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β-amino acid substitutions in β-sandwich model proteins

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A. INTRODUCTION AND AIMS

The function of biological macromolecules, such as DNA, RNA or proteins, depends or their ability to adopt specific, kinetically controlled and thermodynamically stable conformations. This process, referred to as “folding”, ensures the precise arrangement of functional groups required for tasks like molecular recognition or catalysis. A similar ability of self-organization is essential for artificial molecules to be capable of mimicking the diverse structural and functional behavior of proteins. The term “foldamer” is used to describe such molecules: artificial polymers with a strong tendency to adopt compact conformations, i.e. **fold**ing poly**mers**.

The first step in the mimicry of complex protein structures is to design stable secondary structural motifs, like helices, β-sheets, and hairpins. A number of foldameric helices, hairpins, as well as several standalone β-sheets were designed, but the cooperative folding of different elements into a tertiary structure is a current challenge, because of the complexity of the problem. The design of water-soluble, stable β-sheets and β-sandwiches is also hindered by their propensity to form insoluble aggregates. Designing stable scaffolds with β-sheet structure is, however, very much desirable, as many aspects of protein functionality are realized through domains with high β-sheet content, like the variable region of antibodies and a great number of protein–protein interaction (PPI) surfaces.

The pharmaceutical need for such protein-mimicking foldamers is also high, because the number of drugs with proteins as active compounds is continuously increasing, but their application is greatly limited, because of their poor pharmacokinetics, stability and immunogenicity. However, many of these disadvantages can be reduced or eliminated by introducing artificial residues into their backbones.

Our goal was to explore the feasibility of β-amino acid substitutions in a complex, protein-sized β-sandwich model. Betabellins, a model protein family were chosen as templates, which are *de novo* designed, 64-residue β-sandwich proteins stabilized by the dimerization of two identical monomeric chains.

First, we introduced β-amino acid mutations in the hydrophobic core of the β-sandwich to investigate the effects and tolerability of diverse side chains by using homologous β^3- and conformationally constrained cyclic β-amino acids. We also studied the effect of cyclic β-amino acid substitutions in the peripheral strands of the protein templates with a focus on multiple aspects of structural stability: thermodynamic stability (free energy of reversible unfolding), thermal stability (thermal and cold denaturation), and stability against uncontrolled aggregation.

We employed circular dichroism (CD) spectroscopy to assess the overall and temperature-dependent folding propensity, NMR chemical shift analysis for residue-level structural information, and molecular dynamics calculations for modeling and explaining the side-chain

dependent folding behavior. Aggregation tendency was monitored using NMR signal levels and transmission electron microscopy experiments.

B. METHODS

Circular dichroism (CD) spectroscopy

The overall folding properties of the compounds were investigated by CD experiments. Spectra were recorded at peptide concentrations of 100 μM and 50 μM for monomeric and dimeric sequences, respectively. The spectra were deconvoluted with the convex constraint algorithm (CCA+) for a quantitative estimation of secondary structural components. Temperature-dependent CD measurements were carried to investigate cooperative folding and stability. The derived stability data was used to obtain thermodynamic parameters.

NMR spectroscopy

NMR spectroscopic methods were used to investigate conformational behavior at residual level. Spectra were recorded at 500 μM and 250 μM for monomeric and dimeric sequences, respectively. Resonance assignment was carried out with the help of 2D TOCSY, NOESY, ^{13}C- and ^{15}N-HSQC experiments. Secondary structure propensity was determined by the comparison of the detected chemical shifts to a reference random coil chemical shift set. These were combined into secondary structure propensity (SSP) score by the SSP software. NMR measurements were also used to monitor aggregation tendency at increasing salt concentrations.

