Research Trip Summary Report

Task 2. Foreign mobility of WUST doctoral students

I. Data of the doctoral student

1.Full name: Manuela Grelich-Mucha

2.Year of studies: III

3. Educational discipline: Chemical Sciences

II. Foreign research trip (research visit)

1. Research institute in which the foreign research was implemented: Université de Strasbourg, Team of Biosystems Chemistry

2. Name and surname of the host person (mentor): Prof. Vladimir Torbeev

3. Dates of the research trip: 01.10.2021 – 18.12.2021

4. Title and date of a seminar delivered during the research trip: Self-assembling peptide aggregates; 24.11.2021

5. Description of work carried out during the research trip:

During the internship I was mainly focused on synthesis of different amyloidogenic peptides. I incubated synthesized peptide (105-115) fragments of human transthyretin (TTR) protein with varying N- and C-termini. I incubated pure peptides, as well as the possible (1/1) mixtures of the above-mentioned peptides. Moreover, I was controlling the ongoing changes of their secondary structure by attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy. The morphology changes were investigated using transmission electron microscopy (TEM). Moreover, I synthesized islet amyloid polypeptide (hIAPP) fragment, namely hIAPP(22-27). Besides, I was continuing studies which I begun during my previous internship at Prof. Torbeev group. I incubated Amyloid β-Protein (Aβ) fragment, namely Aβ(35-42) (MVGGVVIA). In parallel, I was incubating depsiAβ(35-42), in which an ester bond moiety was incorporated between the 35th and 36th amino acid in the sequence. I studied kinetics of the ester bond hydrolysis, the morphology and the ongoing changes in the secondary structure.

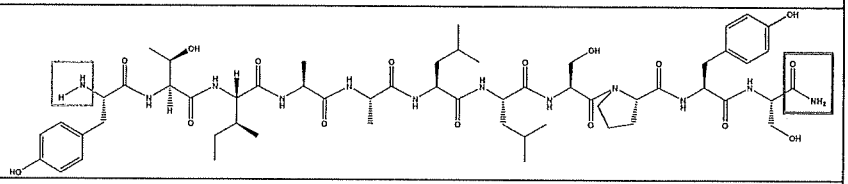
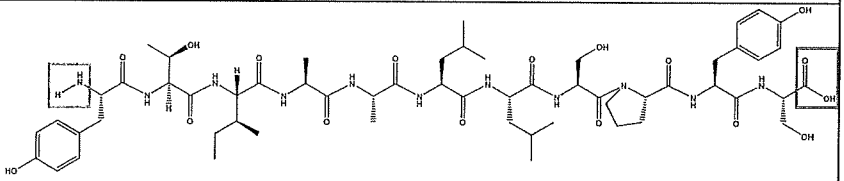
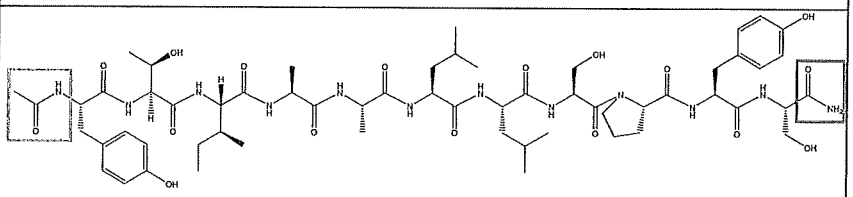
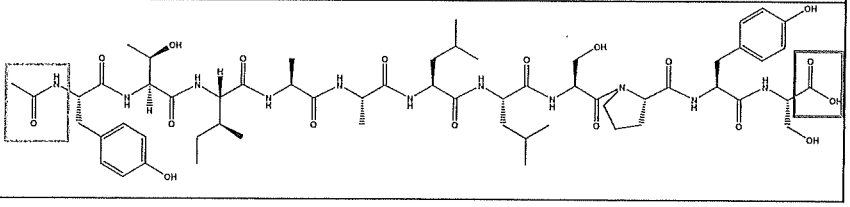
First of all, I synthesized *L*-TTR(105-115) (YTIAALLSPYS) peptide fragments with varying N- and C-termini (**Table 1**). The C-terminus was terminated by a carboxyl group or was amidated. At N-terminus either α-amine group was present or further modifications were performed including acetylation and N,N-dimethylation. I also synthesized *D*-TTR(105-115) termed by α-amine and amide group (MG15) (**Table 1**).

