Report on the scientific results of OTKA PD 124077 grant, entitled "Peptide-based antigen-carrier systems in the development of vaccine candidates"

Efficient vaccination is still challenging in a variety of infectious diseases where the causative agent shows high antigenic diversity due to fast mutation rate or metamorphosis. Subunit vaccines that can combine multiple antigens derived from different stages of a pathogen's life cycle hold promise for overcoming the major obstacles.

After infection, *Mycobacterium tuberculosis* (*Mtb*) can remain in the host phagocytes and renders itself to a phenotypically resistant form [1]. In this latent phase, the pathogen changes its gene expression and consequently the whole antigen repertoire. To enhance the efficacy of a vaccine against tuberculosis (TB), it is essential to combine antigens that can reflect on the different forms and stages of the causative pathogen [2]. In order to produce a multivalent vaccine conjugate, the following protein antigens were considered in this project:

1. Ag85B (325 aa, encoded by the *Rv1886c* gene) is an early secreted antigen that is associated with active bacterial replication [3]. In the C-terminal region, CD8+ T-cell epitope peptide ^{239}KLVANNTRL^{247} (**A**) was reported to lyse BCG-infected human macrophages and Ag85B-pulsed CD8 + T-cells [4].
2. Isoniazid-inducible protein, IniB (479 aa, encoded by the *Rv0341* gene), is strongly upregulated in response to a broad range of inhibitors of cell wall biosynthesis, including Isoniazid, a first-line antitubercular drug [5]. In the 33–45 region, an MHC I-associated ^{33}GLIDIAPHQISSV^{45} peptide epitope (**I**) was identified that can induce the generation of peptide-specific cytotoxic T-cells and lysis of *Mtb*-infected dendritic cells [6].
3. PPE68 protein (368 aa, encoded by the Rv3873 gene) is one of the major antigenic protein associated with dormancy and assists the pathogen in surviving within the host phagocytes during latency [7]. Highly sensitive PPE68-specific T-cell epitope peptides within the 124–156 region were described recently of which the ^{127}FFGINTIPIA^{136} peptide (**P**) was identified as an HLA-DR promiscuous core sequence [8].

Empty MHC molecules on the surface of professional antigen-presenting cells are able to collect peptide antigens from the extracellular milieu for presentation to T-cells. Nevertheless, larger peptides and conjugates that have entered the endocytic pathway of antigen presentation provoke a more efficient immune response. This observation leads to the utilization of synthetic long peptides (SLPs) and branched-chain conjugates that can be produced by using different chemoselective ligation techniques. Nevertheless, the spatial arrangement is an effective way of disrupting peptide aggregation.

Another challenge is to design a conjugate with epitopes that induces efficient cell-mediated immune response and delivers the epitopes to antigen-presenting cells. The elongation of peptide-based conjugates with lipid moieties has been shown to give them function as both adjuvants and delivery vehicles [9]. Previous studies have illustrated a role for the improved immunogenicity of self-adjuvanting lipopeptide delivery systems that contain C16 lipid (palmitic

acid) with the advantage of increased cellular uptake by dendritic cells and activation of Th1/Th2 cytokine response [10].

The main goal of this study was to develop a convenient method to improve the immunogenicity and bioavailability of the selected T-cell epitope peptides.

RESULTS

I. To induce a more potent immune response, a trivalent, branched-chain conjugate was designed. As the core sequence, a Tuftsin derivative was used, which has been reported as a macrophage targeting peptide [9]. The branched-chain was inbuilt to the conjugate with the use of *thiol – maleimide* coupling method, which is a one-pot direct approach to obtain a stable thioether linkage between 2 peptide chains. For that purpose, a maleimide derivative of Tuftsin was synthesized, while the thiol partner was introduced as a *C*-terminal Cysteine residue on the epitope peptide (*Figure 1*).

