been stored 1 month at +4°C.

The chromatograms of TCP-25 in freshly made samples are very similar (***Figure 6a***). While with a longer storage time, several peaks at lower relative retention time (RRT) were observed, indicating degradation of the peptide (***Figure 6b*** and ***c***). Samples stored at RT showed more degradation product than those stored at +4°C. In fact, the main peak for samples with EDTA exhibited higher intensity compared to the control, and several peaks at lower Relative Retention Time (RRT) were there less intense. The analysis of the peaks is shown in the table

[1] Stock solution 1 mM TCP-25 stored at –20°C.

in ***Appendix B***. Epecially from the measurements at RT conditions, there is a clear difference between samples with and without EDTA, from which it seems that samples containing EDTA improves the peptide stability.

### 4.4 Oligomerization

Previously it was shown that TCP-25 oligomerizes when dissolved in 10 mM Tris at pH 7.4, where the oligomerization was both pH and concentration dependent. [31]. Therefore, we decided to investigate whether the oligomerization pattern would be affected by the addition of EDTA. TCP-25 was dissolved in 25 mM NaOAc at pH 5 at different concentrations (50,100, 250,500 µM), with and without 2.5 mM EDTA. Samples were then analysed on BN-PAGE followed by Western blotting. Representative images are shown in ***Figure 7***.

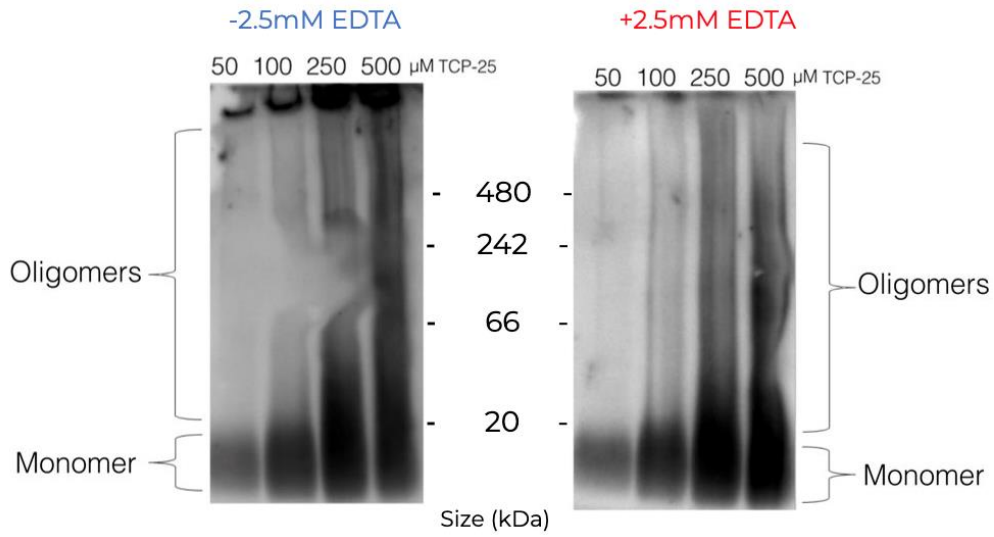



**Figure 7.** Separation on 4-16% (w/v) BN-PAGE followed by Western blot analysis. Increase in oligomerization as the TCP-25 concentration increases from 50 to 500 µM seen in both samples without (left) and with (right) 2.5 mM EDTA. Samples prepared in 25 mM NaOAc buffer at pH 5 with 5% Coomassie dye and 5 µl Native PAGE 4x sample buffer. One representative image of 3 independent experiments is shown (n=3).

As seen in ***Figure 7,*** samples containing 50 µM TCP-25, both with and without EDTA, exhibited a prominent band at the bottom of the membrane, indicating the presence of predominantly monomeric peptide. As the concentration increased, a progressively more pronounced smear along the membrane emerged, suggesting a wide range of oligomer formation. The results are in agreement with previous study [31].

Another way to study oligomerization is to look at the intrinsic fluorescence. It was before demonstrated that TCP-25 in NaOAc buffer was mostly unstructured and when exposed to thermal denaturation its intrinsic fluorescence decreased as the temperature increased [32]. We therefore decided to use the same method to evaluate how TCP-25 was affected by thermal

denaturation in the presence and the absence of 2.5 mM EDTA. Two different concentrations of peptide (10 µM and 100 µM) were tested. Samples were exposed to thermal denaturation by gradually increasing temperature from 20 to 100°C. ***Figure 8a*** and ***b*** show representative fluorescence spectra for the different concentrations tested.