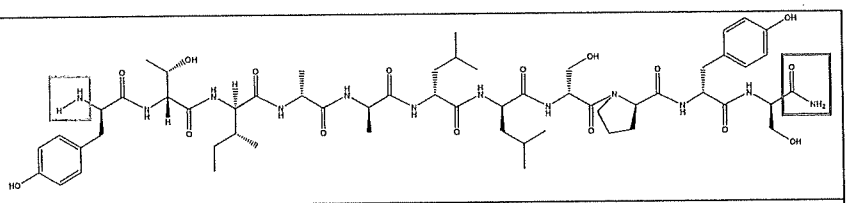
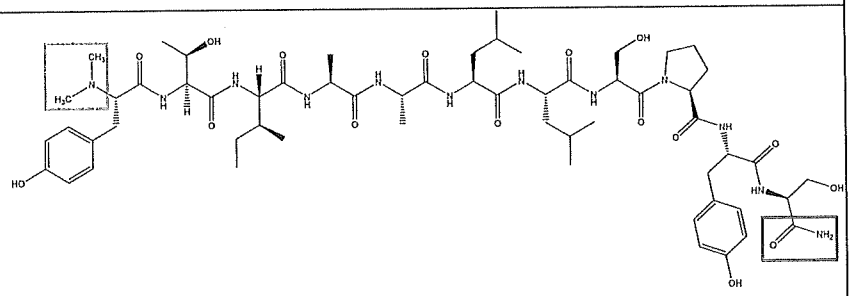
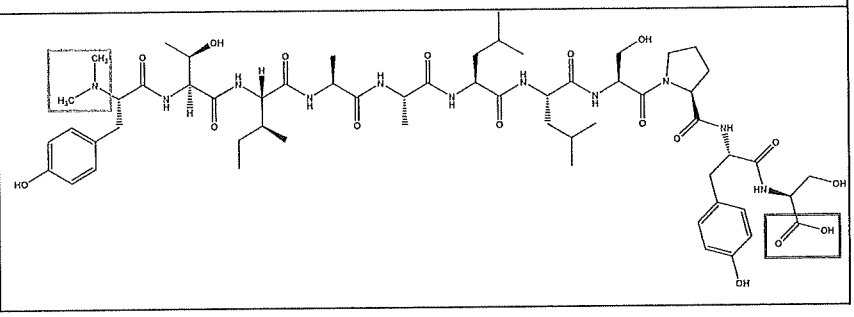
The peptides were synthesized using the Liberty Blue automated microwave peptide synthesizer. Standard 9-fluorenylmethoxycarbonyl (Fmoc) strategy was used. The crude peptides were

purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC). Target fractions were further analyzed using analytical RP-HPLC and their mass was determined by liquid chromatography-mass spectrometry (LC-MS).

C-terminal modifications for TTR(105-115) were achieved using proper resins. In order to provide carboxyl group at C-terminus, a Wang resin preloaded with *L*-Serine was used, namely Fmoc-*L*-Ser(t-Bu)-Wang Resin (100–200 mesh, 0.7 mmol/g). Amidated C-terminus was obtained using Fmoc-Rink Amide AM resin (100–200 mesh, 0.74 mmol/g, 1% DVB). In order to perform acetylation of N-terminus, after the final Fmoc group deprotection, the following procedure was applied: the reaction vessel was filled with N,N-Diisopropylethylamine (DIEA) (200 µl), acetic anhydride (200 µl) and N,N-dimethylformamide (DMF) (1.4 ml). The procedure was performed 2 times for 6 minutes each. In order to confirm the absence of α-amine group at N-terminus, Ninhydrin test was done. N,N-dimethylation of N-terminus was achieved using the following procedure. The purest fractions of peptides termed by α-amine group were dissolved in NaOAc (0.1 M, pH~4) at concentration 1 mg/ml. The mass of the peptide was checked using LC-MS. Then, formaldehyde (1 000 eq.) and $NaBH_3CN$ (30 eq., 0.2 M) were added to the dissolved peptide. After a brief mixing, mass of the peptide was checked using LC-MS. After confirmation of a successful N,N-dimethylation of N-terminus, purification was performed using semi-preparative RP-HPLC (T=40 °C).

Table 1. Synthesized TTR(105-115) peptide fragments with varying modifications of N- and C-termini.

Name	Modification	Structure
MG6	N-terminal α-amine group, amidated C-terminus	
MG7	N-terminal α-amine group, carboxyl group at C-terminus	
MG8	Acetylated N-terminus, amidated C-terminus	
MG9	Acetylated N-terminus, carboxyl group at C-terminus	

MG15	N-terminal α-amine group, amidated C-terminus	
MG18	N-terminal dimethylation and amidated C-terminus	
MG19	N-terminal dimethylation and carboxyl group at C-terminus	

During the internship I incubated TTR(105-115) peptides (MG6, MG7, MG8, MG9) and their possible (1/1) mixtures by mixing the same amount of two different TTR(105-115) peptide fragments (MG67, MG68, MG69, MG78, MG79, MG89) **(Table 2)**.

Table 2. Prepared samples for incubation of TTR(105-115) peptide fragments.

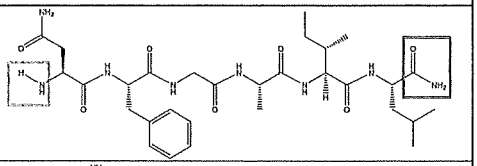
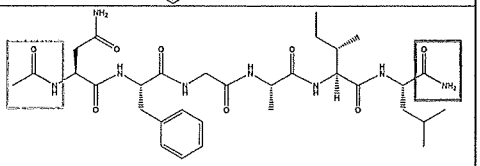
Peptide / Peptide	MG6	MG7	MG8	MG9
MG6	MG6	MG67	MG68	MG69
MG7		MG7	MG78	MG79
MG8			MG8	MG89
MG9				MG9

In order to remove residual TFA (trifluoroacetic acid), target peptides were dissolved in 10 mM HCl and then lyophilized. The procedure was 4 times repeated. The following incubation protocol was used: target peptides (MG6, MG7, MG8, MG9) were dissolved in DMSO. Then, a mixture of H_2O/MeCN (9/1) pH~2 was added. The final content of DMSO was 2% (v/v). The concentration of peptides was 1 mM. In order ensure the same peptide content, peak areas of the peptides were checked using analytical RP-HPLC. Absorbance was set to 280 nm. Purity of the peptides was ≥ 98 %. Afterwards, equimolar

mixtures of the peptides were prepared. The peptides, as well as the (1/1) mixtures were incubated at 37°C. During the incubation period, the secondary structure was investigated using ATR-FTIR spectroscopy. The morphology was studied using TEM.

