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PhD Thesis

Strategies for development of antimicrobial peptides and proteins

by

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VI. **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.

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LIST OF ABBREVIATIONS

Alloc	N-Allyloxycarbonyl
AMPs	Antimicrobial peptides
BSA-NPs	Bovine serum albumin nanoparticles
CD	Circular Dichroism
CMAs	Critical Material Attributes
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
CS	Chitosan
DLS	Dynamic Light Scattering
DoE	Design of the Experiments
DS	Design Space
DSC	Differential Scanning Calorimetry
EE	Encapsulation efficiency
Fmoc	9-Fluorenylmethyloxycarbonyl
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
LBL	Layer-By-Layer
LYZ	Lysozyme
NPs	Nanoscale Particles
PEG	Polyethylene Glycol
QbD	Quality by Design
QTPP	Quality Target Product Profile
RA	Risk Assessment
tBoc	Tert-Butoxycarbonyl
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TPP	Tripolyphosphate
WHO	World Health Organization

1. INTRODUCTION AND AIMS

According to the World Health Organization's (WHO) report, antimicrobial resistance is one of the main global threats [1]. 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 [2,3]. 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 [4,5]. 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 [6,7]. Several thousands of AMPs have been isolated from various natural sources such as microorganisms, plants, insects, crustaceans, animals, humans, etc. 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 [8]. 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 [9–12].

However, 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 in the selection of optimal structural features of AMPs, the best possible modification strategy for AMPs, and the best possible nanocarrier system for them.

After our preliminary above mentioned studies, analysis of the potential risks in the peptide PEGylation process was performed through the example of PGLa and in the next phase of the project, 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) [13].

As can be seen in ***Figure 1*** in the first part of our project we investigated the risk factors that influence the PEGylation process of PGLa by the application of the QbD concept. PGLa is a 21-residue amphipathic antimicrobial peptide-amide (GMASKAGAIAGKIAKVALKAL-NH2), isolated from the African clawed frog Xenopus leavis, that can destroy bacteria by interacting with their lipid membrane [14,15]. Analysis of the risk factors lead to optimized formulation of PGLa with increased half-life, reduced toxicity, improved permeability, selectivity, viscosity and synergic effect for a potential drug delivery system.

In the second part of our project, we investigated the effective delivery of proteins with antimicrobial activity through the example of LYZ. LYZ is a harmless natural antimicrobial enzyme can be derived from the plants, animals, and microorganisms as a single chain polypeptide having a globular shape constructed of 129 amino acids with an approximate molecular weight of 14 kDa, with a quite alkaline nature as its isoelectric point is 11, and its main physiological role is performing the host`s natural immune defence effect.

For the purpose of this research, we carried out pre-formulation experiments by varying factors such as the concentration of the alginate, mixing time, and the pH according to the full factorial design. Based on these variations, different formulations of the LYZ were prepared, tested and optimised and the obtained NPs were comprehensively characterised. Furthermore, analytical measurements and assessments were carried out using the different values for the alginate concentration, mixing, and the sodium sulphate content which served as determinant factors for the particle size and secondary structure of the enzyme nanoparticle solutions (***Figure 1***).