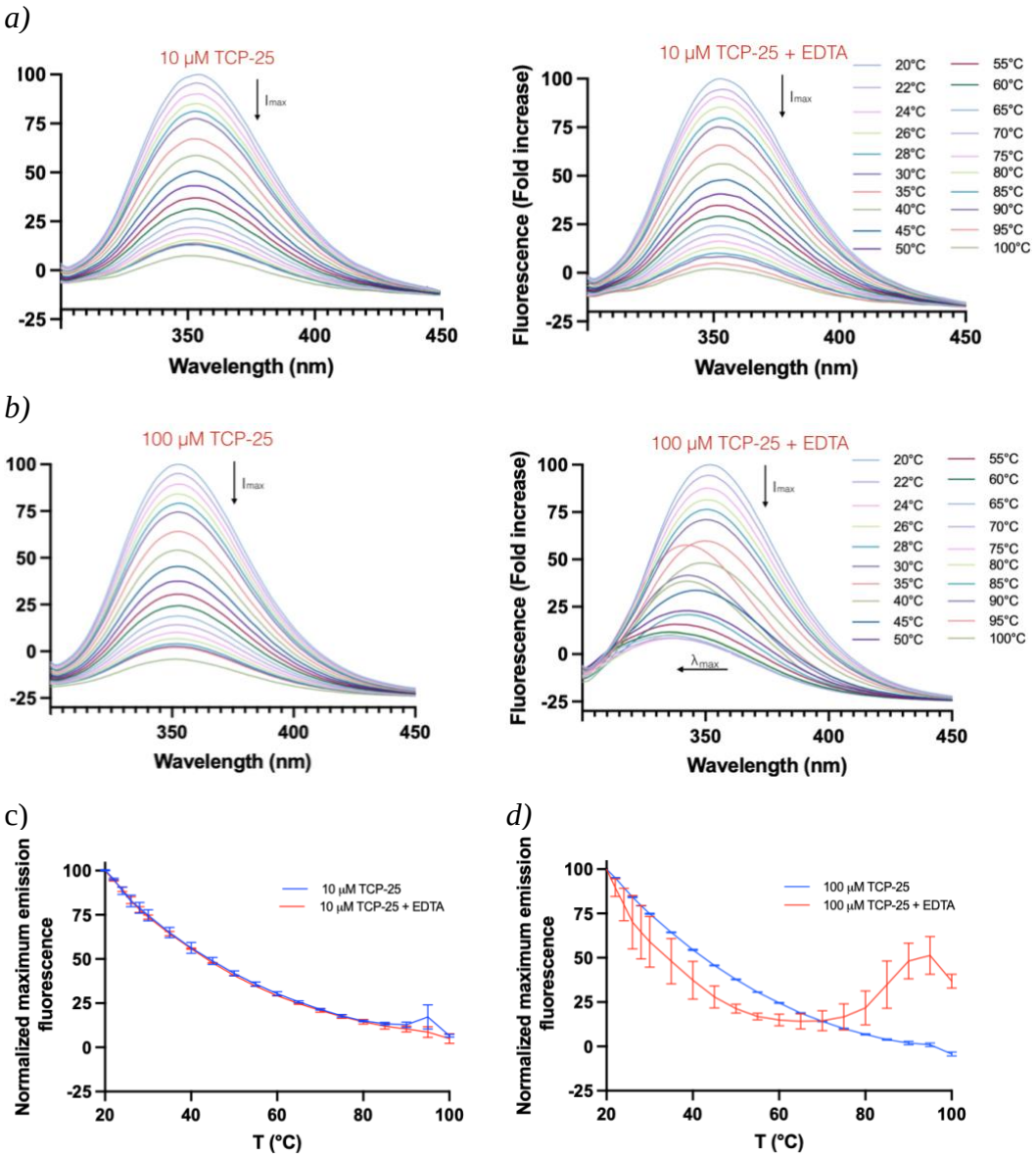



**Figure 8.** Thermal denaturation of TCP-25. **(a)** 10 µM TCP-25 in 25 mM NaOAc at pH 5 with (left panel) or without (right panel) 2.5 mM EDTA was denatured by increasing the temperature**. (b)** 100 µM TCP-25 in 25 mM NaOAc at pH 5 with (left panel) or without (right panel) 2.5 mM EDTA was denatured by increasing the temperature**.** For **(a)** and **(b)** representative spectra are shown of triplicate measurements (n=3) and ←λmax indicates a shift in maximum emission fluorescence; ↓Imax indicate increase in maximum fluorescence intensity. In **(c)** the denaturation curves from **(a)** are shown, and **(d)** gives the denaturation curve of **(b)**. For **(c)** and **(d)**, data was obtained by fitting normalized maximum emission fluorescence as a temperature function.