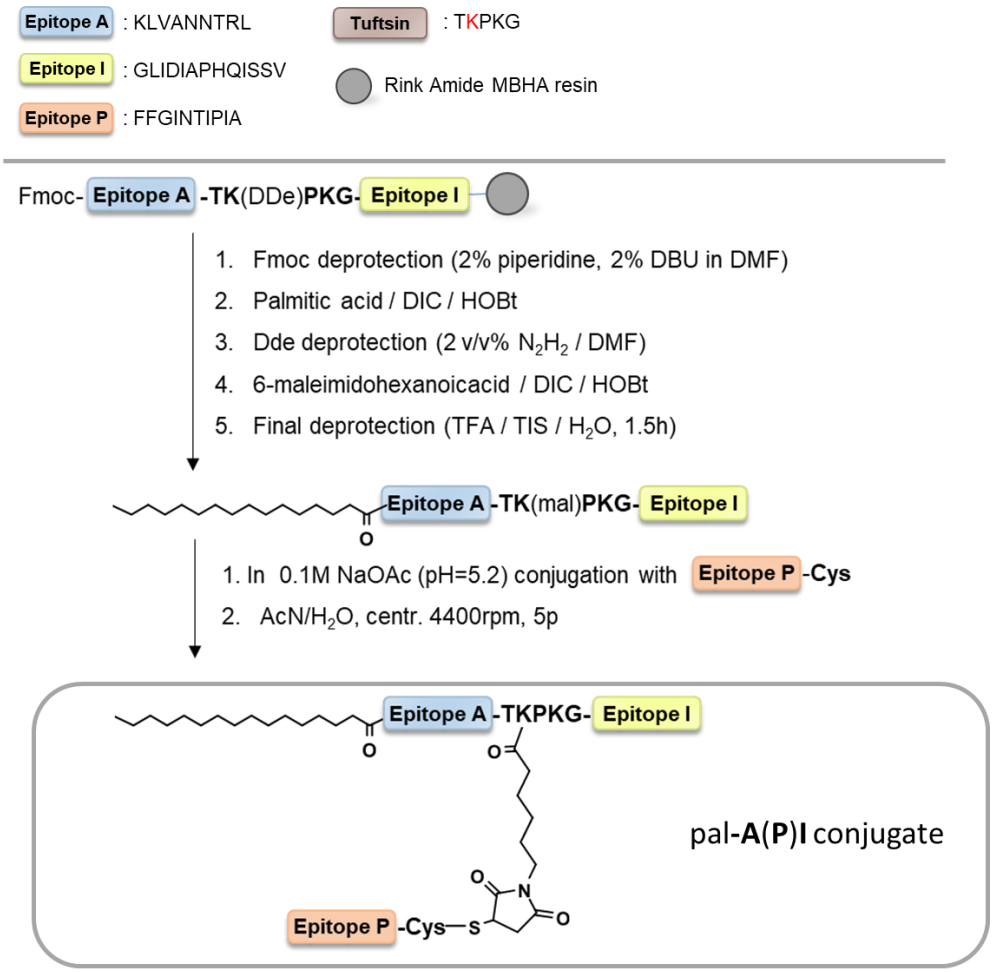

Figure 1. The synthetic strategy to produce a trivalent, branched-chain epitope conjugate.

After synthesis and purification, peptides and conjugates were characterized by high-resolution mass spectrometry and analytical HPLC chromatography (*Table 1*).

Table 1. Analytical characteristics of the peptides and peptide conjugates.

Compound	Sequence	M_{mo} [1]	Rt (min) [2]
P/**A**/**I** mix	^{239}KLVANNTRL^{247} (*Rv1886c)*	1026.6295	8.4
	^{33}GLIDIAPHQISSV^{45} (*Rv0341)*	1347.7508	10.7
	^{124}FFGINTIPIA^{133} (*Rv3873)*	1090.6174	12.7
AI	KLVANNTRL-TKPKG-GLIDIAPHQISSV	2868.6679	10.2
A(**P**)**I**	KLVANNTRL-TKmal(FFGINTIPIAC)PKG-GLIDIAPHQISSV	4297.3649	13.0
pal-**A**(**P**)**I**	palmitoyl-KLVANNTRL-TKmal(FFGINTIPIAC)PKG-GLIDIAPHQISSV	4493.5757	24.1*
Cf-**P**/**A**/**I** mix	*Cf*-KLVANNTRL	1384.6778	11.3
	Cf-GLIDIAPHQISSV	1705.7986	13.3
	Cf-FFGINTIPIA	1448.6654	14.8
A(*Cf*-**P**)**I**	KLVANNTRL-TKmal(*Cf*-FFGINTIPIAC)PKG-GLIDIAPHQISSV	4655.4147	15.5
pal-**A**(*Cf*-**P**)**I**	palmitoyl-KLVANNTRL-TKmal(*Cf*-FFGINTIPIAC)PKG-GLIDIAPHQISSV	4851.6357	25.9 *