Besides the synthesis of TTR(105-115) with different N- and C-termini, I also wanted to synthesize islet amyloid polypeptide (hIAPP) fragment, namely hIAPP(22-27) which has the following sequence: NFGAIL. I synthesized manually the peptide sequence with amidated C-terminus and α-amine (MG12) or acetyl group at N-terminus (MG14) (**Table 3**).

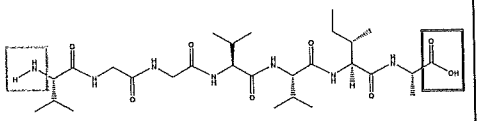
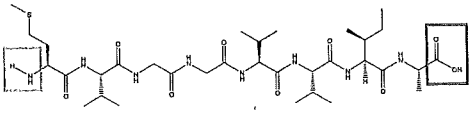
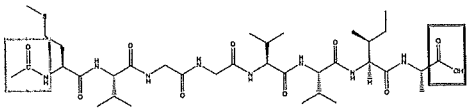
Table 3. Synthesized hIAPP(22-27) peptide fragments with different N-termini and amidated C-termini.

Name	Modification	Structure
MG12	N-terminal α-amine group, amidated C-terminus	
MG14	Acetylated N-terminus, amidated C-terminus	

MG12 is a very hydrophobic peptide and it was difficult to find conditions leading to dissolution of the peptide. Acetylation of N-terminus caused increase in hydrophobicity and it was impossible to dissolve and further purify the peptide. Therefore, the acetylated peptide was not purified.

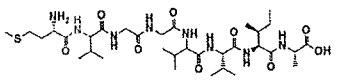
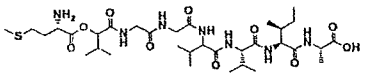
During my internship, I also synthesized Amyloid β-Protein (Aβ) fragments (**Table 4**). I synthesized Aβ(36-42) (VGGVVIA) (MG16). The peptide was termed by α-amine group and carboxyl group at N- and C-terminus, respectively. The C-terminus was provided using Fmoc-*L*-Ala-Wang Resin (100–200 mesh, 0.8 mmol/g, 1% DVB). Moreover, Aβ(36-42) (MVGGVVIA) possessing α-amine group and carboxyl group at N- and C-termini (MG3) was synthesized. Additionally, acetylated analogue was synthesized (MG17). For MG3 and MG17 crude peptides mass spectra were recorded and confirmed the presence of target peptides. However, due to later problems with LC-MS and unavailability to check mass spectra, MG3 and MG17 were finally left as crude peptides.

Table 4. Synthesized Aβ peptide fragments with different N-termini and carboxyl group at C-terminus.

Name	Modification	Structure
MG16	N-terminal α-amine group, carboxyl group at C-terminus	
MG3	N-terminal α-amine group, carboxyl group at C-terminus	
MG17	Acetylated N-terminus, carboxyl group at C-terminus	

In the meantime, I was also continuing research regarding peptides that I synthesized during my previous stay at prof. Torbeev group (10.2019-12.2019) financed within the frames of Erasmus+ 2014-2020. I was incubating Aβ(35-42) (MG3) and depsiAβ(35-42) (MG4) peptides. In MG4 an ester bond was incorporated between the 35th and 36th amino acid in the sequence (**Table 5**). To ensure an effective disaggregation, the peptides were treated using HFIP (1,1,1,3,3,3-Hexafluoropropan-2-ol). After HFIP evaporation, the peptides were dissolved at c=1.5 mM in sodium phosphate buffer (pH~2). The samples were incubated at room temperature (r.t.). At target time points chromatograms (using analytical RP-HPLC) were recorded at absorbance set to 220 nm. Moreover, for the samples ATR-FTIR spectra were recorded and the morphology was studied using TEM.

Table 5. Studied Aβ peptide fragments, synthesized during my previous internship.

Name	Structure
MG3	
MG4	

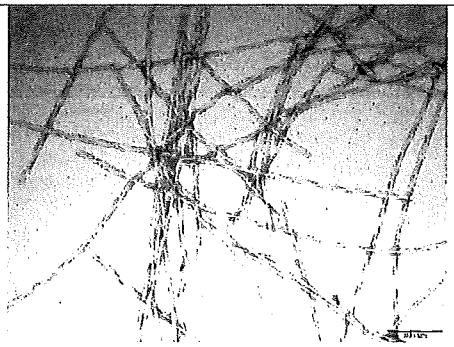
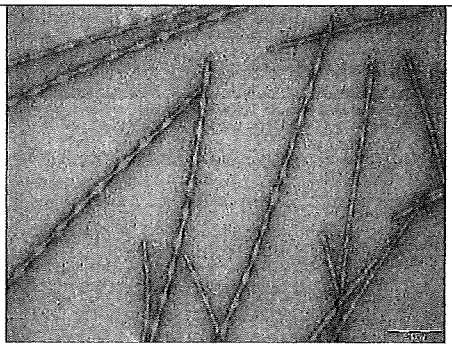
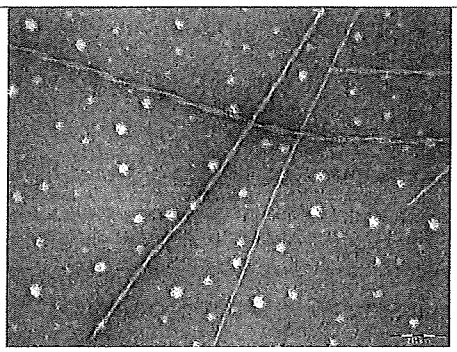
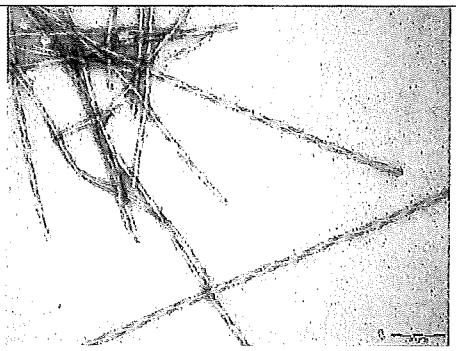
In summary, during the internship I synthesized peptide sequence of *L*-TTR(105-115) with different termini. At N-terminus either acetyl or α-amine group, whereas at C-terminus carboxyl or amide group was present. Additionally, *D*-TTR(105-115) with α-amine and amide group at N- and C-terminus was synthesized. Moreover, I successfully obtained peptides with N,N-dimethylated N-terminus. In total, 7 different analogues of TTR(105-115) were synthesized. For 4 analogues (MG6,