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

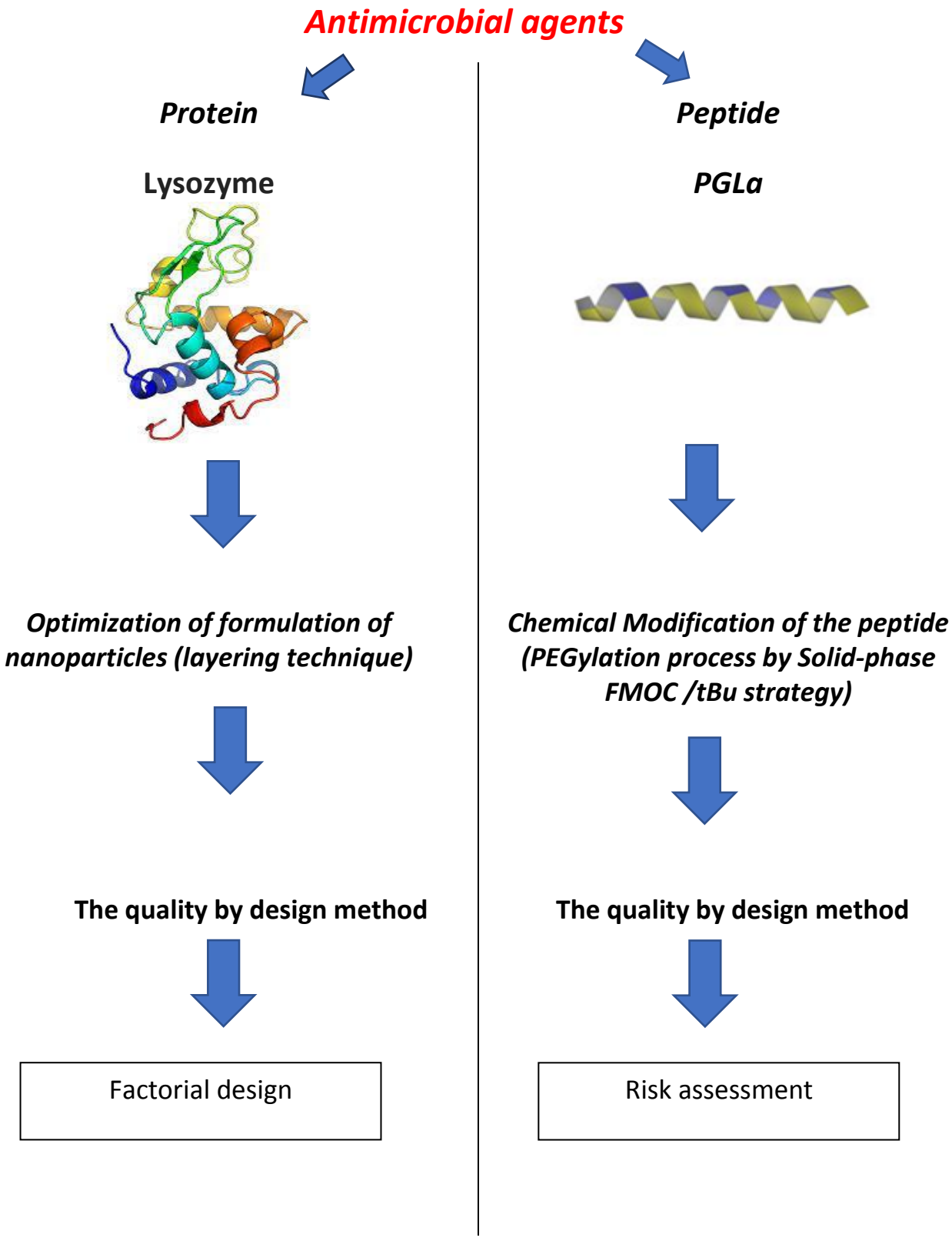

Figure 1. The main strategic QbD based developments of our project

2. LITERATURE SURVEY

2.1. Antimicrobial peptides and their potential in antibiotic therapy

2.1.1 Definition, history, source, target and mechanism of action

The relatively small size of antimicrobial peptides (<10kDa) made their isolation possible only in the 1980s. This was initially achieved in frogs, insects and granules of human and rabbit granulocytes. Since then, a large number of additional antimicrobial peptides has been found virtually everywhere in nature, amounting to over 800 peptides known at present [16, 17]. Antimicrobial peptides seem to have effector functions in innate immunity and can upregulate the expression of multiple genes in eukaryotic cells [18]. They represent a wide range of short, cationic or anionic, gene-encoded peptide antibiotics. Despite sharing a few common features (such as cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups have been proposed to incorporate the diversity of the observed AMP conformation. As shown in ***Figure 2,*** AMPs are produced by bacteria and eukaryotes, such as protozoa, fungi, plants, insects and several types of vertebrate and invertebrate animals. They show a variety of targets, including Gram-positive and Gram-negative bacteria, parasites, fungi and some viruses [19–23]. AMP genes are present in the genetic material of a number of mammals. The expression of these genes has been detected in different cells, including neutrophils, monocytes, macrophages, epithelial cells, keratinocytes and mast cells. AMPs are synthesized as pre-pro-peptides and a post-translational process allows their final maturation into active peptides [24, 25].

AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation methods. A schematic representation of different classifications of AMPs is shown in ***Figure 2***. According to our present knowledge, this is a new overview of various AMP classifications.