Molecular modeling

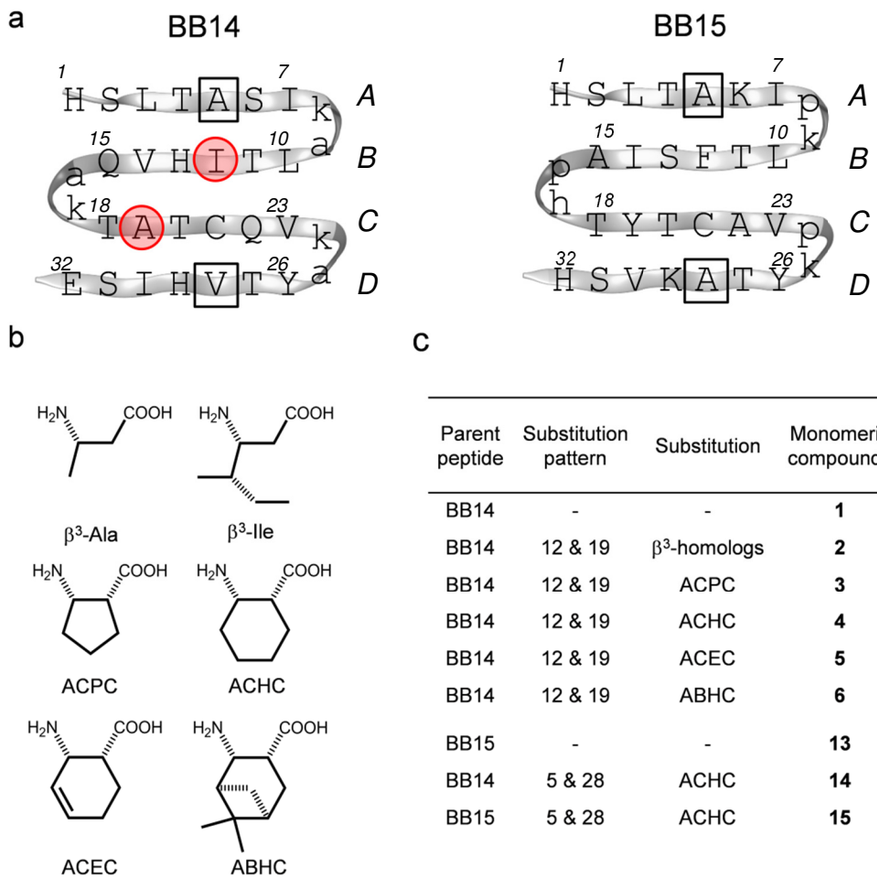
Molecular structures were generated and aligned according to literature data for betabellin-14. Molecular dynamics simulations were run using GROMACS with AMBER ff03 – TIP3P force field. Production runs of 150 ns were used to investigate the flexibility and folding properties of the structures. β-sheet propensity was characterized by calculating solvent-accessible surface areas and number of backbone hydrogen bonds.

Transmission electron microscopy

Aggregation propensity was investigated by electron microscopy. 250 μM solution of the peptide in phosphate buffer was placed on carbon-coated grid and stained with uranyl acetate. The aggregates were characterized on a JEOL JEM-1400 transmission electron microscope operating at 120 kV. Images were taken routinely at magnification of ×25000.

C. RESULTS AND DISCUSSION

1. In order to investigate the feasibility and the rules of β-amino acid replacements in a protein-sized β-sandwich scaffold, we designed and synthesized seven foldameric analogs of two betabellin model proteins, betabellin-14 (BB-14) and betabellin-15 (BB-15), containing β-amino acids, in 32-residue monomeric and in 64-residue dimeric forms. The first design strategy involved substitution of core hydrophobic residues in BB-14 with homologous β^3-amino acids and with different conformationally constrained cyclic β-residues. In the second part of the work we introduced cyclic β-amino acid substitutions in the peripheral strands of betabellin-14 and betabellin-15 to investigate the effects on stability and edge-to-edge aggregation (Figure 1).