[1] Exact M_{mo} (monoisotopic molecular mass) measured on a Thermo Scientific Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. [2] Retention time on an Agilent Eclipse XDB C8, 4.6 × 150 mm column, gradient: 5% B, 2 min; 5%–100% B, 20 min. * Phenomenex Jupiter C4, 4.6 × 250 mm column, gradient: 25% B, 5 min; 5%–100% B, 25 min. *Cf* is for 5(6)-carboxyfluorescein, the *C*-terminus of the peptides are in amidated form.

Next, the conformation of the peptides and conjugates was studied by electronic circular dichroism (ECD) in collaboration with Dr. Viktor Farkas (MTA-ELTE Protein Modelling Research Group). Results showed that the short, linear epitope peptides had highly dynamic conformation, even in trifluoroethanol (TFE). In contrast, branched-chain conjugates had a much higher tendency to fold. The palmitoylated pal-**A(P)I** conjugate gave a more intense spectrum in the range of 185–210 nm, indicating a more partially folded state (*Figure 2*). These results reveal that branched conjugates were able to form an ordered structure (e.g., turn or helical) in a non-hydrophilic medium.

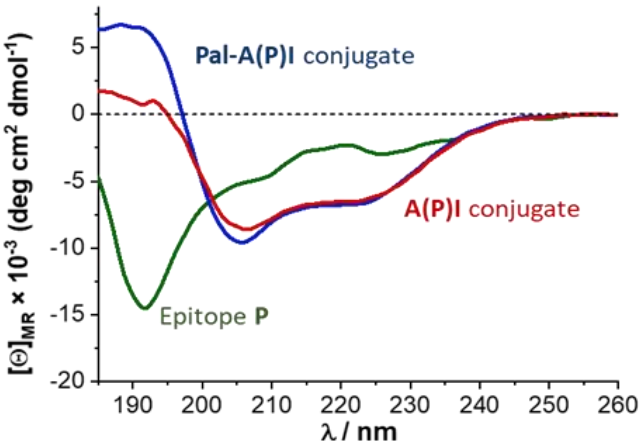

Figure 2. Electronic circular dichroism (ECD) spectra of the peptide and conjugates in trifluoroethanol (TFE).

The role of conjugation and the role of palmitoylation on the cellular uptake and localization was studied on two macrophage model cell types (human MonoMac-6 and murine BMDM) using flow cytometry and confocal laser scanning microscopy. The cellular uptake of the **A(P)I** conjugate was higher than the uptake of the short epitope peptides (**P/A/I** mix). Furthermore, palmitoylation dramatically improved the internalization rate to human and to murine cells. The highest response was measured for pal-**A(P)I**. The epitope peptide mixture (**P/A/I** mix) principally showed colocalization with lysosomes that was even more pronounced in case of **A(P)I**. Pal-**A(P)I** conjugate could be detected both in lysosomes and in the cytosol, which might indicate that this compound internalizes not only through the endo-lysosomal pathway, but also through direct cell penetration.

To determine whether lipo-tuftsin conjugation could successfully improve the immunogenicity of the selected T-cell epitope peptides, T-cell proliferation and cytokine release was measured using immunized CB6F1 mice (a cross between female BALB/c and male C57BL/6). Animal experiments were performed at the Animal House of the National Public Health Center in collaboration with Dr. Kinga Fodor and Ervin Varga. A group of animals was immunized *s.c*. with 50 µg of pal-**A(P)I** three times, two weeks apart. For comparison, another group was immunized with the mixture of the epitopes (**P/A/I** mix) using equivalent amount of peptides. Splenocytes from immunized animals were re-stimulated, and cell proliferation was assayed by CFSE staining method. Simultaneously, supernatants were tested for cytokine production using a LEGENDplex cytokine multiplex kit. Splenocytes from mice that had been vaccinated with pal-**A(P)I** conjugate showed a high proliferative response and the T-cell response to the pal-**A(P)I** conjugate was characterized by a Th1/Th2 cytokine pattern with prominent expression of IFN-γ, IL-2, and IL-10 cytokines. Immunization with free epitope peptides resulted in very low proliferative or cytokine response in all mice. These results indicate that the applied conjugation approach greatly enhanced the T-cell response to the epitopes.