We observed that as the temperature increased, the intrinsic fluorescence decreased, indicating higher exposure of Trp residues to the polar environment. At 10 µM, the presence of EDTA did not affect the intrinsic fluorescence of the peptide (***Figure 8a***). However, at 100 µM of TCP-25 the addition of EDTA (***Figure 8b***) caused a pronounced blue shift of the $\lambda_{max}$, from

350 nm at 35°C to 335nm at 70°, which indicates peptide aggregation [31]. This is not surprising, since tryptophan's absorbance is also sensitive to the local electrostatic field [32] and EDTA is a negatively charged molecule. The change in pattern of emission as the thermal denaturation proceeded is illustrated in ***Figure 8c*** and ***d***. For all conditions apart from 100 µM TCP-25 + EDTA, the fluorescence emission decreases similarly. While for 100 µM TCP-25 + EDTA the emission started to increase from around 70°C, confirming the aggregation of the peptide.

Unfolding of a peptide from increasing temperature is often paired with irreversible aggregation [31] and if this was the case also for TCP-25 in the presence of EDTA was unknown. To study this, samples used for thermal complete denaturation were cooled down to 20°C and then the intrinsic fluorescence was recorded again. As we can see in ***Figure 9a***, all samples were showing higher intrinsic fluorescence after denaturation than at starting point, indicating the non-reversibility of the process. This was even more pronounced for the samples containing higher concentration of TCP-25 and EDTA (***Figure 9b*, right panel**).

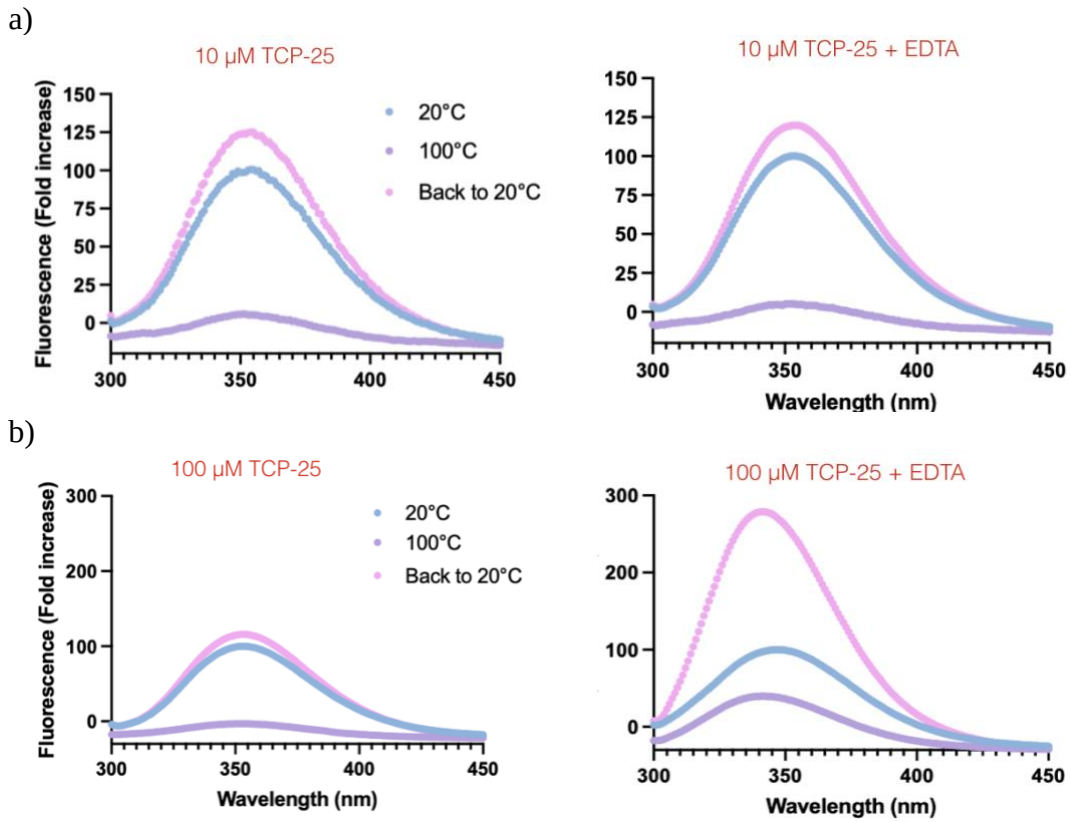



**Figure 9.** Reversibility of thermal denaturation of TCP-25 at pH 5. TCP-25, diluted to 10 (**a**) and 100 (**b**) µM in 25 mM NaOAc at pH 5 with and without 2.5 mM EDTA, was denatured by heating it up to 100°C and then lowering the temperature to 20°C. Possible refolding was seen by measuring the intrinsic fluorescence of the peptide. Spectra were collected at 20 (blue), 100 (purple) and 20°C after denaturation (pink). Each graph is representative of 3 independent experiments (n=3).