Parent peptide	Substitution pattern	Substitution	Monomeric compounds	Dimeric compounds
BB14	-	-	**1**	**7**
BB14	12 & 19	β^3-homologs	**2**	**8**
BB14	12 & 19	ACPC	**3**	**9**
BB14	12 & 19	ACHC	**4**	**10**
BB14	12 & 19	ACEC	**5**	**11**
BB14	12 & 19	ABHC	**6**	**12**
BB15	-	-	**13**	**16**
BB14	5 & 28	ACHC	**14**	**17**
BB15	5 & 28	ACHC	**15**	**18**

Figure 1. Amino acid sequence and secondary structure representation of model betabellin structures (**a**). The residues are coded with standard one-letter α-amino acid notations; lower-case letters indicate D-α-amino acids. Circles and squares represent the positions of central and peripheral replacements, respectively. β-amino acids utilized in the study (**b**). Design strategies of the betabellin analogs studied (**c**).

2. Overall folding behavior of the foldamers was analyzed by circular dichroism (CD) spectroscopy. Dimerization-induced folding similar to the parent sequences was observed for all analogs except **12** containing bulky ABHC residues (Figure 2a-b) Deconvolution of the CD spectra was carried out for a quantitative estimation of the secondary structural content, revealing differences in the induced β-sheet contents of the analogs (Figure 2d). All foldamers exhibited less ordered structure compared to the parent sequence. The highest β-sheet content was achieved with 1*R*,2*S*-amino-cyclohexanecarboxylic acid (ACHC) substitutions. Compared to core mutations, peripheral substitutions resulted in higher overall β-sheet content (Figure 2c).

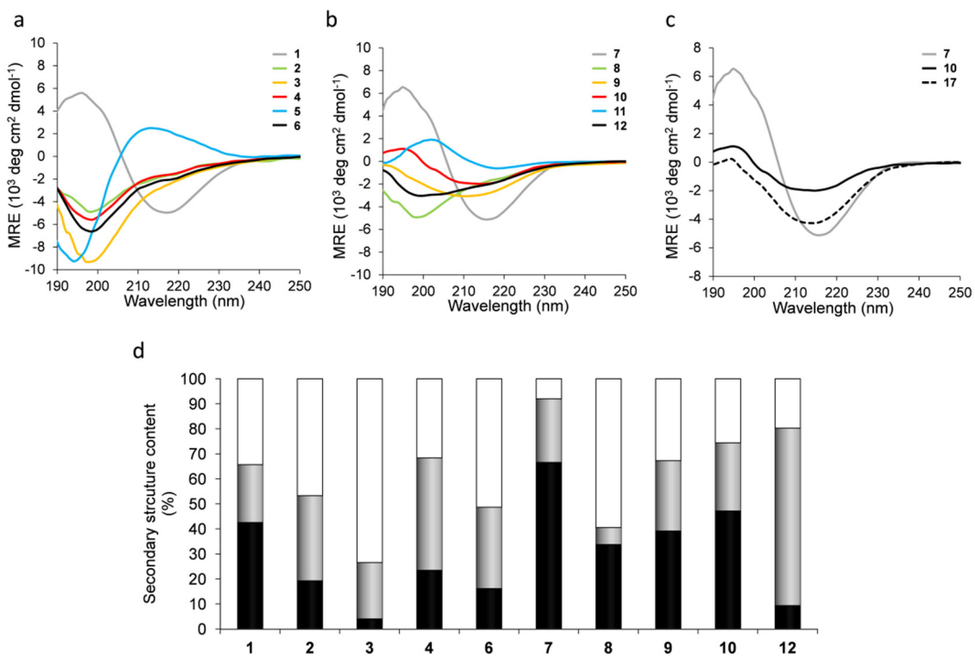


Figure 2. Mean residue ellipticities (MRE) obtained for monomeric (**a**) and dimeric (**b**) compounds with central substitutions. CD spectra comparison of central and peripheral ACHC-substituted BB–14. (**c**) Secondary structure content in percentages of BB–14 and its analogs with central substitutions (**d**).

3. Temperature-dependent CD measurements were carried out to investigate the stability and cooperativity of the folding. Protein-like cold and thermal denaturations were observed in the parent sequences and the analogs containing ACHC residues. (Figure 3a-b) The stability curve of the peripheral-substituted compounds was calculated from the CD data and compared to those of the parent sequences, revealing a decrease in the thermodynamic stability, but not in the temperature of maximum stability (Figure 3c).