For the vaccine efficacy study, a group of five BALB/c mice were immunized with 50 µg pal-**A(P)I** *s.c.* three times, two weeks apart. Then, mice were infected *i.p.* with 200 µL *Mycobacterium tuberculosis* $H_{37}Rv$ (10^6 CFU/mL). Seven weeks after *Mtb* infection, mice were euthanized and autopsy was performed. Lung and spleen homogenates were cultured on Löwenstein–Jensen medium to determine the CFU. In histologic sections prepared from the spleen of unvaccinated control animals, rod-shaped acid-fast bacteria were observed within small groups of epitheloid macrophages. This indicates that the infection method used was successful in modelling experimental tuberculosis. When mice were immunized with the pal-**A(P)I** conjugate, lower numbers of bacteria were enumerated compared to the unvaccinated control group. Tissue sections of pal-**A(P)I**-vaccinated animals were free of visible tuberculosis-related granulomatous lesions. With this experiment we have demonstrated the utility of this branched-chain conjugation technique to improve the immunogenicity and protective efficacy of T-cell epitope peptides.

Results were presented on 3 conferences and published in 1 paper:

- Horváti K. et al. (2019) A convenient synthetic method to improve immunogenicity of *Mycobacterium tuberculosis* related T-cell epitope peptides. *Vaccines* 7(3), 101. [IF:4.760]

- Horváti K. et al. (2019) How to improve the immunogenicity of T-cell epitope peptides. *48th Annual Meeting of the Hungarian Society for Immunology*.16-18 October 2019. Bükfürdő, Hungary, *oral presentation*
- Horváti K. et al. (2019) *Mycobacterium tuberculosis* related T-cell epitope peptide-based vaccine candidates. *18th International Congress of the Hungarian Society for Microbiology*. 3-5 July, 2019. Budapest, Hungary, *oral presentation*
- Horváti K. et al. (2019) Experimental design on slow progressing murine tuberculosis model to evaluate vaccine and treatment efficacy. *14th FELASA (Federation of European Laboratory Animal Science Associations) Congress*, Prague, Czech Republic, 10-13 June 2019, *poster*

All works with infectious Mtb were performed at the National Biosafety Laboratory (National Public Health Center) under biosafety level 3 (BSL-3) conditions in collaboration with Dr. Zoltán Kis and Bernadett Pályi. Prior to BSL-3 experiments, a personnel biosafety training program was provided. For in vivo experiments, a FELASA accredited course on laboratory animal science was completed that gave me license to independently design and implement animal experiments.

II. In collaboration with Prof. Éva Kiss and Dr. Gergő Gyulai (Laboratory of Interfaces and Nanostructures, ELTE) pal-**A(P)I** conjugate was encapsulated to biodegradable polymeric nanoparticles (PLGA, poly(D,L-lactic-co-glycolic acid). Nanoparticles (NPs) were prepared by nanoprecipitation method and to improve the immunogenicity trehalose-6,6-dibehenate (TDB) adjuvant was also incorporated to the NPs. The characterization (dynamic light scattering and atomic force microscopy) revealed that the size of the NPs was between 100-120 nm with low polydispersity and the loading efficacy of the conjugate was up to 80% (*Figure 3*). This construct – together with relevant controls (PBS, mix of epitopes, non-particulated pal-**A(P)I** conjugate) was administered to BALB/c female mice sc. 3-times (spaced 2 weeks). Peptide content of the injections was 50 µg in each treatment groups. Six weeks after the last immunization splenocytes were prepared and the epitope-specific immune response was measured by CFSE proliferation assay using flow cytometry. The comparison of the constructs showed that encapsulation of the conjugate and the addition of the TDB adjuvant further improved the immunogenicity of the peptides. Therefore, we can conclude that the applied lipo-conjugation technique and PLGA encapsulation efficiently improved the immunogenicity of the T-cell epitopes.

