University of Szeged

Faculty of Pharmacy

Institute of Pharmaceutical Technology and Regulatory Affairs

Summary of PhD thesis

# Strategies for development of antimicrobial peptides and proteins

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Doctor of Pharmacy

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University of Szeged

Graduate School of Pharmaceutical Sciences

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# Strategies for development of antimicrobial peptides and proteins

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## 1. INTRODUCTION

According to the WHO report, antimicrobial resistance is one of the main global threats. Therefore, there is a need for the development of other agents, such as antimicrobial peptides (AMPs). AMPs are small molecules with less than 50 amino acids, having activity against a wide range of microorganisms and showing less immunogenicity compared to recombinant proteins and antibodies. Recent researches have demonstrated that in addition to the antimicrobial functions of AMPs, these peptides also play an important role in the complex pathogenesis of several inflammatory diseases. Peptide therapeutics has considerable advantages in terms of safety aspects. Since the products resulting from their degradation are natural amino acids with a short half-life, only a small quantity of peptides is accumulated in the tissues. The result is a reduction in the safety risks caused by metabolites. Less immunogenicity is another advantage of therapeutic peptides. Several thousands of AMPs have been isolated from various natural sources such as microorganisms, plants, insects, crustaceans, animals, humans. However, only a few of them have been translated commercially to the market so far. This is because of drawbacks of the naturally obtained AMPs like the susceptibility to protease degradation, inactivity at physiological salt concentrations, cytotoxicity to host cells and lack of appropriate strategies for sustained and targeted delivery of the AMPs. These initial barriers are being increasingly overcome with new chemical modification strategies such as N- and C-modifications, incorporation of non-natural or D-amino acids, cyclization and the attachment of the polyethylene glycol (PEG) polymer to peptides (PEGylation). These approaches as well as strategies for delivery of peptides allowed several researchers to enhance the bioavailability of AMPs and improve their bio-distribution and rate of clearance.

## 2. AIMS

Generally peptides and proteins modifications and formulation of their delivery systems are challenging tasks and hide several risks. The aim of this PhD thesis project is to understand and evaluate these risks through a quality by design (QbD) based antimicrobial peptide and protein modification and formulation design. It will lead to develop more stable agents with efficient delivery to the target site. We started our project by collecting and evaluating the results of most recently published researches about antimicrobial peptides and protein modification and formulation. It led us to obtain narrowed and specified knowledge and directed us on appropriate strategies in designing

a high-quality modified AMP formula with the most influence on bioavailability and antimicrobial activity enhancement. Therefore, this knowledge can help us with the selection of optimal structural features of AMPs, the best possible modification strategy for AMPs and the best possible nanocarrier system for them. After preliminary studies, analysis of potential risks in the peptide PEGylation process was performed through the example of PGLa and in the next phase the effective delivery of proteins with antimicrobial activity was accomplished through the example of Lysozyme (LYZ) in a novel formulation strategy (layer-by-layer polyelectrolyte core-shell nanoparticle)

**PART A: PEGylation and Formulation of Anti-Microbial Peptide (PGLa) according to the Quality by Design approach**

- ✓ identifying the critical factors with the highest effect on the quality of a final modified AMP
- ✓ determining the priority ranking of critical factors
- ✓ The selection of the right methodologies and materials in the synthesis of the PEGylated AMPs and their formulation development

**PART B: Optimization of layering technique and the secondary structure analysis during formulation of nanoparticles containing lysozyme by Quality by Design approach**

- ✓ preparation of core-shell NPs containing LYZ
- ✓ Determination of the secondary structure of the all samples
- ✓ Determination of the correlation between optimization parameters

## 3. MATERIALS AND METHODS

### 3.1 Materials (Part A)

PGLa:(H-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-AlaLys-Val-Ala-Leu-Lys-Ala-Leu-NH2) is a 21-residue amphipathic antimicrobial peptide amide. Its net charge is +5 at physiological pH. It has good water solubility, and shows only limited haemolytic activity.

### 3.2 Methods (Part A)

After the analysis of the relevant scientific literature collected data were structured and visualized. Ishikawa diagram was prepared for categorization of the influencing factors (causes), flow charts were prepared for PEGylation process description. QTPP in this study was defined as the end-product of a pre-formulation process, namely the

modification procedure itself, where the targeted end-product was the PEGylated AMP. QTPP selection happed according to ICH Q8 (R2) guideline. CQAs are factors which have critical influence on the QTPP according to the safety, quality or efficacy aspects. They are generally associated with the substances, in-process materials and final product. CQAs were determined as physical, chemical, biological, or microbiological properties or characteristics of the output material (product), that should be within an appropriate limit, range, or distribution to ensure the desired product quality. The selection of CQAs is based on a holistic view of the formulation development and is based on previous knowledge and experience. CMAs are critical material attributes, physical, chemical, biological, or microbiological properties or characteristics of an input material. CPPs are process parameters whose variability has a critical effect on the aimed product performance. CPPs and CMAs are linked to the selected production/formulation process. CMA and CPP selection were based on prior knowledge resulting from the knowledge space development phase of the study. The initial RA was performed by means of the Lean QbD Software. The connections between the QTPP elements, the CQAs and CPPs were thoroughly evaluated. The interdependence between QTPPs and CQAs, as well as between CQAs and CPPs were structured and evaluated one by one, then rated on a three-level scale. This scale reflects the impact of the parameters' interaction on the product as high (H), medium (M) or low (L). The probability of the occurrence of the critical factors were also estimated with the software using the same three-grade scale. As the output of the RA evaluation, Pareto diagrams were generated showing the ranked parameters according to their critical effect on the aimed PEGylated AMP as end-product. The relative occurrence-relative severity chart was also prepared, presenting the critical factors in four different quarters according to their estimated occurrence and severity (or the degree of their impact if they occur). This allows a different presentation manner of the RA results, where the upper right corner of the generated figure needs the highest attention as it represents those critical factors which have the highest risk of occurrence and have great impact on quality. 2.2.6. Preparation process: PEGylation Preparation of the PEGylated PGLa by the solid-phase FMOC/tBu strategy was selected in this study as a model process to perform the initial RA. FMOC was selected as amino protecting group, solid phase strategy was selected for the design.

### 3.3 Materials (Part B)

Lyophilized LYZ stored at freeze (-20°C) was used as a model protein, lyophilized *Micrococcus lysodeikticus* used as a gram-positive bacterium for layered NPs activity investigation, sodium sulphate was used as precipitating agent, Alginic acid sodium salt was utilized as layering polymer, sodium hydroxide and hydrochloric acid were used as pH ad justifiers and the other all reagent were of analytical grades.