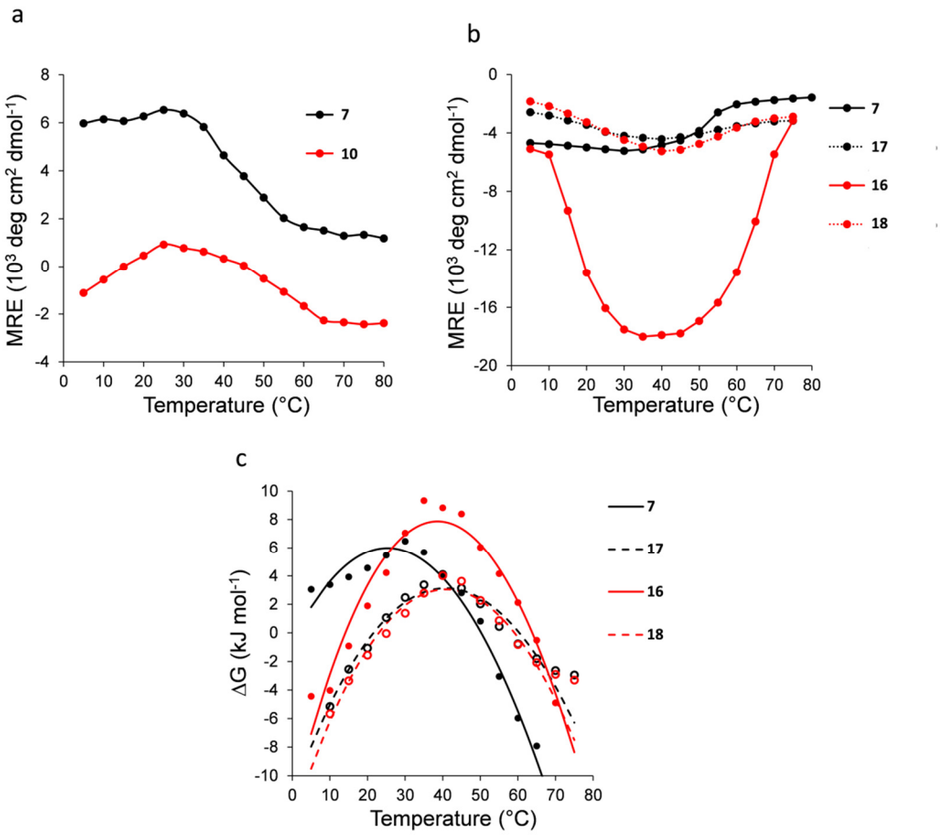


Figure 3. Cold and thermal denaturation according to temperature-dependent CD experiments. MRE values at 195 nm for **7** and **10** (**a**). MRE values at 216 nm for betabellins and peripheral substituted analogs (**b**) Experimental and fit protein stability curves (free energy of unfolding) for betabellins and peripheral substituted analogs (**c**).

4. NMR spectroscopic experiments were run and secondary chemical shift analysis was performed to obtain residue-level structural data. The chemical shifts of all compounds could be successfully assigned and SSP scores were calculated. A local distortion of the structure was observed around the β-residues. Dimerization induced the β-sheet content mostly in the strand containing the Cys residue, propagating to the other strands. The induced β-sheet content was the highest for analog **10**, while other analogs showed varying folding behaviors and a lower extent of inducibility. (Figure 4a-c) Peripheral mutations also had a marked effect on the folding propensity of the core strands, suggesting the presence of shielding contacts between the edge and core strands. (Figure 4d-e)