# 5. Discussion

Understanding chronic wounds and discovering new ways to combat them has sparked significant interest in the recent years, particularly due to the increasing population affected by such wounds. It is now estimated that 1-2% of the World population will experience a chronic wound during their lifetime [33]. Especially as antimicrobial resistance is growing, new alternatives are needed. The TCP-25 peptide has been shown to have antimicrobial and anti-inflammatory effects but is unable to disrupt biofilms. The biofilms give the bacteria higher treatment resistance and are a large issue with chronic wounds. EDTA has been shown to have biofilm disrupting abilities [27]. Therefore, incorporating EDTA into a formulation of TCP-25 as a topical gel has potential to enhance its antibacterial action to help promote wound healing. *In vitro* studies of this new formulation have shown positive results, indicating is efficacy in combating biofilms. Additionally, the biophysical properties investigation is of utmost importance as it paves the way for further clinical development of the formulation. Understanding the formulation's physical characteristics is vital to ensure stability, efficacy and suitability for clinical applications.

EDTA is an acid with multiple dissociation constants (pKa) at different pH values: 2.0, 2.7, 6.2 and 10.31 [34]. The pH of the final TCP-25 formulation was set to 5. At this pH, two out of four hydroxyl groups in EDTA will be deprotonated, resulting in the molecule carrying partial negative charge. This partial negative charge contributes to overall electrostatic properties and behaviour of EDTA in solution. One of the major actions of TCP-25 is its ability to bind LPS through electrostatic force. Since LPS also carries a negative charge, there was a concern that the presence of EDTA could potentially interfere with LPS binding to TCP-25. However, the study revealed that EDTA did not interfere with this interaction. The binding affinity ($K_D$) between LPS and TCP-25 remained within the same range, indicating that EDTA did not disrupt the binding process.

Furthermore, the secondary structure investigation confirmed that EDTA did not induce any significant changes. The secondary structure of TCP-25 remained unaffected regardless of peptide concentration even when EDTA was added. This observation suggests that EDTA does not alter the functional conformation of TCP-25, further supporting its compatibility and effectiveness when combined with the peptide. Overall, the study demonstrated that EDTA does not interfere with the binding of TCP-25 to LPS and that it does not induce structural

changes in the peptide. With these findings, confidence is gained for the potential use of TCP-25 in combination with EDTA for its antimicrobial and wound-healing properties without functionality compromises. To confirm these findings, employing other laboratory methods such as NMR and 3D *in silico* modelling could be useful.

Apart from structure and interaction with LPS, also the stability of the formulation was investigated. Peptides are generally quite unstable [35], where especially oxidation is a primary degradation pathway [36]. Amino acids such as cysteine (C), methionine (M), histidine(H), tryptophan (W) and tyrosine (T) are prone to oxidation because of their reactivity with oxygen species [36]. EDTA is a promising agent to improve peptide stability as it has been shown to be effective in oxidation reduction of peptide based formulations [37]. Also, $Na_2EDTA$ was shown to protect monoclonal antibodies against iron contamination, but only when the ratio of $Na_2EDTA$ to iron ions was above one [38]. Contradictory, in another study it was seen that EDTA can increase oxidation. In particular, in a study on Human Insulin-Like Growth Factor I, it was shown that oxidation of iron ions was only happening when EDTA was present [39]. Preliminary results from this project show that EDTA improves the stability of the peptide in solution after one month storage. This is especially evident when TCP-25 is stored at RT. However, to investigate this further a longer stability study is needed. In addition, since TCP-25 will be formulated in a gel, it is important to conduct further stability studies in a setting corresponding to the final product that is going to be used in wounds.

Since EDTA seemingly improves the stability of TCP-25 in solution at RT, it was interesting to investigate if increased oligomerization was the cause. Oligomerization is known to provide stability to the TCP-25 peptide at pH 7.4 [31]. Oligomers can be both positive or negative for the formulation of a drug since it could mean reduced activity, but could also be part of the intrinsic action of the peptide [31]. Oligomerization could also provide a slower active molecule release [31], which could be useful for control of release dynamics. Previously, it was shown that TCP-25 oligomerizes, and that oligomerization is pH dependent, i.e. higher at pH 7.4 than at pH 5 [31]. In the current project, addition of EDTA did not appear to affect the oligomerization pattern of TCP-25. Therefore, the findings from previous studies investigating the oligomerization of TCP-25 under different pH conditions [31] remain relevant and applicable to this application. In particular, since in the thermal stability studies it appears that EDTA causes a lot of irreversible aggregation at higher temperatures with higher peptide content, indicating that EDTA could affect oligomerization at higher temperatures. One

possible explanation is that as the thermal denaturation exposes amino acids that were previously concealed within the peptide structure, they become able to bind to EDTA, ultimately resulting in the formation of aggregates. However, this is just an idea and needs to be studied more in detail to understand the interaction and which amino acids that could be interacting with EDTA. One way to investigate this can be to study a possible structural shift after heating it the solution up to 100°C using CD spectroscopy or other techniques to investigate structure such as Mass spectrometry or NMR.