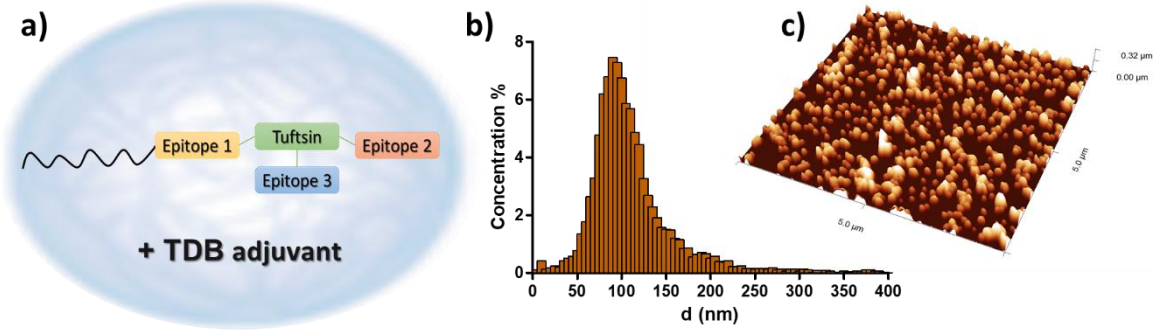

*Figure 3. Schematic representation of pal-**A(P)I** conjugate loaded PLGA nanoparticles (**a**). Dynamic light scattering (**b**) and atomic force microscopic image of the NPs (**c**).*

Results were presented in two international conferences and in 1 national journal:

- Horváti K. et al. (2018) Novel nanoparticulated conjugates comprising T-cell epitopes in branched chain arrangement on lipo-Tuftsin platform, *Elsevier 12th Vaccine Congress*, 2018
- Horváti K. et al. (2018) Nanoparticulated multi-epitope conjugates as vaccine candidates against tuberculosis, *16th Naples Workshop on Bioactive Peptides*, 2018
- Horváti K (2019) Peptid-konjugátumok: új típusú fegyverek a multirezisztens baktériumok ellen. Biokémia: A Magyar Biokémiai Egyesület internetes folyóirata XLIII. évfolyam 4. szám 35-44.

III. PLGA nanoparticles can be efficiently targeted to different cells by the modification of the surface with targeting peptides. A convenient synthetic method was developed to covalenlty couple targeting peptides to the surface layer of PLGA NPs without the use of coupling reagents or additional chemicals. Previous methods found in the literature involve three or more synthetic steps and the coupling agents and other reactants can remain in the polymer product and contaminate the NPs. To overcome the obstacles, a new synthetic route was designed based on a one-step quantitave reaction of aminooxy-compounds with aldehydes. This conjugation technique does not require pre-activation and water can be used as reaction media.

To improve biocompatibility and to protect PLGA NPs against particle aggregation, Pluronic F127 surfactant is often used as a compound of the surface layer of NPs. Pluronic F127 is a non-ionic poloxamer type surfactant consisting of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks. Pluronic F127 has been evaluated for numerous biomedical applications and due to terminal hydroxyl groups, which are available for conjugation, further functionalities can be added to the pharmaceutical perspectives. Transformation of Pluronic F127 hydroxyl groups - which have limited reactivity - into aldehyde groups provide a convenient way to bind aminooxy-peptide derivatives in a one-step reaction (*Figure 4)*.

As targeting moiety, $[Thr\text{-}Lys\text{-}Pro\text{-}Lys\text{-}Gly]_2$-Thr-Lys-Pro-Pro-Arg (T10Tp) peptide was used which a synthetic derivative of Tuftsin. To prepare an aminooxy-peptide, a convenient solid-phase synthetic route is available, namely coupling Boc-aminooxyacetic acid to the peptide-resin followed by a standard cleavage procedure. Resulted aminooxy-derivative was then dissolved in dist. H_2O and allowed to react with Pluronic F127-aldehyde. To prove the appearance of the oxime bond between Pluronic F127-aldehyde polymer and aminooxy-peptide NMR spectroscopy was evaluated that provided unambiguous evidence for the formation of the oxime bond. To provide the proof of principle, an antitubercular drug candidate was encapsulated into PLGA NPs and into Tuftsin peptide-targeted PLGA NPs and the internalization rate together with the intracellular killing of *Mtb* was compared. Significantly higher uptake rate was measured for the peptide-conjugated NPs than for unconjugated NPs, which result indicates that Tuftsin-peptide labeling increases the internalization of the PLGA NPs. Furthermore, intracellular killing efficacy of the drug-loaded NPs was improved by the Tuftsin-peptide conjugation that can be the consequence of the higher internalization rate observed for the peptide-coated NPs.