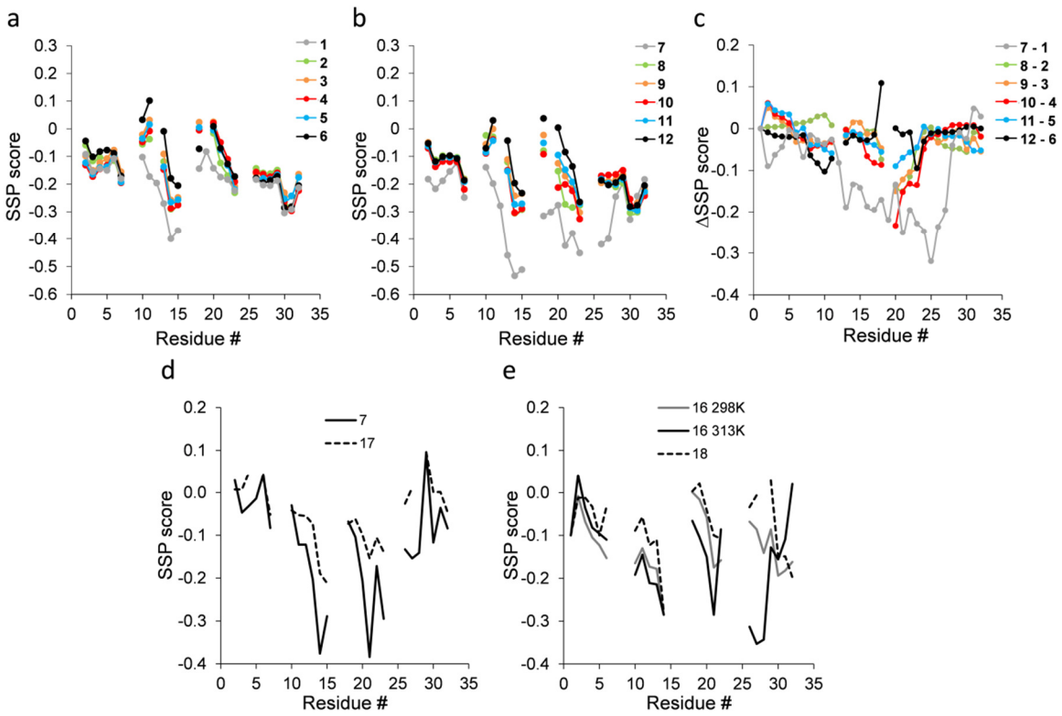


Figure 4. Residue-level secondary structure propensity (SSP) scores obtained monomeric (**a**) and dimeric (**b**) BB-14 and its core-substituted analogs. SSP-score differences including D-α-residues (**c**). SSP scores of parent sequences and peripheral substituted analogs for dimeric BB-14 (**d**) and BB-15 (**e**).

5. Molecular dynamics calculations were performed to gain information on the side-chain-dependent folding behavior. In line with the experimental data, the simulations predicted **10** as the most rigid among the α/β analogs, as well as having the smallest deviation from the structure of the parent sequence (Figure 5a-b). We analyzed the solvent-accessible surface of the hydrophobic core and the number of backbone H-bonds as markers of compact folding, and found that **10** displayed values closest to BB–14 (Figure 5c-d). The lack of cooperative folding observed for the other analogs could be explained either with insufficient fitting into the hydrophobic core (**8** and **12**) or a conformational mismatch disrupting the H-bond network (**9** and **11**).