Overall, one of the main limitations to this study is that the current project was performed using TCP-25 in solution, whereas the final formulation for clinical use will be in hydrogel form. The reason not to employ the gel format was primarily due to the limitations of the methods used in this study for evaluating gel formulations. As a result, the data obtained from this study provides insight to how EDTA affects TCP but may not fully represent the behaviour or performance of TCP-25 in a gel formulation. Besides, aspects such as rheology of TCP-25 gel with EDTA are moving forward important to study to understand the properties of the formulation. Furthermore, testing other batches of the peptide and EDTA will help establish the reproducibility and robustness of the observed effects, enhancing the overall reliability and validity of the study findings.

# 6. Conclusion

Based on the experiments performed, EDTA does not seem to have an impact on the structure of TCP-25 nor does it interfere with the LPS binding. As for stability measurements, it seems that EDTA improves the stability of TCP-25 when stored at RT for one month. The stability increase may be explained by the suggested presence of oligomers during thermal denaturation measurements from which structural shifts were seen with high peptide concentrations and added EDTA.

Overall, EDTA addition appears to give the TCP-25 solution biophysical properties that are promising for further clinical development, but further characterization is needed to fully understand the applicability of the formulation.

# 7. Future work

To further investigate the formulation with TCP-26 and EDTA, it would be interesting to investigate the following:

- Use *in silico* modelling to investigate the 3D interaction of TCP-25 and EDTA in the formulation;
- Use NMR or Mass spectrometry to investigate structural properties of the formulation;
- Perform a longer stability study;
- Use CD after thermal denaturation to detect possible structural shifts;
- Use other batches of TCP-25 and EDTA to be more certain of measurements not being related to a certain batch;
- Investigate how the rheological properties of TCP-25 gel are affected by the addition of EDTA;
- Investigate if EDTA affects the release profile of TCP-25 from the gel.

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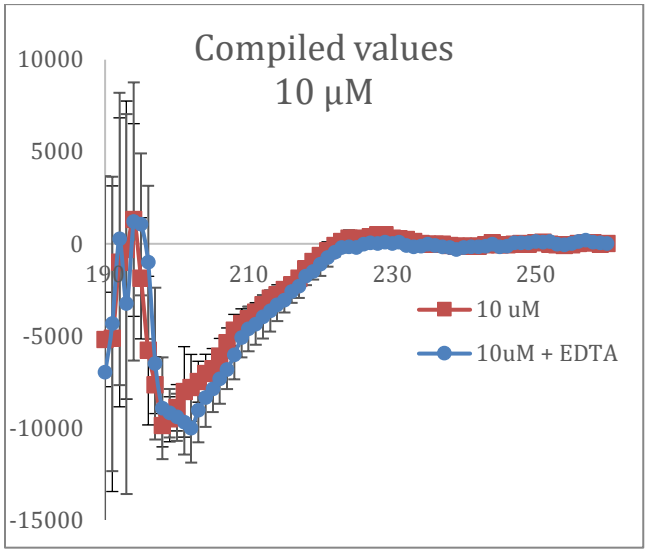
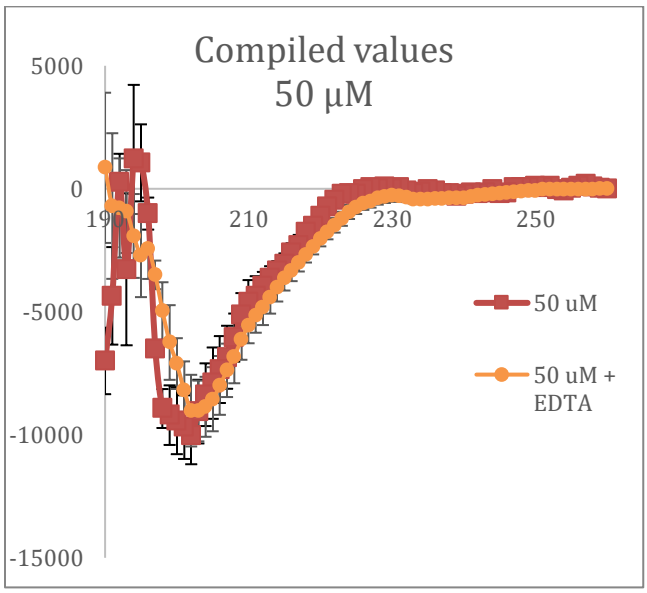
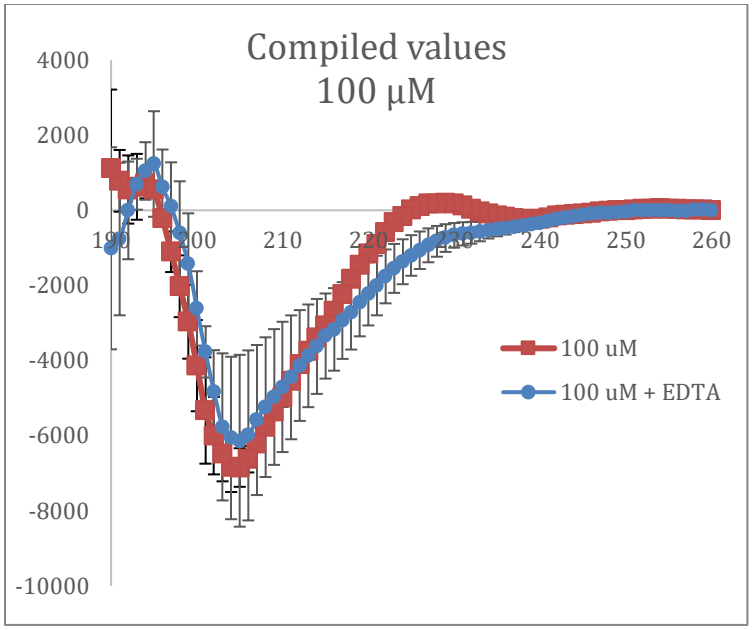
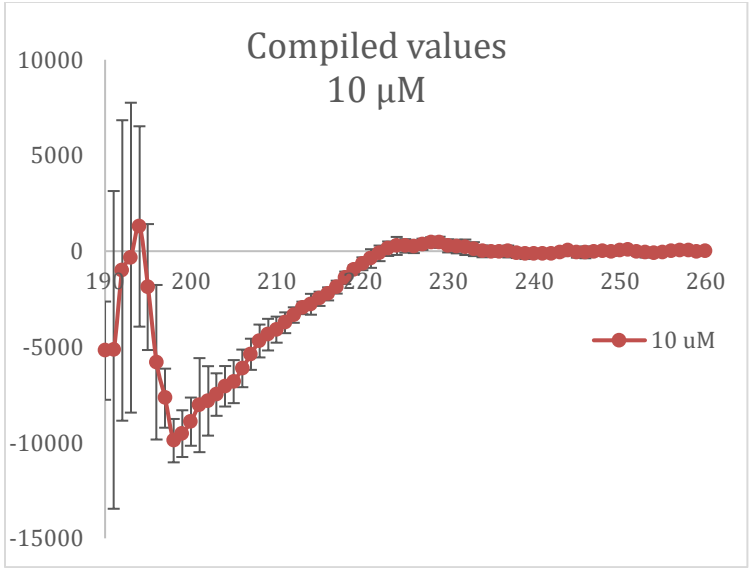
# Appendix