### 3.4 Methods (Part B)

The experiments were conducted according to $2^3$ full factorial design, the pH value 6 (-1) and 10 (+1), alginate concentration (0.004% w/v (-1) and 0.006% w/v (+1) and mixing time 1(-1) and 2 (+1) hour) were considered as variable factors. Whereas, the enzyme activity, particle size, encapsulation efficiency, precipitation percent and zeta potential were set as optimization parameters. The preparation of LYZ NPs was made according to $2^3$ full factorial design, 8 samples were prepared. 0.6 g of lyophilized enzyme was dissolved in purified water to obtain 19.4 g homogenous aqueous solution, then each sample was mixed with 4ml of 2M $Na_2SO_4$ solution by using a magnetic stirrer for different period of time (1 and 2 hours). The 8 samples were centrifuged at 5000 rpm for 15 minutes by using high performance refrigerated centrifuge. The obtained supernatants were carefully removed from the precipitated NPs, then diluted to a suitable range with purified water and the absorption was measured by using UV-Vis spectrometer at lambda max 281nm. Based on the absorbance the concentration of unprecipitated NPs enzymes were used to calculate the precipitation efficiency. The obtained supernatants were carefully separated from the encapsulated NPs and the absorption was measured at 281nm for each sample, then the concentration of free enzyme NPs was measured for all samples, from which the encapsulation efficiency was determined. The precipitated NPs were adequately diluted, and the particle size of the sample was measured with Malvern Mastersizer. The Zeta potential of the same sample was measured with a Malvern Zetasizer apparatus with three parallel measurements. Layering with alginate to the redispersed precipitants aqueous alginate solutions (250 ml) of conc. 0.004 and 0.006 w/v% and pH 6 and 10 were added to each sample according to the factorial design, the samples then mixed by Ultra-Turrax high shear mixer for 15 seconds, then followed by re-centrifugation with same parameters as mentioned before. The structure and the morphology of the precipitants NPs (after layering) were described with transmission

electron microscopy (TEM). The TEM images were made with HRTEM microscope with accelerating voltage of 200 kV in bright field mode. Sample were suspended in water and dropped onto a carbon film-coated coper grid. The activity of the prepared layered nanoparticle samples was carried out by measuring the degradation of lyophilized *Micrococcus lysodeikticus* by using UV spectrometer. 25 mg of lyophilized bacterial cells was dispersed in 100ml of phosphate buffer (pH 6.8); the basic absorption at 450 nm was around 0.7. The absorptions of bacterial suspension were measured for 5 minutes before each test to reduce the error raised from bacterial sedimentation. 10 mg of the layered NPs or 10 mg of crude LYZ were dissolved in 25 ml phosphate buffer. 0.1 ml of layered NPs/or crude enzyme solution has been added to 2.5 ml of bacterial suspension and shaked for 20 seconds in quartz cuvette, then the change in the bacterial absorption was measured for 5 minutes. The pellet's activity was calculated from the percentage degradation of the bacterial cells relative to crude enzyme activity as a reference. Infrared spectra for the prepared samples and the other excipients were obtained by using FT-IR apparatus, by using potassium bromide disc method, the scanning was run at wavelength range 600 to 4000 $cm^{-1}$, the spectra were collected from 64 scans to obtain smooth spectra, at the spectral resolution of 4 $cm^{-1}$ and applying $CO_2$ and $H_2O$ corrections. The SpectraGryph 1.2 software was used for the second derivation of spectra. For deconvolution of second derivatives spectra was used the Fityk software. After assigned of peaks the area was calculated. From these data the α-helix content was determined. To determine the α-helix content of the initial LYZ and the synthesized NPs, circular dichroism (CD) spectra were recorded on an spectrometer between 250–190 nm. For the measurements, a 4-opened quartz cuvette with 1 cm optical length was used and the solid samples were dissolved in PBS buffer applying 0.04 mg/mL protein concentration. The spectra were corrected with the PBS buffer background. The α-helix content was calculated.

## 4 RESULTS AND DISCUSSION

### 4.1 Results (Part A)

The basis for initial RA was an evaluation (***Table 1**.)* of the present knowledge in the different limits of PEGylation, how these barriers can lead to risks, how these risks can be overcome by novel opportunities offered by chemistry or biochemistry for achieving desirable bioactive AMP.

***Table 1.*** Limitations, risks and opportunities in AMP PEGylation process

| Limitation | Risk | Opportunity |
|---|---|---|
| Random PEGylation | Reduced antimicrobial activity | development of new site-specific protein PEGylation strategies |
| Low Mw | Lack of specificity, reduced conjugate activity | higher Mw of PEG, more selective PEG reagents |
| High Mw | Reduced antimicrobial activity | Using PEG molecules with lower Mw |
| Masking the binding (active) site of AMP | Reduced antimicrobial activity | PEG-Linker-Drug strategy |
| The interference of PEG molecule with the mechanism of action of AMPs | Reduced antimicrobial activity | development of new reagents and methodologies with not disturbing biological activity of the peptide |
| Non-hydrolysable chemical bond between PEG and AMPs | Low or reduced functional activity | development of new reagents such as degradable linkers |
| PEG-specific immunity | Accelerated blood clearance | Understanding mechanisms of anti-PEG immunity, monitoring patients before and during PEGylated drug treatment, less immunogenic delivery approaches |

The initiative step of the RA process of the preparation of the PEGylated PGLa by the solid-phase FMOC/tBu strategy was the construction of the Ishikawa diagram (***Fig. 1.***), where the different factors and possible associated risks in selection, modification and formulation of in a suitable delivery system are highlighted. This gives the basis for the selection of the CQAs during modification and formulation of PGLa. These parameters were ranked into six groups: AMP characteristics, chemical modification method, final product characteristics, AMP formulation, patient acceptance, therapeutic aim, and administration route. The solid phase strategy for PEGylated PGLa preparation including the possible CPPs and CMAs is presented in ***Fig. 2***. These graphical representations (***Figs. 1 and 2***) aimed the selection of the CQAs that could critically affect the desired QTPP and also helped in selection of the CMAs/CPPs that may have a significant effect on the CQAs of PEGylated PGLa. After the systemic collection and evaluation of all the potential influencing factors, the QTPP elements, the CQAs, and the CPPs/CMAs for of the PEGylated PGLa were defined.