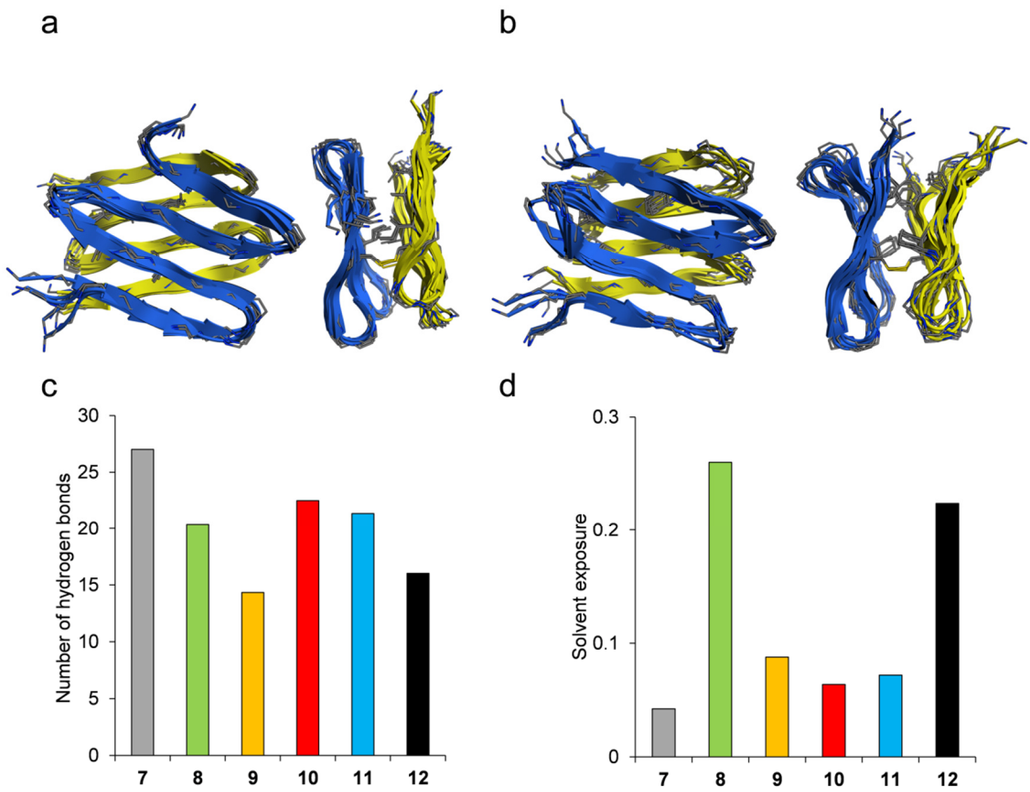


Figure 5. Overlay of the five lowest-energy structures obtained in molecular dynamics simulations for sequences 7 (**a**) and 10 (**b**). Average solvent-exposure ratio of the core hydrophobic residues in strands B and C (**c**). The number of existing H-bonds in the β-sheets (theoretical maximum: 36) (**d**).

6. NMR and TEM measurements were employed to study the aggregation properties under high salt concentrations. The parent BB-15 sequence (**16**) was found to form fibrils, but no aggregation was observed for **18**, suggesting that peripheral ACHC-substitution is an efficient method to prevent edge-to-edge association (Figure 6).

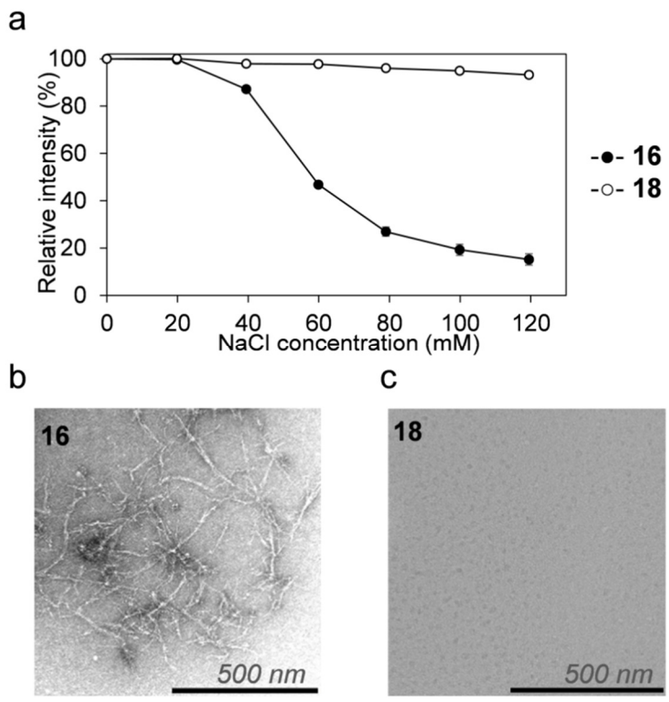


Figure 6. Reduced fibrillization tendency of **18** compared to **16**. The NMR-visible fraction of the peptides assessed by the integration of ^{1}H NMR signals at various NaCl concentrations (**a**). Integrals were carefully corrected for dilution effects and the possible salt-dependent sensitivity loss of the NMR probe. The TEM images of **16** and **18** are given in panels (**b**) and (**c**), respectively.