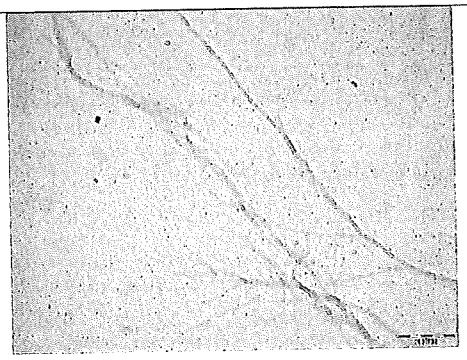
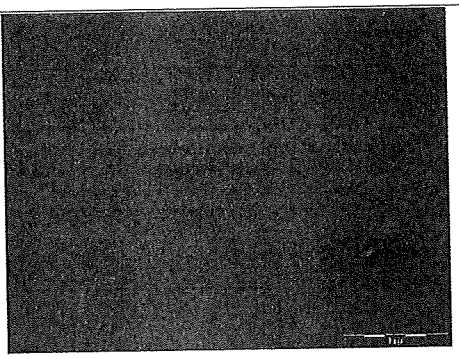
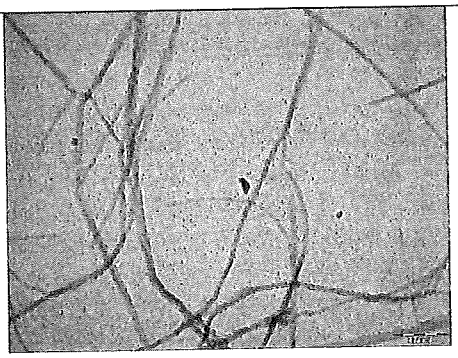
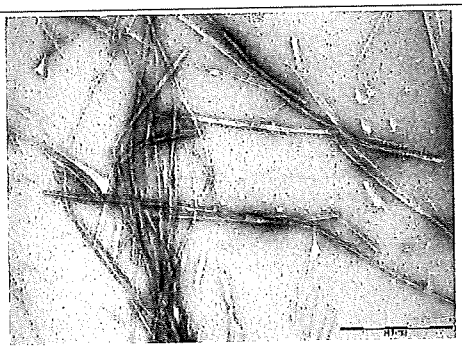
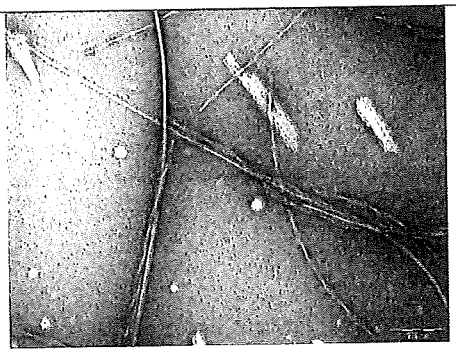
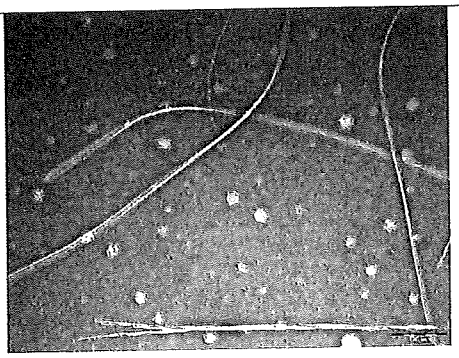
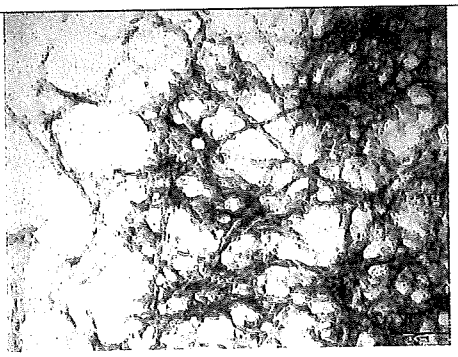
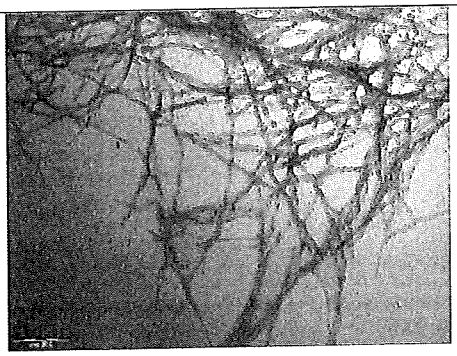
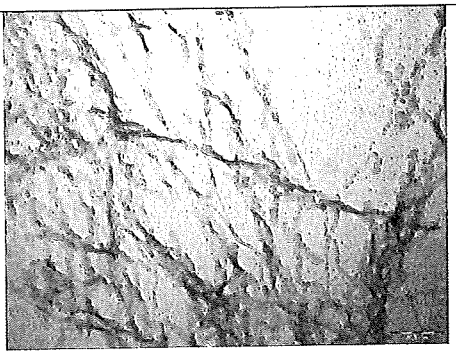
MG7, MG8, MG9) and their equimolar mixtures during the incubation time morphology and structural properties were studied. Finally, optical properties of incubated samples were not investigated. I will analyze them at Advanced Materials Engineering and Modelling Group (Wroclaw University of Science and Technology). The optical properties will be studied for all synthesized TTR(105-115) peptide variants. I synthesized hIAPP(22-27) peptide fragments, as well as Aβ peptide fragments. Finally, I also investigated morphology and structure of Aβ(35-42) and depsiAβ(35-42). For depsiAβ(35-42) kinetics of ester bond hydrolysis was investigated.