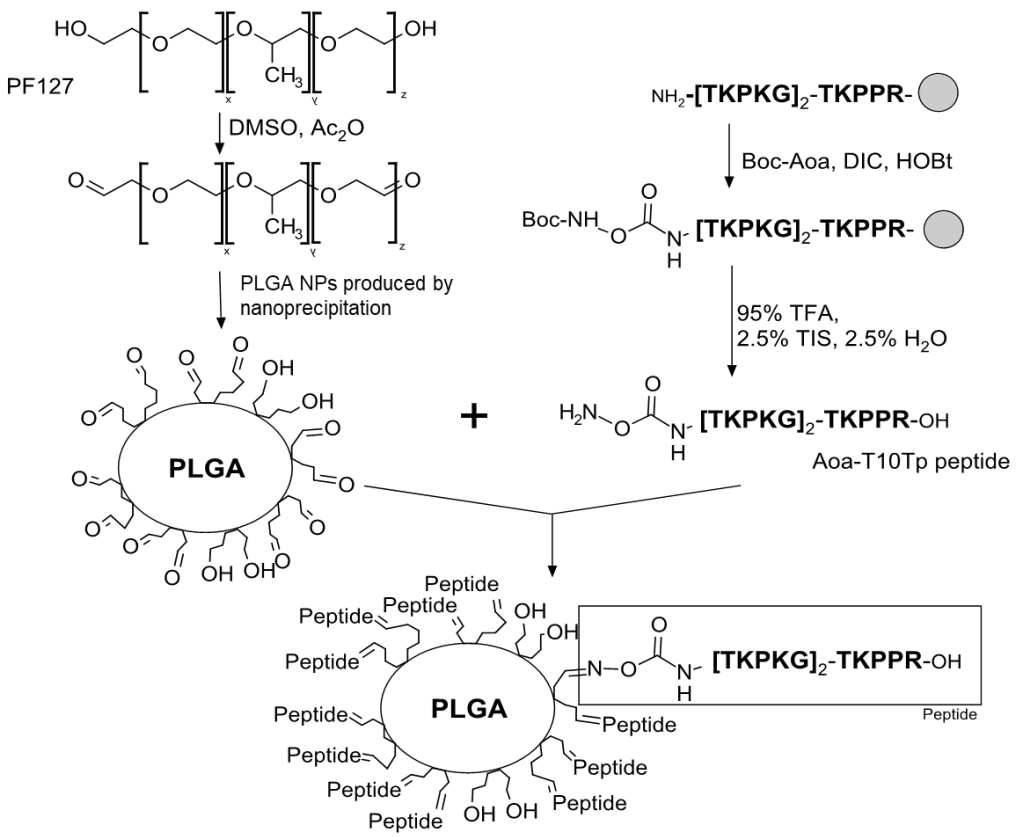

Figure 4. Outline of the synthesis of PF127-aldehyde and aminooxy-Tuftsin peptide and the oxime bond formation on the surface of PLGA nanoparticles.

Results were summarized in a publication:

Horváti K. et al. (2018) Surface layer modification of PLGA nanoparticles with targeting peptide: a convenient synthetic route for Pluronic F127 - Tuftsin conjugate (2018) *Bioconjugate Chemistry* 29(5):1495-1499, [IF: 4.485]

IV. New type of nanoparticulated carriers were also considered for vaccine application and peptide-based targeting to immune cells. Dr. Gergő Gyulai (Laboratory of Interfaces and Nanostructures, ELTE) has developed a convenient method to produce luminescent carbon quantum dots which have a promising potential for bio-imaging. With further surface modifications these particles could serve as nano-carriers for drug/vaccine delivery. Future studies will focus on elucidating the effect of surface modifications on the mechanism of cellular uptake, immunogenicity and biodistribution.