Full papers related to the thesis

I. Olajos, G., Hetényi, A., Wéber, E., Németh, L. J., Szakonyi, Z., Fülöp, F., Martinek, T. A. (2015). Induced folding of protein-sized foldameric β-sandwich models with core β-amino acid residues. *Chemistry – a European Journal*, **21** (16), 6173-6180.

IF: 5.160

II. Olajos, G., Hetényi, A., Wéber, E., Szögi, T., Fülöp, L., Martinek, T. A. (2018). Peripheral cyclic β-amino acids balance the stability and edge-protection of β-sandwiches. *Organic & Biomolecular Chemistry*, **16**, 5492-5499

IF: 3.423

Other full papers

I. Mándity, I. M., Wéber, E., Martinek T. A., Olajos, G., Tóth G., Vass, E., Fülöp, F. (2009). Design of Peptidic Foldamer Helices: A Stereochemical Patterning Approach. *Angewandte Chemie International Edition*, **48** (12), 2171-2175

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II. Olajos, G., Bartus, É., Schuster, I., Lautner, G., Gyurcsányi, R. E., Szögi, T., Fülöp, L., Martinek, T. A. (2017). Multivalent foldamer-based affinity assay for selective recognition of Aβ oligomers. *Analytica Chimica Acta*, **960**, 131-137

IF: 5.123

III. Lázár, V., Martins, A,. Spohn, R., Daruka, L., Grézal, G., Fekete, G., Számel, M., Jangir, P. K., Kintses, B., Csörgő, B., Nyerges, Á., Györkei, Á., Kincses, A., Dér, A., Walter, F., Deli, M. A., Urbán, E., Hegedűs, Z., Olajos, G., Méhi, O., Bálint, B., Nagy, I., Martinek, T. A., Papp, B., Pál, C., (2018). Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. *Nature Microbiology*, **3**, 718-731

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IV. Bartus, É., Olajos, G., Schuster, I., Bozsó Z., Deli M. A., Veszelka, S., Fruzsina R. Walter, F. R., Datki, Z. Szakonyi, Z., Martinek, T. A., Fülöp, L., (2018). Structural Optimization of Foldamer-Dendrimer Conjugates as Multivalent Agents against the Toxic Effects of Amyloid Beta Oligomers. *Molecules*, **23** (10), 2523-2536

IF: 3.098

Scientific lectures related to the thesis

1. G. Olajos, A. Hetényi, T.A. Martinek
   Tervezett β-szendvics foldamerek: egy protein méretű modell
   28-30. May 2014., Peptidkémiai Munkabizottsági Ülés, Balatonszemes

2. G. Olajos, A. Hetényi, E. Wéber, J. L. Németh, T. A. Martinek
   β-sandwich foldamers: A protein sized model
   poster presentation
   7th Central Europe Conference, Chemistry Towards Biology, Katowice
   12-14. September 2014

3. T. A. Martinek, G. Olajos, Z. Hegedüs
   Foladmerek sötét oldala. Béta-redők és szendvicsek
   28-30. May 2018, Peptidkémiai Munkabizottsági Ülés, Balatonszemes

Other lectures

1. G. Olajos, E. Wéber, J. L. Németh, T. A. Martinek
   Designig β-solenoid foldamers: stereochemistry, chain length and hydrophobic packing
   Symposium on Foldamers, Paris,
   12-14. April, 2013

2. R. Nagy, G. Olajos., V. Hársfalvi, Ü. Murvai, R- H. Pires, G. Ferenczy, L. Fülöp, B. Penke, T. A. Martinek, M. S. Kellermayer
   Highly Oriented, Epitaxially Generated Beta-amyloid-based Nanoarray for Nanobiotechnological Applications
   Polymers and Self-assembly: From Biology to Nanomaterials, Rio de Janeiro
   25-30. October 2015

3. G. Olajos, R. Spohn, C. Pál, T. A. Martinek
   Rationally designed antimicrobial foldamers against antibiotic resistance
   9th Central Europe Conference, Chemistry Towards Biology, Budapest
   24-27. September 2018