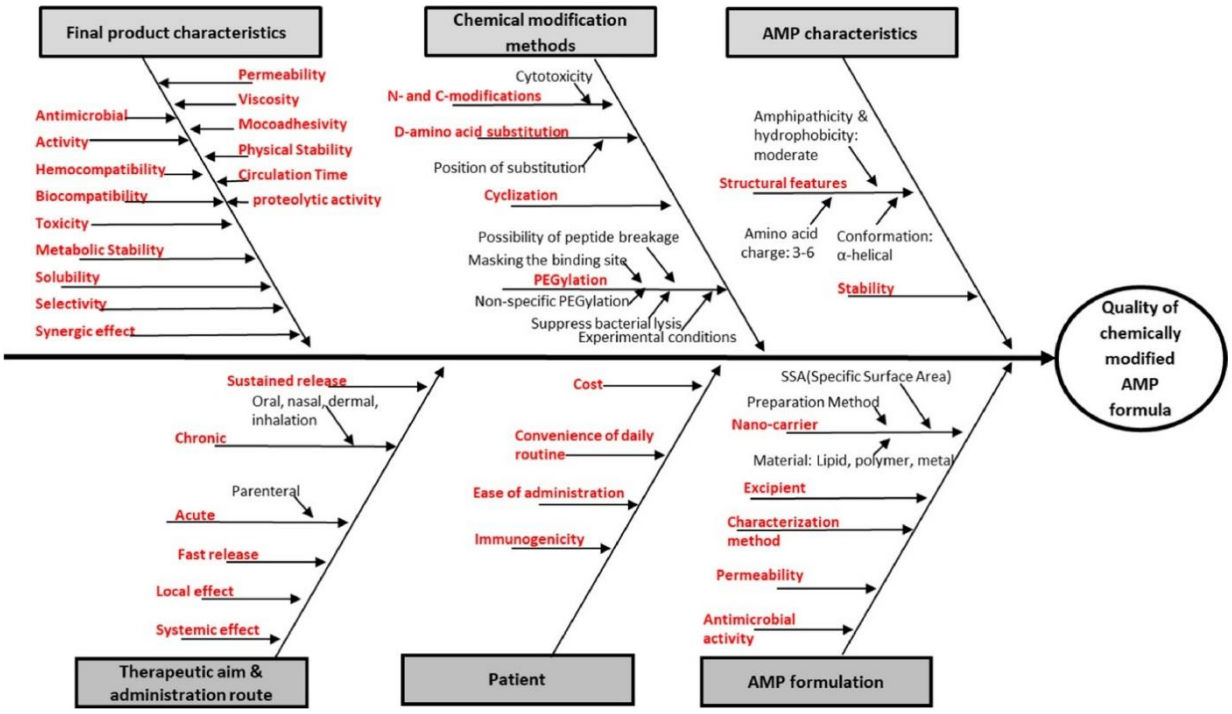



***Figure 1.*** Ishikawa diagram including all the parameters influencing the desired chemically modified AMP formula

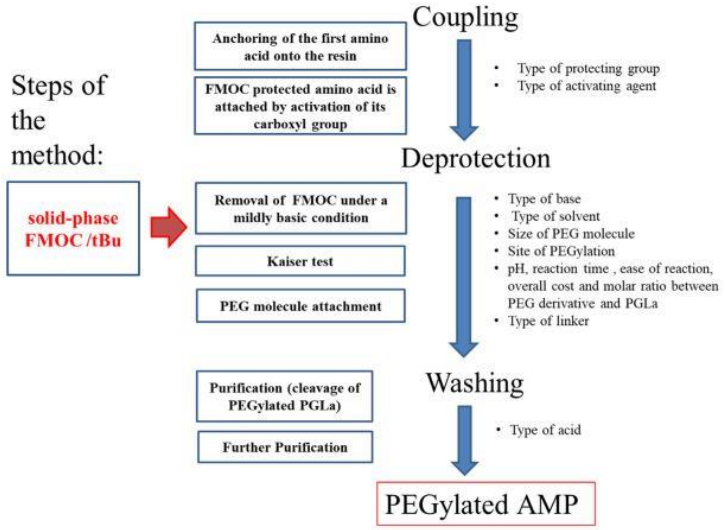
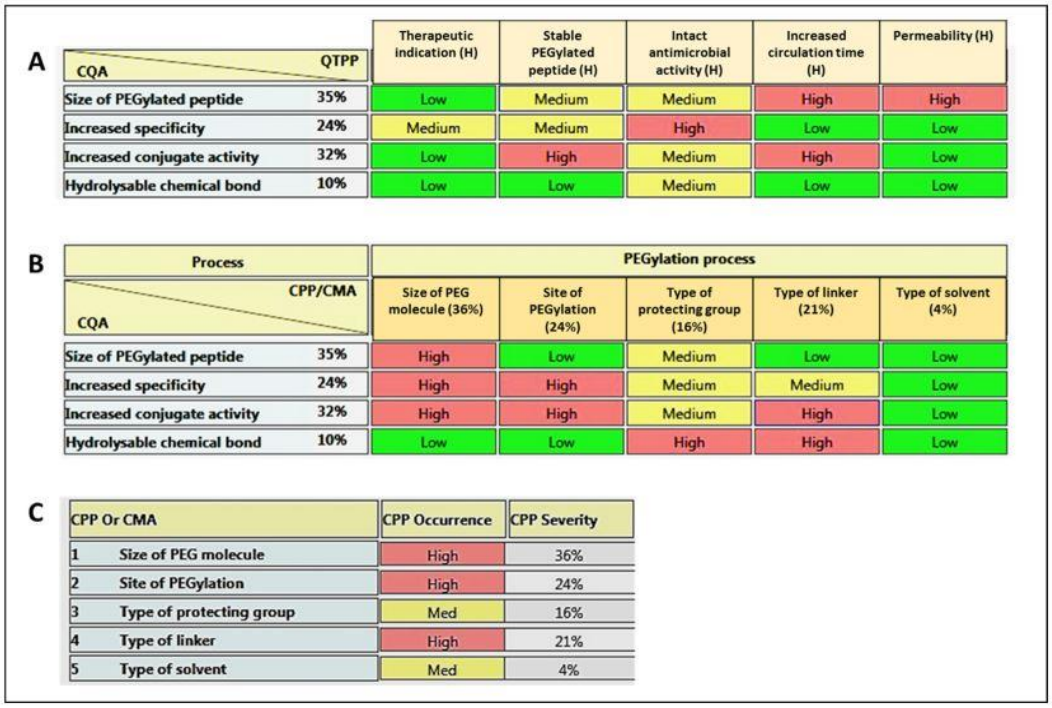



***Figure 2*** Flow-chart of solid-phase PEGylation process of the selected AMP (PGLa)

The evaluation of the interdependences among the QTPP elements and CQAs, as well as the CQAs and CMAs/CPPs and the occurrence estimation is shown in ***Fig. 3***. As it can be seen, the size of the final PEGylated peptide as one of the CQAs of the final product has the highest influence on circulation time and permeability according to the theoretical knowledge-based interdependence estimation (***Fig. 3A***). The specificity of the PEG

reagent significantly affects the antimicrobial activity of the final product; while it has less important effect on circulation time and permeability of the peptide. Lack of selectivity can cause random PEGylation and increase the risk of losing the antimicrobial activity of AMPs. PEGylations with increased conjugation activity related to stable product with high circulation time. Hydrolysable chemical bond between PEG and AMPs displays lowest influences on QTPPs. The interactions among the CMAs/CPPs and CQAs are displayed in ***Fig. 3B***. The size of PEG molecule is highly related to the increased specificity PEG molecule, size of the final PEGylated peptide and increased conjugated activity of it while it has low relation with hydrolysable chemical bond. If PEG molecule is too low in Mw it can be related to low selectivity and reduced conjugate activity of it. The results of the occurrence rating are shown in the ***Fig. 3C.*** Its interpretation is, that the size of PEG molecule and PEGylation site are more risky factors and have highest occurrence potentials comparing to other parameters. Both size of PEG and site of PEGylations highly related to specificity and conjugate rate of PEG.



A

| CQA \ QTPP | | Therapeutic indication (H) | Stable PEGylated peptide (H) | Intact antimicrobial activity (H) | Increased circulation time (H) | Permeability (H) |
|---|---|---|---|---|---|---|
| Size of PEGylated peptide | 35% | Low | Medium | Medium | High | High |
| Increased specificity | 24% | Medium | Medium | High | Low | Low |
| Increased conjugate activity | 32% | Low | High | Medium | High | Low |
| Hydrolysable chemical bond | 10% | Low | Low | Medium | Low | Low |

B

| Process | | PEGylation process | | | | |
|---|---|---|---|---|---|---|
| CPP/CMA \ CQA | | Size of PEG molecule (36%) | Site of PEGylation (24%) | Type of protecting group (16%) | Type of linker (21%) | Type of solvent (4%) |
| Size of PEGylated peptide | 35% | High | Low | Medium | Low | Low |
| Increased specificity | 24% | High | High | Medium | Medium | Low |
| Increased conjugate activity | 32% | High | High | Medium | High | Low |
| Hydrolysable chemical bond | 10% | Low | Low | High | High | Low |

C

| CPP Or CMA | | CPP Occurrence | CPP Severity |
|---|---|---|---|
| 1 | Size of PEG molecule | High | 36% |
| 2 | Site of PEGylation | High | 24% |
| 3 | Type of protecting group | Med | 16% |
| 4 | Type of linker | High | 21% |
| 5 | Type of solvent | Med | 4% |

***Figure 3*** Interdependence rating results among the QTPP elements and CQAs (A), as well as among the CPPs and CQAs (B) and the results of the occurrence rating as steps of the RA

The ***Figure 4*** demonstrates the severity scores calculated by the software for the CQAs and CPPs and these scores and their ranking are visualized in Pareto charts. These charts show the theoretical hierarchy of the influencing factors (CQAs and CPPs) of the

PEGylated AMP due to their criticality. The factors having the highest impact scores are the most highly critical and need emphasized attention during the peptide modification process. In this special case the most critical quality related factors of the PEGylated PGLa product are the following: its final size, its conjugate activity (increased) and its specificity (increased) (***Fig. 4***).