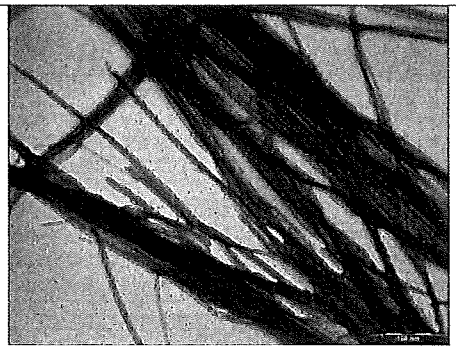
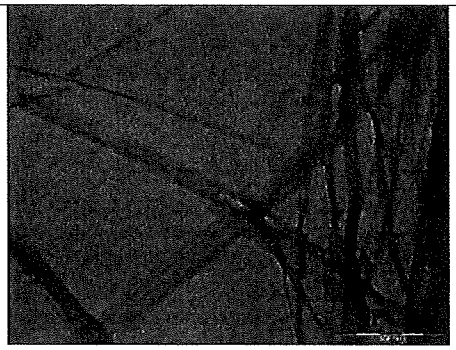
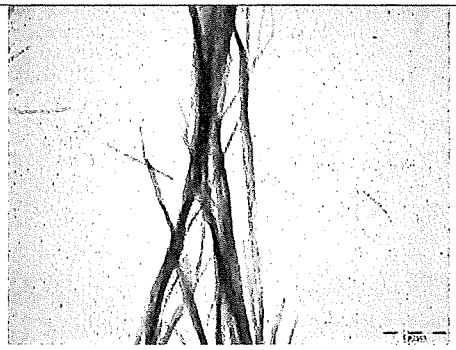
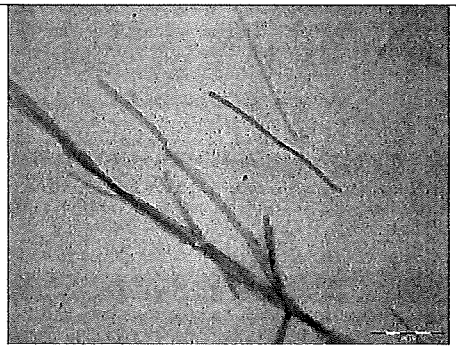
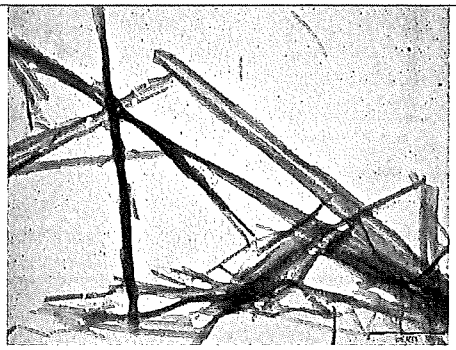
6. Description of the main results obtained:

Incubation of *L*-TTR(105-115) peptides with different N- and C-termini, as well as their possible (1/1) mixtures lead to the formation of amyloid fibrils. The presence of fibrils was confirmed by TEM images recorded at two different time points: day 8 and 20 of incubation (**Table 6**).

Table 6. TEM images obtained for *L*-TTR(105-115) samples at day 8 and 20 of incubation period. Scale bar on the images is set to 200 nm, except for MG68 (day 20) where the scale bar is set to 1 μm, MG8 and MG9 (day 8 and 20) where the scale bar is set to 500 nm.

Sample	Day 8	Day 20
MG6		
MG67		

MG68		
MG69		
MG7		
MG78		
MG79		

MG8		
MG89		
MG9		

TEM images (**Table 6**) evidence the presence of amyloid fibrils among all of the samples on day 8 and 20 of the incubation period. However, there were differences in morphology when comparing day 8 and 20 of incubation. For example, for sample MG67 on day 8 besides helical fibrils, also many spherical aggregates were present. On day 20 in the sample the small spherical structures were still present, but in a lower amount. Additionally, there were more amyloid fibrils present than on day 8. The fibrils on day 20 of incubation were characterized by a shorter helical pitch (205.0±15.1 nm on day 20 and 249.2±23.7 nm on day 8).

For MG69, totally different morphology was observed on day 8 and 20. On day 8 many tiny fibrils were predominating, whereas on day 20 spherical aggregates and wider, but shorter amyloid fibrils were present.