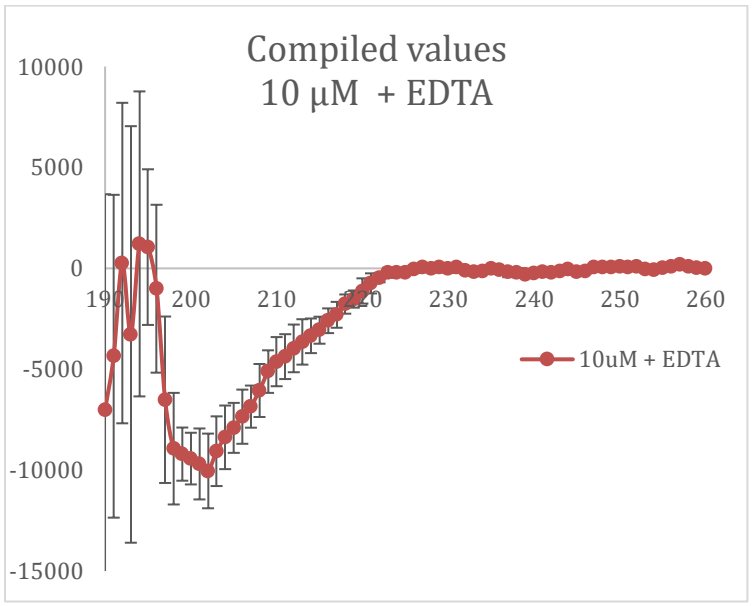
## A. CD analysis – compiled values

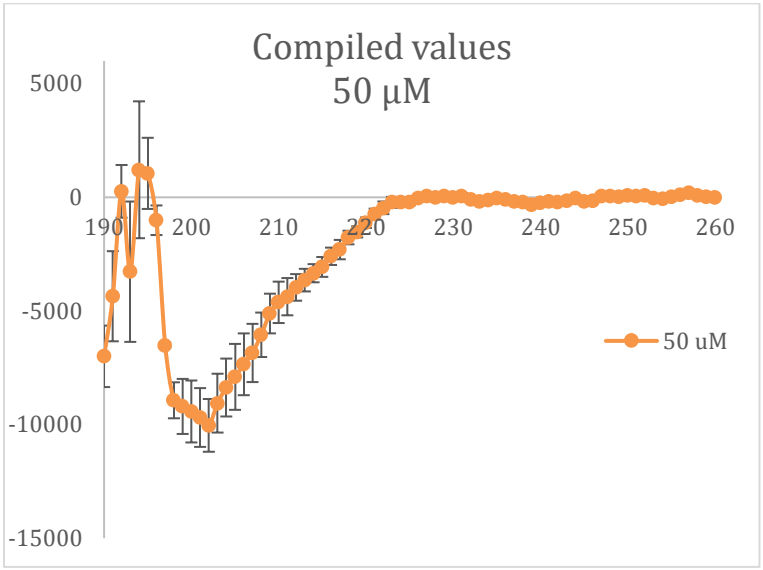


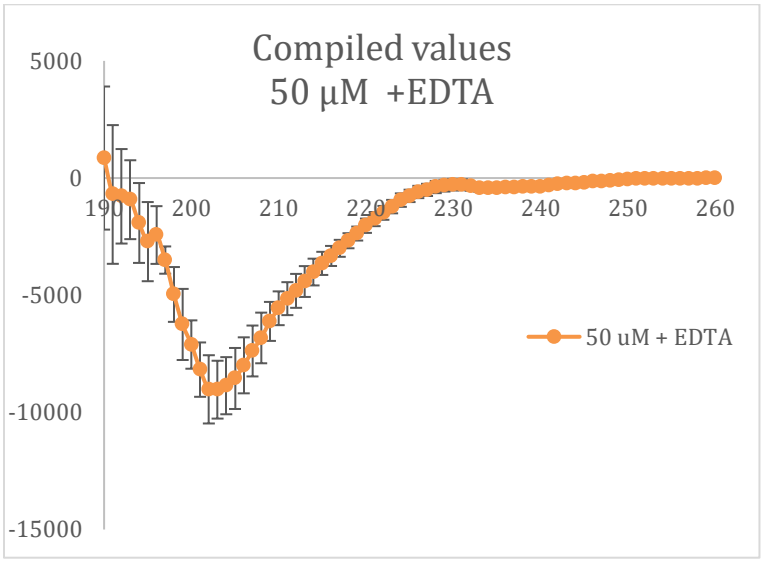



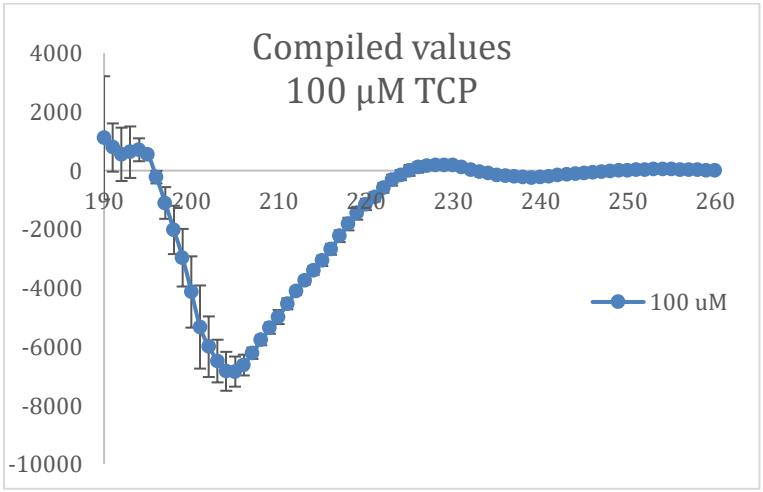

Compiled values
100 µM
4000
2000
0
-2000
-4000
-6000
-8000
-10000
190
200
210
220
230
240
250
260
100 uM
100 uM + EDTA

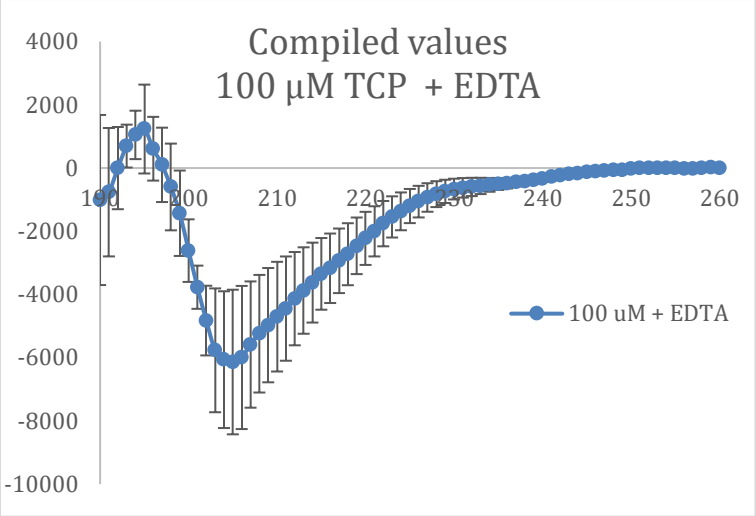

Compiled values
10 µM
10000
5000
0
-5000
-10000
-15000
190
200
210
220
230
240
250
260
10 uM