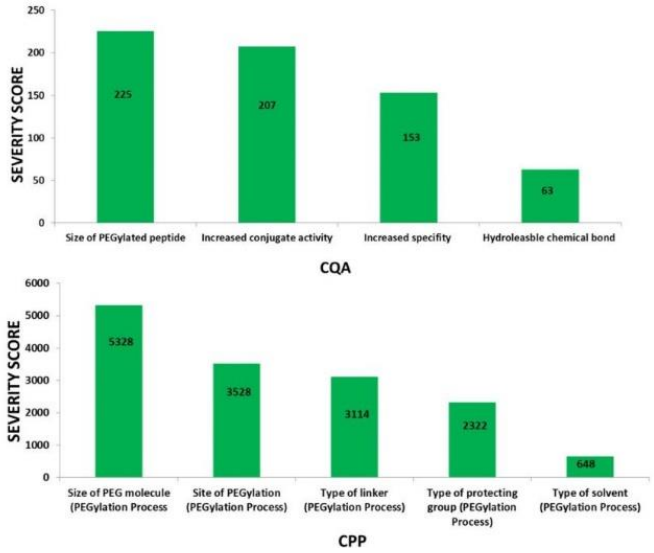



***Figure 4.*** Pareto charts presenting the ranking of the selected CQAs and the CPPs as results of the RA.

On the other hand, in relation to the PEGylation process, as the most critical influencing factors the following were found: the highest critical effect on final product has the size of the starter material (size of the PEG molecule), the next is the proper selection of the site of the PEGylation, and also has high critical effect, but lower than the previously presented two others, is the type of the linker in the PEGylation process. Thy type of the protecting group and solvent have lower effect. In the ***Fig. 5*** the most potential process factors, as CPPs with the highest estimated or relative occurrence and relative severity rate can be seen. ***Fig. 5*** presents the same results as the Pareto chart of the CPP previously, but this interpretation can be useful as well, especially by extended processes with several steps and factors, as those factors which can be found in the right upper quarter of the graphic need corrective actions, or their risk have to be eliminated, or decreased during the continuous quality improvement tasks on site. On the other hand, after such a theoretical RA based experiment design like it was made in this study, these factors found in the right upper quarter will form the basis of the factorial DoE and having the most accentual part in the research executed in practice.

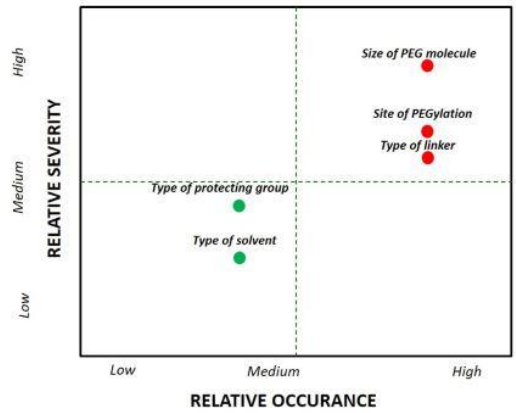



***Figure 5.*** The relative occurrence and relative severity diagram of the CPPs.

### 4.2 Discussion (Part A)

The main focus in this study was on evaluating the risk factors and the required decision points in PEGylation process of PGLa. From the proposed structure and mechanism of action of PGLa, we suggested two possible ways of PEGylation process. N-terminal PEGylation or PEGylation at specific positions. The second approach is worth to try, since it can slow the degradation process and increase bioactivity of PGLa. However, the attachment of PEG molecule in different positions can cause PGLa to lose its positive charges and reduce the antimicrobial activity. Several limitations that result in significant risks influencing final product in both PEGylation manners: large PEG molecule, the interference of PEG molecule with the mechanism of action of PGLa, non-hydrolysable chemical bond between PEG and peptide influence the biological activity of PGla etc. Moreover, during synthesis of PEGylated PGla by FMOC strategy different factors such as the selection of protecting group, acids and bases uses for deprotection and washing steps, linkers, solvents and activating agents, the rate of FMOC hydrolysis and occurrence of side reactions, should be considered for enhanced pharmacokinetic properties of PGLa. According to the result of RA, the size of PEG molecule, the site of PEGylation and the type of the linker were found as having the most critical impact among the process related parameters. So, it is crucial to consider them more carefully before designing the experiments and performing them in practice. They display greater potential to enhance the use of PGLa as therapeutics. These factors can significantly influence PGLa formula by affecting the half-life and antimicrobial activity and overall efficacy and quality of it. The selection of protecting groups and solvents during the synthesis of PEGylated is also affecting the QTTPs of final products but are leading to less risks comparing to other mentioned parameters. In this study the risk factors that influence the PEGylation process

of PGLa were investigated by the application of the Quality by Design (QbD) concept. This approach is resulted in identifying the critical factors with the highest effect on the quality of a final modified AMP. The priority ranking of these factors is as following: its final size, its conjugate activity (increased) and its specificity (increased). On the other hand, the following critical influencing factors during PEGylation process were found to be important respectively: size of the PEG molecule. PEGylation site and the type of the linker. Other factors such as type of the protecting group and solvent have lower effect comparing to the three others. This strategic QbD based development leads to an optimized formulation of PGLa for a potential drug delivery system. Increased circulation time, reduced toxicity, improved permeability, selectivity, viscosity and synergic effect is achievable by considering all the critical parameters during the strategic and risk assessment-based design of the experiments. The selection of the right methodologies and materials in the synthesis of the PEGylated AMPs and their formulation development is vital in proper optimization. This study confirms that the risk-based approach in PEGylation design and process can help to focus the efforts (human, financial, time) on the factors with most critical effects on final product quality.

### 4.3 Results (Part B)

#### 4.3.1 Enzyme activity

Enzyme activity was measured according to the speed coefficient of the degradation of *Micrococcus lysodeicticus* cell wall. ***Figure 6*** shows the enzyme activity results for all samples prepared according to factorial design. Enzyme activity was between 12.1 and 65.2% in each case. The highest value was at pH 10 (+1 level), 0.006% alginate concentration (+1 level) and with a mixing time of 1 h (-1 level).