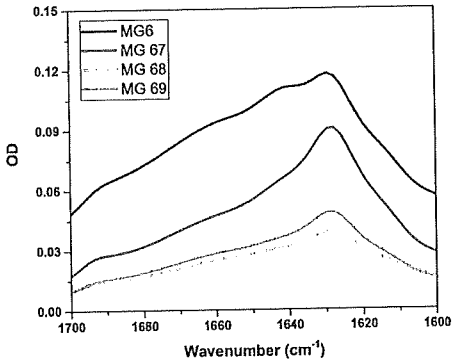
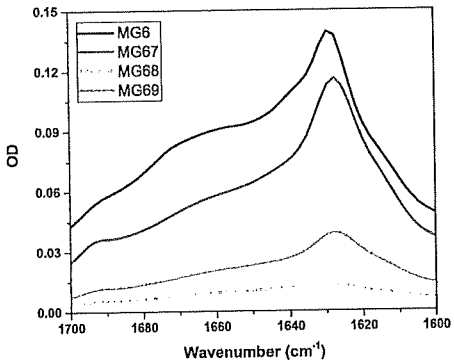
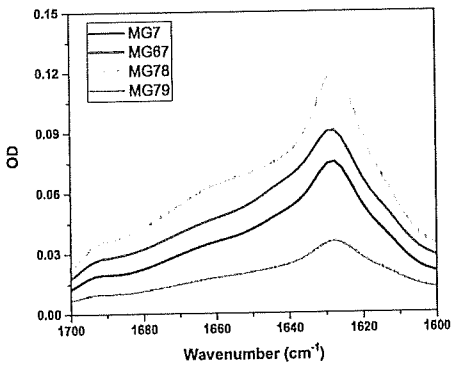
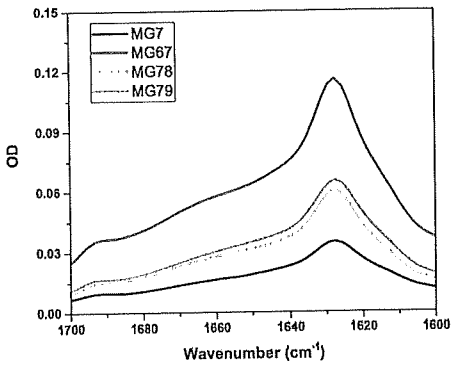
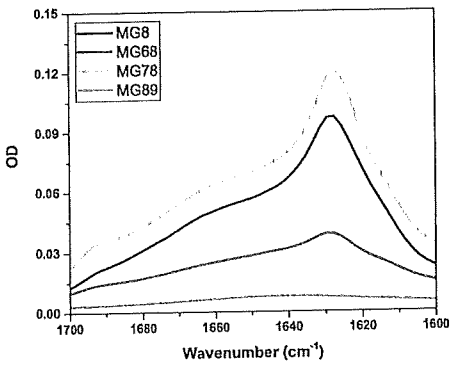
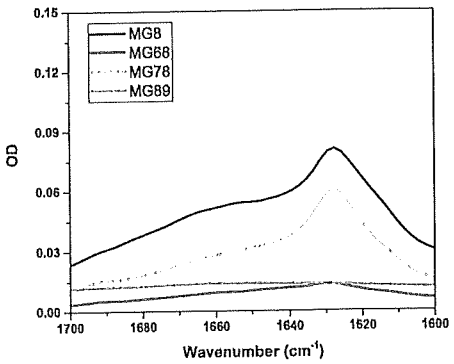
For MG7 on day 8 only flat fibrils were present. However, on day 20 besides flat fibrils, also helical ones were present.

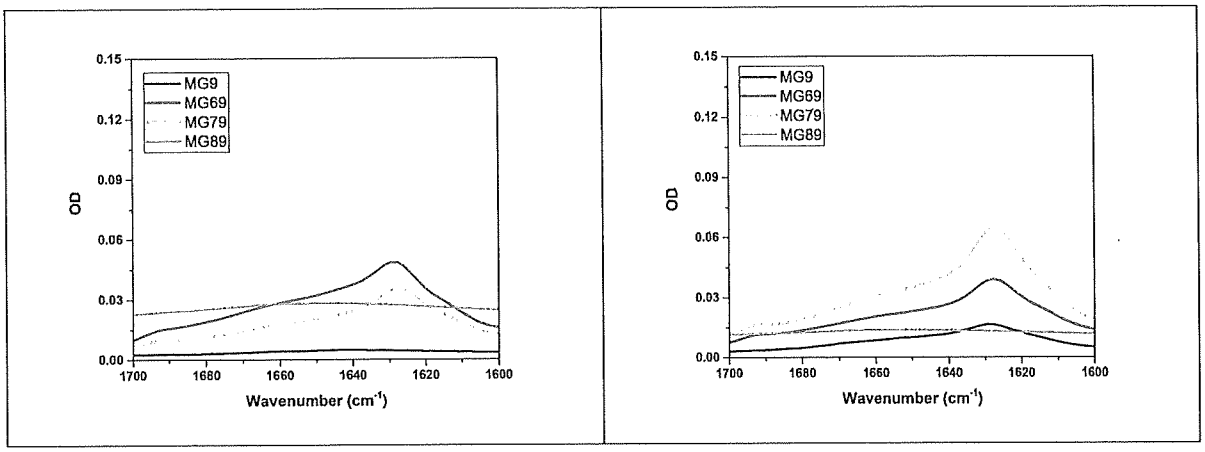
For MG78 on day 8 many spherical aggregates were present. On day 20 fibrils were predominating. However, their morphology was different comparing fibrils observed on day 8 and 20.