Results were summarized in a publication:

Gyulai G., Ouanzi F., Bertóti I., Mohai M., Kolonits T., Horváti K., Bősze S. Chemical structure and in vitro cellular uptake of luminescent carbon quantum dots prepared by solvothermal and microwave assisted techniques. *J. Colloid Interface Sci*. 2019, 549, 150-161. [IF: 6.361]

V. In collaboration with Péter Ábrányi-Balogh (Medicinal Chemistry Research Group, MTA TTK) a broad library of cysteine targeting compounds was explored. For that purpose, cysteine containing model peptides were synthesized and characterized in our laboratory. In a recently published research paper (Ábrányi-Balogh P et al. (2018) A road map for prioritizing warheads for cysteine targeting covalent inhibitors. *Eur J Med Chem*. 160:94-107. [IF: 4.816]) diverse chemical reactions suitable for specific covalent protein/peptide modification were systematically reviewed and the available organic chemistry toolbox were extended with new reactions. Cysteine targeting conjugation techniques described in this paper was further investigated in the recent research project as well.

VI. In collaboration with with Dr. Ferenc Zsila and Dr. Tamás Beke (Biomolecular Self-assembly Research Group, MTA TTK) and Prof. Éva Kiss and Dr. Gergő Gyulai (Laboratory of Interfaces and Nanostructures, ELTE) the secondary structure and monolayer interaction of antimicrobial peptides was studied. Previously, the folding inducing capability of some salts and pharmaceutical agents were observed, therefore systematic studies on the interaction of different compounds with intrinsically disordered cationic peptides were evaluated. Secondary structure analysis based on the CD data and semi-empirical quantum chemical calculations suggested that negatively charged small molecules (such as therapeutic drugs, polysulfonated dyes and protoporphyrin compounds of human origin) induce peptide folding and transient conformations that play a vital role in their biological functions.

In addition, previous observations with 5(6)-carboxyfluorescein (Cf) derivative of membrane active cationic peptides raised the question of whether the fluorescent labeling changes penetration ability of the peptides. Fluorescent labeling is a common approach to reveal the molecular details of cellular uptake, internalization, transport and distribution. Membrane affinity of the peptides and their Cf-derivatives was determined quantitatively and compared applying Langmuir monolayer of zwitterionic (DPPC) and negatively charged (DPPC+DPPG) lipids as cell membrane models. The interaction with neutral lipid layer is mainly governed by the overall hydrophobicity of the molecule which is remarkably increased by Cf-conjugation. Cf-conjugation also improved the penetration ability of some peptides suggesting that both the high charged character (Z/n) and the increased hydrophobicity by Cf-conjugation present important contribution to membrane interaction.

Our results were published in the following papers:

- Quemé-Peña M et al. (2019) Manipulating active structure and function of cationic antimicrobial peptide CM15 by the polysulfonated drug suramin: a step closer to in vivo complexity. *ChemBioChem* 20:1–14 [IF: 2.774]
- Zsila F et al. (2017) Drug and dye binding induced folding of the intrinsically disordered antimicrobial peptide CM15. *RSC Advances* 7:41091-41097. [IF: 2.936]
- Zsila F. et al. (2018) Hemin and bile pigments are the secondary structure regulators of intrinsically disordered antimicrobial peptides. *Chirality* 30(2):195-205. [IF: 1.956]

- Kiss É et al. (2018) Membrane affinity and fluorescent labeling: comparative study of monolayer interaction, cellular uptake and cytotoxicity profile of carboxyfluorescein-conjugated cationic peptides. *Amino Acids* 50(11):1557-1571 [IF: 2.906]

Based on the results of this OTKA grant, an international collaborative network was formed. This new consortium successfully applied for a Marie-Skłodowska-Curie ITN grant within the Horizon 2020 Programme of the European Commission. The project "BactiVax" will focus on developing novel vaccines to tackle the huge challenge of antimicrobial-resistant human pathogens that cause chronic, life-threatening respiratory and/or systemic infections. This network comprises 14 Principal Investigators from 8 European countries and two companies such as Pfizer and CycloLab.

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