Based on the statistical evaluation, the effect of factors on enzyme activity can be seen on the response surface. As the response surface of enzyme activity shows, enzyme activity will increase with increasing pH (***Figure 6***), which can be explained by the IEP of LYS (pH 11.1). If the pH is much lower than the IEP, the secondary structure of the protein may change. The amount of α-helix structure correlates well with enzyme activity. The following equation was obtained as the output of the statistical analysis:

$$y=32.92^{*}+7.94x_1+6.96x_2+1.20x_3+8.78x_1x_2-8.19x_1x_3-3.51x_2x_3 \qquad \textit{Eq. 1}$$

$^{*}$ Statistically significant ($p<0.05$)

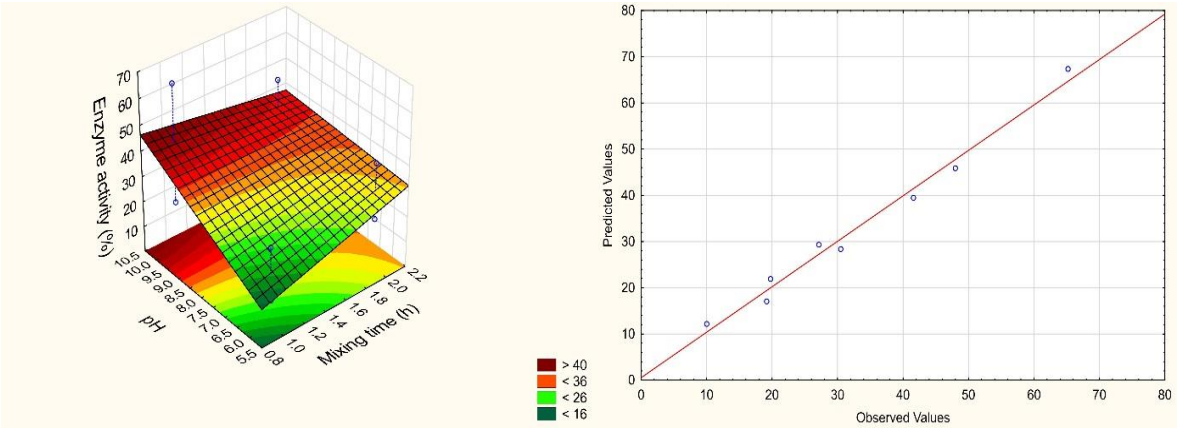



***Figure 6***. The response surface (alginate concentration on zero level) and the predicted values of enzyme activity

In this case, only $b_0$ was a statistically significant factor, which means the average value. Alginate concentration ($x_1$) had the largest effect on enzyme activity (7.94), and pH ($x_2$) also had a great effect (6.96) (*Eq.1*). In this range mixing time had no significant effect. The two-way interaction coefficients were also high for $x_1x_2$ and $x_1x_3$. The correlation between the predicted and the observed values can be seen in ***Figure 6***. It can show the accuracy of the calculated mathematical model for enzyme activity. This means that enzyme activity can predict well in this range with the application of this mathematical model.

### 4.3.2 Encapsulation efficiency

After the precipitation step, precipitation efficiency was calculated according to the UV spectra of the supernatant after centrifugation. In this case, average precipitation efficiency was 66.7%, so 0.4002 mg of the precipitated LYS remained in the system. The next step was the layering of alginate with alginate solution of different concentrations and different pH values. These samples were centrifuged again and the supernatant UV-VIS spectra were measured. From these data, the loss of LYS was calculated and summarized with precipitation efficiency, after which encapsulation efficiency can be calculated. EE was between 62.98 and 66.35 % in all cases (***Figure 7***). It is a very narrow range because approximately 97% of the entire loss of LYS was lost during the precipitation step. After the layering step, the concentration of LYS of the supernatant was very low after centrifugation. It can be explained by the electrostatic relationship between LYS and polyanionic alginate because the redispersion procedure was performed directly in the alginate solution and LYS could not solve in the buffer because the

formation of the alginate layer on the surface of the precipitated LYS started immediately. The alginate layer formed can protect LYS.

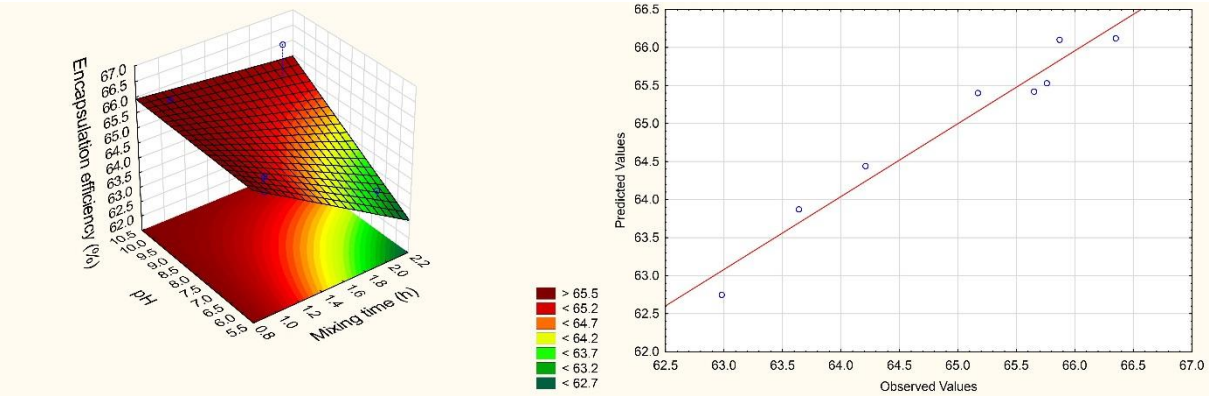



***Figure 7.*** The response surface and the predicted values of encapsulation efficiency

The effects of mixing time and pH were important factors, but statistically not significant. There was no great difference between the results because 97% of the loss of LYS was lost after the first centrifugation (first step of formulation) and the first precipitation step was performed with the same method in all cases. Therefore, the values of the coefficients were very low and statistically not significant. An inverse relationship can be seen between mixing time and EE (***Figure 7***). This can be explained by the starting of the dissolution of LYS from NPs. Therefore, increasing mixing time is not recommended. During a mixing time of 1 h the alginate layer can be formed, which was confirmed by the negative Zeta potential values in all cases. The other important factor is pH, in this case the coefficient was +1.19 (*Eq. 2*). Fig 2 reveals that this factor had an effect on EE only in the lower pH range. In the higher pH range dissolution did not start after a mixing time of 2 h. It can be explained with the isoelectric point (IEP) of LYS (pH 11.1) because at around pH 10 near the IEP, the charge difference between LYS and alginate is lower, therefore the degree of the diffusion of LYS is lower in the polyanionic alginate solution. The third factor was alginate concentration, but this effect was very low (0.85). In this case, a low linear relationship was detected between the factor and EE.

$$y=64.95^{*}+0.85x_1+1.19x_2-1.32x_3+0.28x_1x_2+0.55x_1x_3+0.78x_2x_3 \qquad Eq.\ 2$$

The predicted and the observed values can be seen in ***Figure 7***. The predicted values correlate well with the observed values. This mathematical model can be used to show that EE can be predicted well in this range.

### 4.3.3 Particle size

Particle size was measured freshly before lyophilisation with the laser diffraction method. The results were between 164±1 and 207±3 nm in all cases (***Figure 8***). After the precipitation step, the average particle size was 233±3 nm. In each case, it can be seen that the final particle size was smaller than after the first step of preparation. The reason for this is that the polymer layer can result in a more compact NP structure.