Among the above-mentioned samples, *i.e.* MG67, MG69, MG7 and MG78, the most spectacular differences in morphology were observed.

For the samples also ATR-FTIR spectra on day 8 and 19 of the incubation period were recorded (**Table 7**).

Table 7. ATR-FTIR spectra recorded for *L*-TTR(105-115) samples at day 8 and 19 of the incubation period.

Day 8	Day 19
	
	
	



Recorded ATR-FTIR spectra (**Table 7**) evidence the presence of a β-sheet structure (maximum of absorption band at ~1630 cm^{-1}) in amide I region among almost all of the samples. For MG89 during the subsequent scans differences in the secondary structure were observed. During the final scans, obtained spectra were almost flat. The same tendency was observed on day 8 and 19 of incubation. For MG9 almost flat spectrum was recorded on day 8. However, on day 19 recorded spectrum indicated the presence of a β-sheet structure.

The TEM images indicate that from the small spherical aggregates at later time point amyloid fibrils are forming. They also suggest different kinetics of fibril formation depending on N- and C-termini. Recorded ATR-FTIR spectra are consistent with the TEM images, as amyloid fibrils are characterized by the presence of a secondary β-sheet structure. For MG89 and MG9, which possess acetylated N-terminus, a flattening in amide I region was observed. However, on day 19 for MG9 a spectrum evidencing a presence of a β-sheet structure was recorded. The above-mentioned statements correspond to the initial studies performed during the internship. In order to gain more information regarding the ongoing differences in morphology and the structural properties, TEM imaging and ATR-FTIR spectra measurements should be performed also at another time points. Moreover, the experiment should be repeated in order to verify whether the results are repetitive.

During the internship I incubated at r.t. MG3 (Aβ(35-42)) and MG4 (depsiAβ(35-42)) samples in sodium phosphate buffer (pH~2). Using analytical RP-HPLC, the changes in chromatograms at different time points (λ=220 nm) for MG4 were recorded. At acidic pH ester bond hydrolysis is enhanced. Therefore, at later time points the area corresponding to depsiAβ(35-42) was decreasing and for the target hydrolysis product was increasing. In order to confirm the presence of hydrolysis product, on day 4 of incubation mass spectrum was recorded for the sample (**Figure 1a, b**); m/z=746.500 and m/z=615.083 correspond to depsiAβ(35-42) and its hydrolysis product, respectively. According to obtained results, the area of MG4 divided by area of the formed hydrolysis product decreased around 2 times already on the second day of incubation (**Figure 1c**).