Compiled values
10 µM + EDTA
10000
5000
0
-5000
-10000
-15000
190
200
210
220
230
240
250
260
10uM + EDTA


Compiled values
50 µM
5000
0
-5000
-10000
-15000
190
200
210
220
230
240
250
260
50 uM


Compiled values
50 µM +EDTA
5000
0
-5000
-10000
-15000
190
200
210
220
230
240
250
260
50 uM + EDTA


Compiled values
100 µM TCP
4000
2000
0
-2000
-4000
-6000
-8000
-10000
190
200
210
220
230
240
250
260
100 uM


Compiled values
100 µM TCP + EDTA
4000
2000
0
-2000
-4000
-6000
-8000
-10000
200
210
220
230
240
250
260
100 uM + EDTA

## B. Stability study – table

**Table 6.** Shows the normalized % areas of the smaller peaks in relation to the main peak of TCP-25 and the change from in initial (fresh) values to values after 1 month of storage at 4C or RT.

| | Fresh | | | | 4°C 1 mon | | | | RT 1 mon | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No EDTA | | With EDTA | | No EDTA | | With EDTA | | No EDTA | | With EDTA | |
| | RRT | Norm area w/ main (%) | RRT | Norm area w/ main (%) | RRT | Norm area w/ main(%) | RRT | Norm area w/ main (%) | RRT | Norm area w/ main (%) | RRT | Norm area w/ main (%) |
| TCP-25 | 1 | 100 | 1 | 100 | 1 | 100 | 1 | 100 | 1 | 100 | 1 | 100 |
| Degradation product | 0,56 | 0,04 | 0,56 | 0,04 | 0,49 | 0,12 | 0,56 | 0,25 | 0,55 | 0,78 | 0,56 | 0,64 |
| | 0,94 | 0,06 | 0,94 | 0,05 | 0,56 | 0,11 | 0,63 | 0,06 | 0,61 | 0,06 | 0,84 | 0,03 |
| | 0,95 | 0,28 | 0,95 | 0,17 | 0,94 | 0,47 | 0,93 | 0,12 | 0,66 | 0,05 | 0,94 | 0,08 |
| | 0,97 | 0,16 | 0,97 | 0,15 | 0,95 | 0,19 | 0,95 | 0,26 | 0,72 | 0,05 | 0,95 | 0,11 |
| | 0,98 | 0,25 | 0,98 | 0,21 | 0,97 | 0,11 | 0,97 | 0,07 | 0,76 | 0,04 | 0,95 | 0,04 |
| | 1,03 | 0,35 | 1,03 | 0,41 | 0,98 | 0,09 | 1,03 | 0,62 | 0,78 | 0,04 | 0,97 | 0,07 |
| | 1,04 | 0,14 | 1,06 | 0,23 | 0,98 | 0,14 | 1,07 | 0,05 | 0,80 | 0,04 | 0,98 | 0,10 |
| | 1,06 | 0,28 | 1,07 | 0,05 | 1,03 | 0,26 | 1,08 | 0,07 | 0,81 | 0,06 | 1,03 | 0,18 |
| | 1,08 | 0,18 | 1,08 | 0,06 | 1,04 | 0,08 | 1,09 | 0,13 | 0,84 | 0,42 | 1,03 | 0,15 |
| | 1,09 | 0,10 | 1,09 | 0,10 | 1,07 | 0,04 | 1,11 | 0,07 | 0,89 | 0,04 | 1,04 | 0,17 |
| | 1,11 | 0,05 | 1,11 | 0,07 | 1,08 | 0,07 | | | 0,93 | 2,34 | 1,08 | 0,06 |
| | | | | | 1,09 | 0,11 | | | 0,95 | 0,21 | 1,09 | 0,12 |
| | | | | | 1,11 | 0,07 | | | 0,97 | 0,15 | 1,11 | 0,07 |
| | | | | | | | | | 0,98 | 0,08 | 1,19 | 0,03 |
| | | | | | | | | | 1,03 | 0,17 | | |
| | | | | | | | | | 1,03 | 0,13 | | |
| | | | | | | | | | 1,04 | 0,14 | | |
| | | | | | | | | | 1,07 | 0,03 | | |
| | | | | | | | | | 1,07 | 0,10 | | |
| | | | | | | | | | 1,09 | 0,10 | | |
| | | | | | | | | | 1,10 | 0,05 | | |

From **Table *6*** it is seen that that number of peaks increases more for the samples with only TCP than for those with EDTA added. This indicates that the amount of degradation product increases without EDTA.