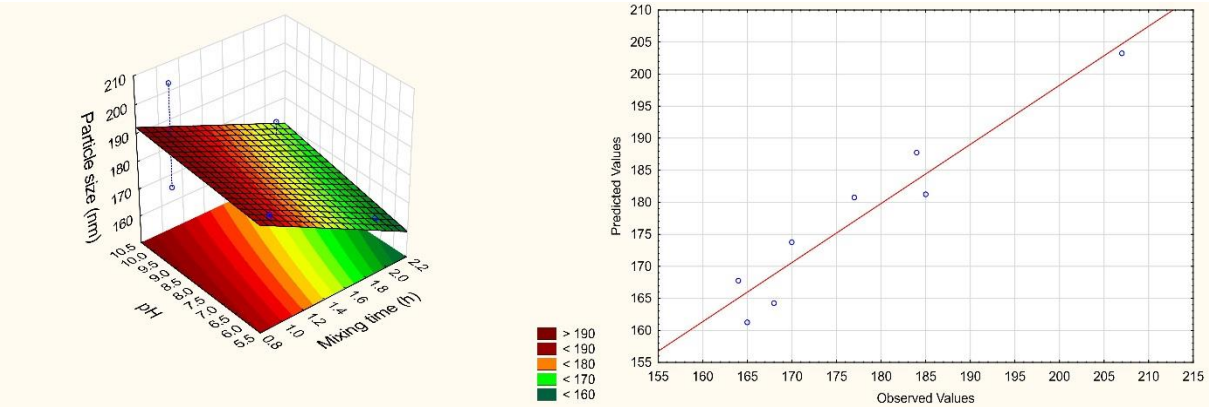



***Figure 8.*** The response surface and the predicted values of particle size

It can be seen in ***Figure 8*** that mixing time had the greatest effect on particle size. During mixing, the dissolution of LYS can start from the NPs, and the degradation of the polymer can also start in parallel with this process. This can cause a decrease in particle size. The EE results confirm this because in the case of higher mixing time, EE was lower because of the dissolved LYS during mixing. In this case ($x_3$) the coefficient was -9 (*Eq. 2*), which means an inverse relationship between particle size and mixing time. The alginate concentration had a smaller effect on particle size. The coefficient was 5.75 (*Eq. 3*) and there was a linear relationship between particle size and alginate concentration. It can be explained by the fact that a higher alginate concentration can result in higher layer thickness, which can lead to larger particle size.

$$y=177.5^{*}+5.75x_1+3.0x_2-9x_3+5.75x_1x_2-3.25x_1x_3+1.0x_2x_3 \qquad Eq.\ 3$$

The coefficient of pH ($x_2$) was 3. The effect of this factor was the lowest, it was not a statistically significant ($p<0.005$) factor. ***Figure 8*** shows that here the predicted value also correlates well with the observed value, therefore this mathematical model is well applicable to predicting particle size in this range of parameter setting.

The alginate layer on the surface of the precipitated LYS can be observed well (***Figure 9***). The particle size correlated with the results determined with the Mastersizer based on the TEM, approximately particles around 170 nm are visible. The core-shell structure is clearly visible in the TEM images, which is also supported by the Zeta potential values.

The Zeta potential value of the LYS solution was 24±2 mV and decreased to -18.2±0.7 mV for LYS NPs layered with alginate in all cases.

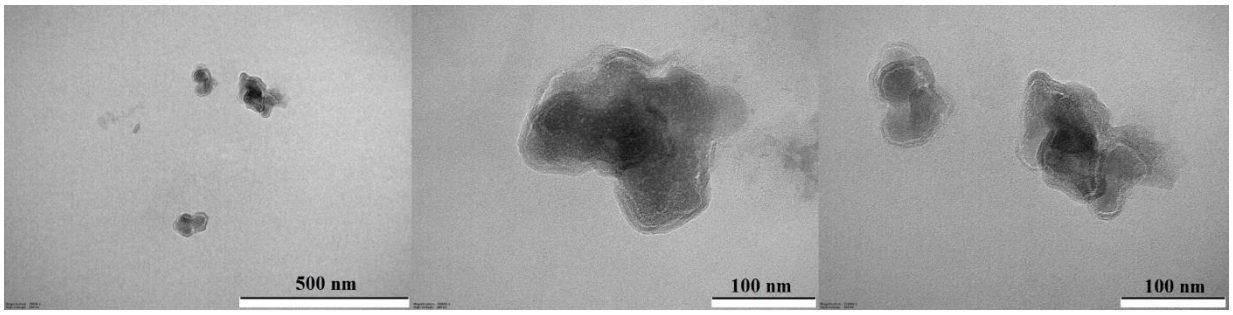



***Figure 9.*** The TEM pictures of alginate layered NPs

### 4.3.4 FTIR and the secondary structure analysis

The samples were analysed with FTIR in KBr pastilles. The amide I, II and III characteristic peaks of proteins can be well assigned in each case (***Figure 10***). The amide I region can be found between 1700-1615 $cm^{-1}$.

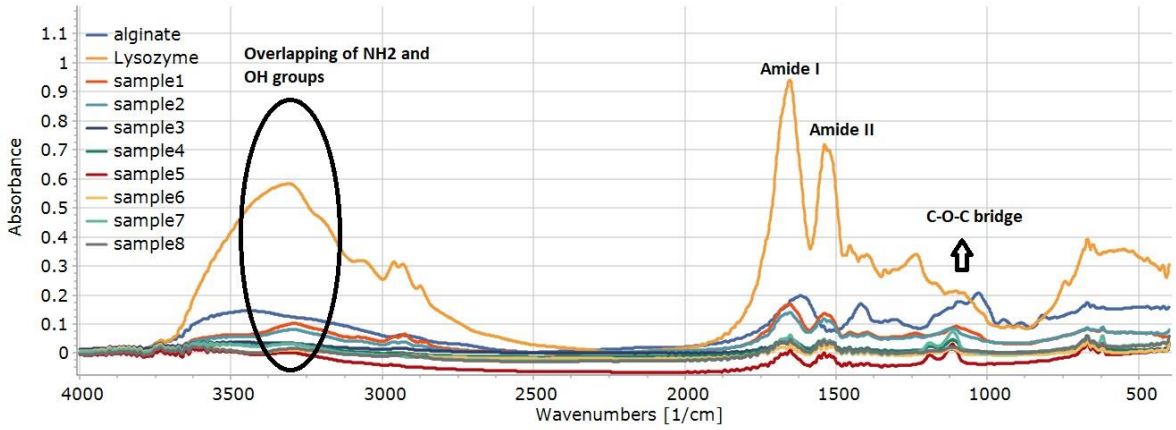



***Figure 10.*** The results of FTIR spectroscopy measurements

After the second derivation of the 1700-1600 $cm^{-1}$ region, the deconvolution of the peaks was performed, the results of which are shown in ***Figure 12***. Seven main peaks were found in this region. At 1685 $cm^{-1}$, 1637 $cm^{-1}$ and 1629 $cm^{-1}$ the β-sheets, at 1672 $cm^{-1}$ and 1666 $cm^{-1}$ the β-turns, at 1654 $cm^{-1}$ the α-helix right next to 1648 $cm^{-1}$ as random, at 1618 $cm^{-1}$ the side chain structure was specific.

The amount of α-helix or other structures can be calculated from the area of the peaks. In ***Figure 11*** the amount of α-helix can be seen. For the raw material LYS, the α-helix content was 22.69%, which is lower than the literature data (40%; 34% in phosphate buffer pH 5.1; 40% in $D_2O$ solution; 30% in water). This may be due to freeze-dried LYS because this product may be more sensitive to environmental parameters than spray-dried

LYS. The α-helix content of precipitated LYS was 19.66% (Table 5). The α-helix content of the samples was higher than this value in all cases except for Sample 1 and Sample 5. In these cases, both alginate concentration and pH were at minimum levels. The reason for this may be that at pH 6 (-1 level) the alginate concentration (-1 level) is too low to stabilize the NPs, but if mixing time increases to 2 h, the α-helix content is also higher (Sample 5). In all cases, if the pH was 6, the α-helix was lower than at pH 10. This can be explained with the IEP of LYS (11.1) because the α-helix content near the IEP can be higher than at lower pH. The effect of pH and mixing time as well as the tendency of the α-helix content can also be observed on the response surface (***Figure 11***).