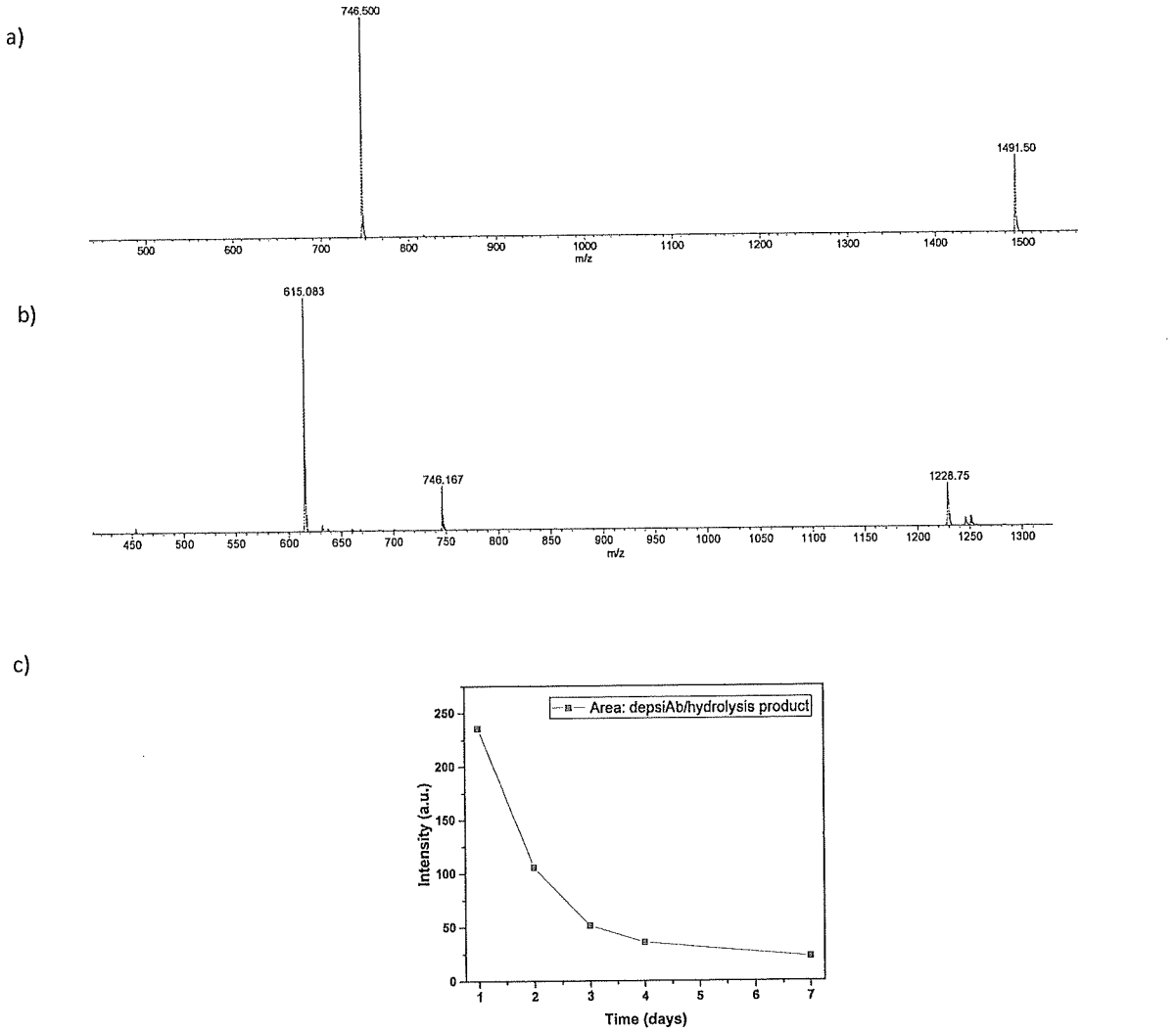


Figure 1. LC-MS spectra recorded on the 4th day of incubation evidencing the presence of depsiAβ(35-42) (a) and its hydrolysis product (b). A plot: area of MG4 divided by area of hydrolysis product as the function of time (c).

Morphology of the samples was studied using TEM imaging (**Figure 2**) which demonstrated the presence of amyloid fibrils in MG3 (**Figure 2a**). However, in MG4 very big aggregates were predominating (**Figure 2b**).

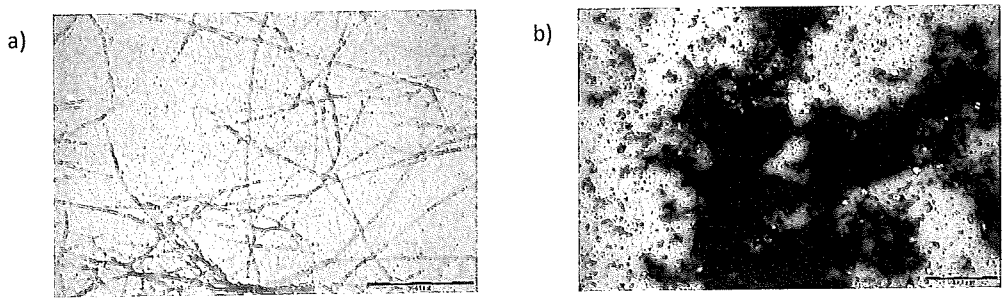


Figure 2. TEM images of MG3 (a) and MG4 (b) on the 10th day of incubation. The scale bar on the images is set to 500 nm.

In addition, ATR-FTIR spectra were recorded for the samples on day 3 of incubation period (**Figure 3**).

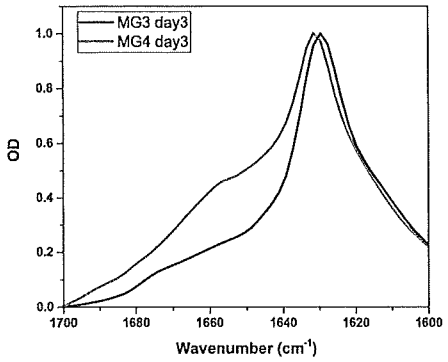


Figure 3. Normalized ATR-FTIR spectra recorded for MG3 (black) and MG4 (red) on the 3rd day of incubation.

Recorded ATR-FTIR spectra evidence the presence of a β-sheet structure in both, MG3 and MG4 samples on day 3 of the incubation period. Moreover, they demonstrate that in MG4 sample more content of random coiled structure was present, which is probably due to the formation of bigger peptide aggregates observed on TEM images (**Figure 2b**).

In summary, initial results obtained during the internship are very promising. The experiments have to be repeated in order to verify their repeatability.

7. Future collaborations (if applicable):

Future collaboration will include continuation of performed experiments. The experiment regarding incubation of *L*-TTR(105-115) peptides and the equimolar mixtures will also be performed for the other synthesized analogues, including MG15, MG18, and MG19 peptides. For target samples morphology, structural and optical properties will be studied. The optical properties measurements will include UV-Vis spectrophotometry, fluorescence spectroscopy and most probably fluorescence lifetime decays.

In order to better understand the phenomenon of amyloid fibrils autofluorescence and the role of proton transfer, the peptides will be dissolved and lyophilized in deuterated solvent (D_2O or diluted DCl solution). The process will be repeated several times. Afterwards, the peptides will be incubated in the same conditions as mentioned above, but using deuterated solvents. The procedure will allow H/D exchange of exchangeable protons in amide, hydroxyl, amine and carboxylic groups. For as-formed fibrils optical properties will be studied and compared to the samples incubated in protonated solvents.

The results will be presented during some conferences. If they will have a great potential, they will be possibly published in the form of a scientific article.

8. Title and date of a seminar presenting the results of the trip delivered at Wroclaw University of Science and Technology after returning from the research trip: Synthesis and initial studies of amyloidogenic peptides with modified N- and C-termini; 14.01.2022.

III. Doctoral student's signature

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(Date) (doctoral student's signature)

IV. Confirmation and information from the host

1. Confirmation of compliance of the information contained in the report: I CONFIRM / DO NOT CONFIRM. (*In justified cases, the confirmation of the host may be sent by e-mail to the Dean's Office of the Doctoral School email: interdocschool@pwr.edu.pl)*

2. Additional information and comments

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(Date) (signature(s) of Host)