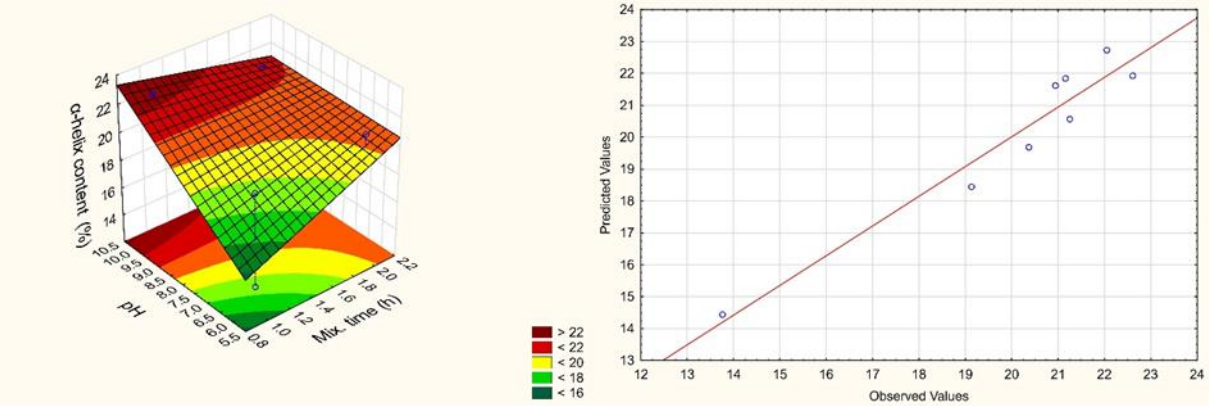



***Figure 11.*** The response surface of α-helix content (alginate concentration on zero level) and the predicted value

This tendency correlates very well with the enzyme activity results (***Figure 6***). It can be seen that enzyme activity increases with the α-helix content. In the course of the statistical evaluation, there was no statistically significant ($p<0.05$) factor. The effects of all factors were positive (Eq. 4), which means a linear relationship between the factors and the optimization parameter. The coefficient of pH was the highest value (+1.61), which can be explained by the fact that the secondary structure of proteins may change with changing pH. We found that the amount of α-helix increases slightly with increasing alginate concentration and mixing time.

$$y=20.16^{*}+0.99x_1+1.61x_2+0.46x_3-1.11x_1x_2-0.52x_1x_3+1.02x_2x_3 \quad \text{Eq. 4}$$

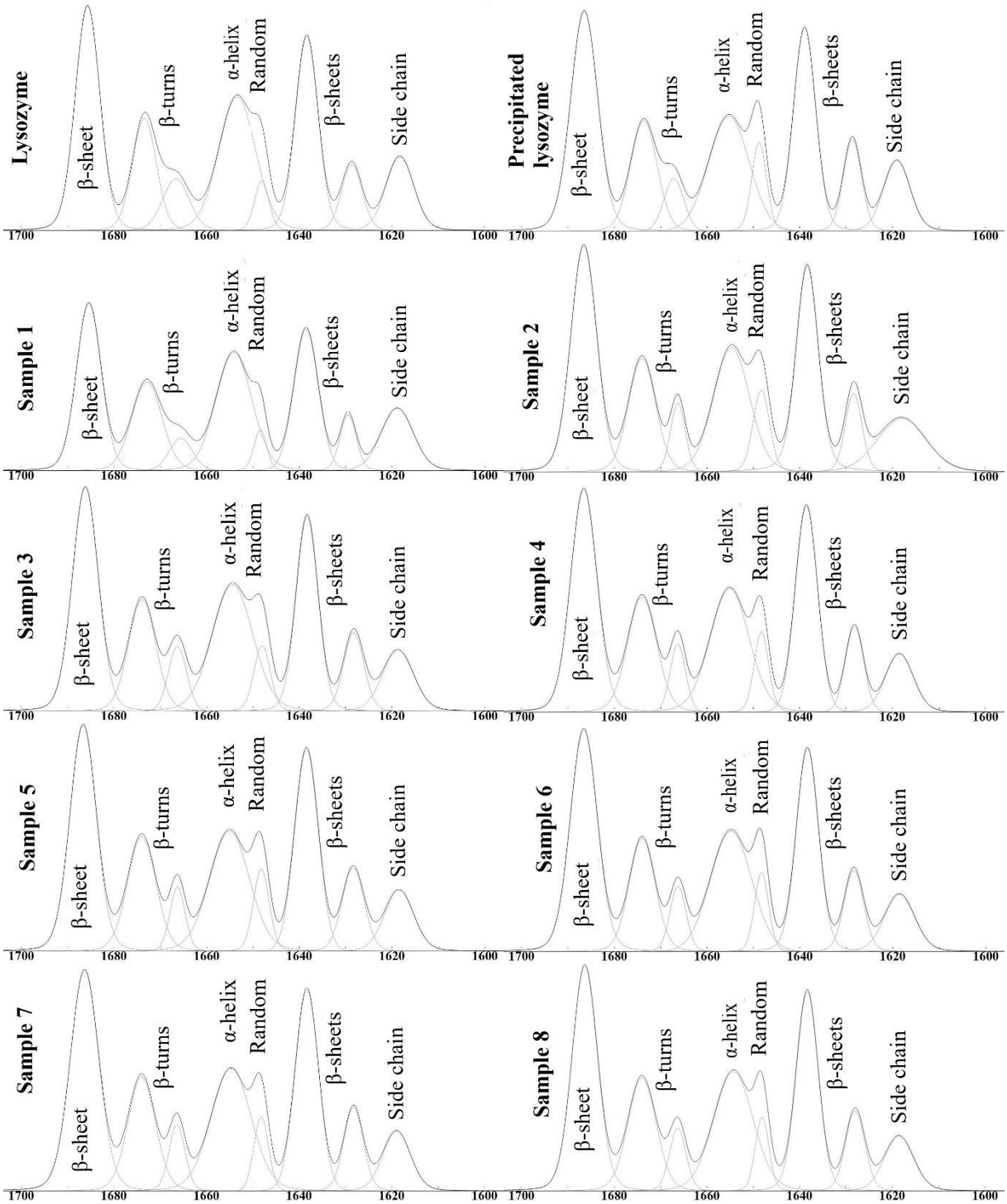



***Figure 12.*** Deconvolution of infrared spectrum of LYZ, precipitated LYZ and the samples

### 4.3.5. CD spectroscopy

Namely, the α-helix content is 41.79 %, 22.75 % and 35.12 % for LYS, precipitated LYS and core-shell NPs, respectively. Based on the CD measurements, the protein chain unfolds during the synthesis of LYS-based NPs, while the alginate shell causes a more compact structure because it wraps and compresses the chains of the precipitated protein.

Comparing the results obtained from the FTIR spectra, it can be seen that there is only a small difference between the precipitated LYS and a major difference between the α-helix results for the starting LYS (22.69%) and NPs (21.16%). The reason for this may be that the FTIR measurement was performed in solid state of protein, while CD spectroscopy was measured in liquid. Therefore, only the precipitated LYS had a similar value for the α-helix contents (3% difference) because in this case the precipitated LYS was also present as solid particles in the liquid during the CD measurements.

### 4.4 Discussion (Part B)

In this study, a simple procedure and analysis for the preparation of core-shell NPs containing LYS were presented. The secondary structure of all samples was determined and statistically evaluated. As regards enzyme activity and the content of α-helix, pH was the most important factor because the α-helix secondary structure is present to a greater extent close to that of IEP of LYS. These optimization parameters correlate well each other. During the formulation of NPs containing LYS pH 10 is recommended. The coefficient of the effect of mixing time was the highest for encapsulation efficiency and particle size, since the dissolution of LYS started during mixing, therefore a mixing time of 1 h is recommended during formulation. The results of the α-helix content of FTIR and CD measurements were very similar for the precipitated LYS due to the solid state of LYS. In the case of alginate layered and raw material LYS, the difference was very high because of the liquid form during the CD measurements. Mathematical models were set up successfully in accordance with the QbD guidelines, which can be used to predict future optimization parameters and design space determination in this range. In summary, this information may help the design of the formulation in the future because it was a very simple composition with a minimal number of excipients applied, therefore only the factors can affect the optimization parameters no other effects should be considered.

## 5. SUMMARY

Peptide modifications and formulation of peptide delivery systems are challenging tasks and hide several risks. Understanding and evaluating the cause - effect relations within the initial Risk Assessment (RA) step in case of all attributes is novelty since it gives the basis for the experimental design as the next step, and aids the formulation development in order to get the final product in the targeted quality range. It also helps to focus on the resources (human, financial, time) related to the final product quality aimed at. By means

of RA method within QbD approach of early pharmaceutical development we monitored the factors with highly risk potential in the PEGylation process and risks such as loosing antimicrobial activity of peptide are prevented. The selection of CQAs, CQAs, QTPPs, CQAs and CPPs/CMAs of a PEGylated PGLa formula was performed and interdependence rating among the QTPP elements and CQAs, as well as among the CPPs and CQAs was performed. This careful theoretical study led to the selection of the right methodologies and materials in the synthesis of PEGylated AMPs and their formulation and consequently resulted in obtaining optimized formulation. In our second work LYZ encapsulated in a novel polyelectrolyte core- sell nanoparticles through the LBL technique utilized as a carrier system to control the release of protein. The preparation of LYZ NPs was made according to $2^3$ full factorial design with QbD approach. Our aim was to understand the effect of process parameters through the determination of mathematical equations, based on which the optimization parameters can be predicted under different process parameters. The optimization parameters were encapsulation efficiency, particle size, enzyme activity, and the amount of α-helix structure. The NPs were analyzed with TEM, FTIR, and CD spectroscopy. Based on our results, we found that pH was the most important factor and pH 10 was recommended during the formulation. Enzyme activity and α-helix content correlated with each other very well, and particle size and encapsulation efficiency also showed a very good correlation with each other. The results of the α-helix content of FTIR and CD measurements were very similar for the precipitated lysozyme due to the solid-state of lysozyme. The mixing time had the best influence on the encapsulation efficiency and the particle size, which leads to the conclusion that a mixing time of 1 h is recommended. The novelty in our study is the presentation of a mathematical model with which the secondary structure of the lysozyme and optimization parameters can be controlled in the future during the development of nanoparticle-based on the process parameters.

## 6. PRACTICAL USEFULNESS

Following the literature evaluation as a preliminary step of our project, our knowledge specified on optimal structural features of antimicrobial peptides and proteins, mechanism of action, therapeutic aim, advantages and limitations, novel modification methods and novel carrying opportunities of them.

- Risk factors that influence the PEGylation process of PGLa were investigated by the application of the Quality by Design (QbD) concept.

- Identifying the critical factors with the highest effect on the quality of a final modified AMP.
- The priority ranking of critical factors: its final size, its conjugate activity (increased) and its specificity (increased).
- The following critical influencing factors during PEGylation process were found to be important respectively: size of the PEG molecule, PEGylation site and the type of the linker. Other factors such as type of the protecting group and solvent have lower effect comparing to the three others.
- Optimized formulation of PGLa for a potential drug delivery system: Increased circulation time, reduced toxicity, improved permeability, selectivity, viscosity and synergic effect.

In the second phase, core-shell nanoparticles containing lysozyme were formulated with precipitation and layering self-assembly. Factorial design (DoE) was applied by setting the process parameters during the preparation with the Quality by Design (QbD) approach.

- In the case of the enzyme activity and the content of α-helix the pH was the most important factor because of near iep of LYZ to a greater extend the α-helix secondary structure.
- During the formulation of NPs containing LYZ pH 10 is recommended.
- The coefficient of effect of the mixing time was the highest in the case of the encapsulation efficiency and the particle size because of starting of dissolution of LYZ during the mixing therefore 1 h mixing time can be recommended during the formulation.
- The α-helix content of FTIR and CD measurement resulted were very similar in the case of the precipitated LYZ because of the solid state of LYZ. In the case of the alginate layered and the raw material LYZ the difference was very high because of the liquid form during the CD measurements.

# LIST OF PUBLICATIONS AND CONFERENCE PROCEEDINGS

## List of Publications Related to the Thesis

**Manteghi R,** Pallagi E, Olajos G, Csóka I. (2020): Pegylation and formulation strategy of Anti-Microbial Peptide (AMP) according to the quality by design approach. European Journal of Pharmaceutical Sciences 144 105197 **(IF: 4.23), Q1**

Kristó K, **Manteghi R,** Ibrahim Y**,** Ungor D**,** Csapó E, Berkesi D, Kónya Z, Csóka I.: Optimization of layering technique and secondary structure analysis during the formulation of nanoparticles containing lysozyme by quality by design approach. PLoS ONE 16(12): e0260603

**(IF: 3.04), Q1**

**Manteghi R**, Kristó K, Szakonyi G, Csóka I. (2022): Recent insight into strategies for the design of antimicrobial peptides (AMPs)/ Acta Pharm Hung 92; 20-37 **(IF: -), (Q4)**

## List of Conference Proceedings

**Manteghi R**, Kristó K, Csóka I. Optimization of layering technique and the secondary structure analysis during formulation of nanoparticles containing lysozyme *IV. Symposium of Young Researchers on Pharmaceutical Technology,Biotechnology and Regulatory Science,* Szeged, Hungary, 2022

**Manteghi R**, Kristó K, Szakonyi G, Csóka I Strategies for development of antimicrobial peptides and proteins *III. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science,* Szeged, Hungary, 2021

**Manteghi R**, Szakonyi G, Csóka I. PEGylation and formulation strategies of antimicrobial peptides and proteins development *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science*, Szeged, Hungary, 2020

**Manteghi R**, Szakonyi G, Csóka I. Design and Development of a novel modified anti-microbial peptide (AMP) formula: evaluation of different parameters and risks influencing AMP effectiveness *I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science,* Szeged, Hungary, 2019

**Manteghi R**, Csóka I, Katona G, Dorina D, Ismail R, Pallagi E. Colloidal systems as carriers for peptide drugs: possibilities and challenges. *EUFEPS,* Frankfurt, Germany, 2019.

**Manteghi R** and Csóka I. Pharmaceutical applications of colloidal drug delivery systems: case
studies for biological drugs. *11th Conference on Colloid Chemistry*, Eger, Hungary,2018.

**Manteghi R** and Csóka I. Formulation strategy of antimicrobial peptide (AMP) delivery systems. *12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs*, Szeged, Hungary,2018.