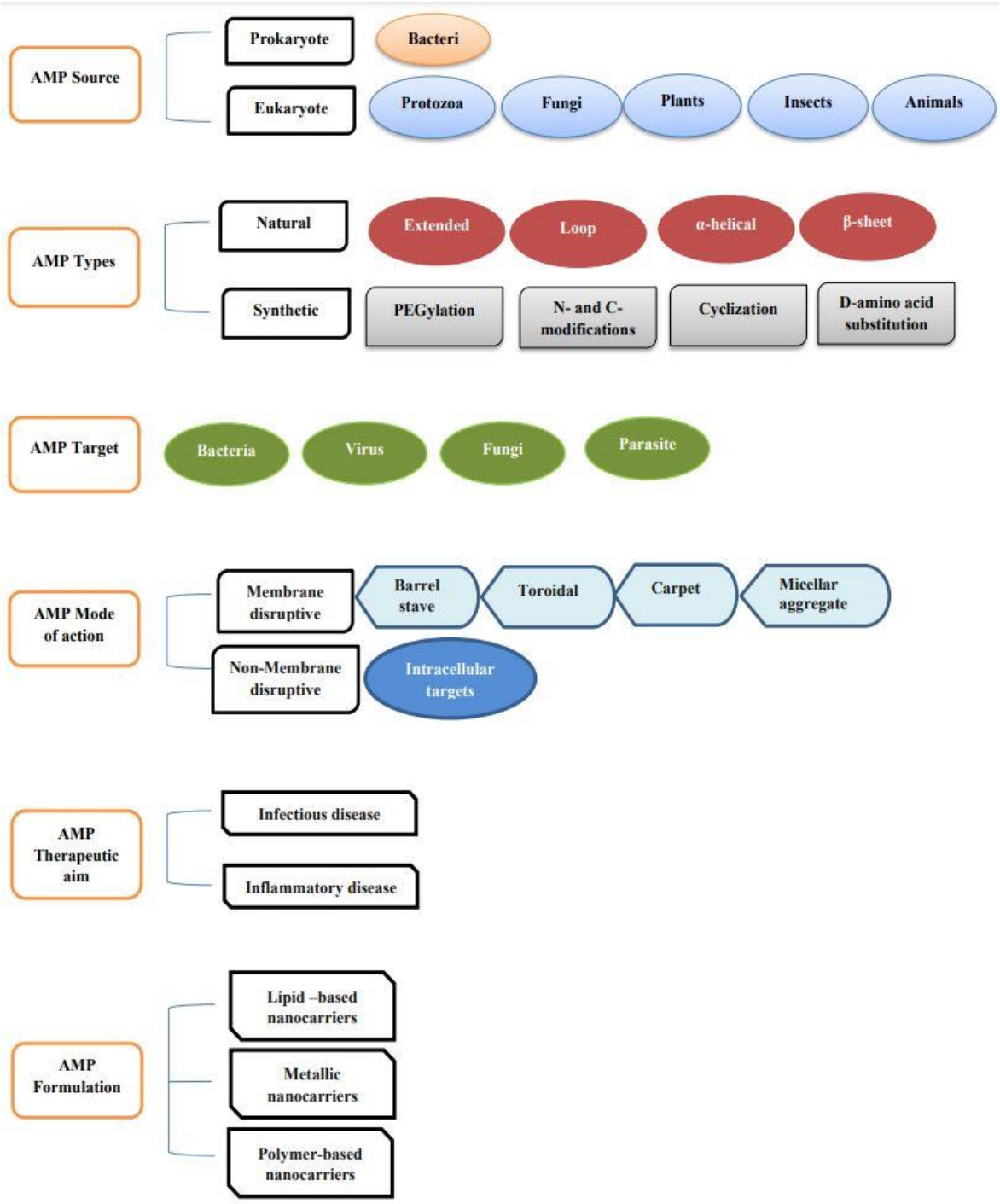

Figure 2. Schematic representation of different classifications of antimicrobial peptides. AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation type

An insight into the mechanism of action of AMPs is essential for the further development and design of optimized AMPs that could be efficiently used as therapeutic drugs. Thus, a

broad range of researches are assigned to study the mechanism of action of AMPs [26-28]. According to these researches, AMPs are divided into two main groups based on their mode of action: membrane disruptive AMPs and non-membrane disruptive AMPs [29].

2.1.2 Structural and physicochemical features

The antimicrobial activity and selective toxicity of AMPs are significantly influenced by their structural and physicochemical features. Furthermore, studying different structural parameters of AMPs is a vital part of the design and development of novel antimicrobial agents with enhanced antimicrobial activity [30].

2.1.2.1 Conformation (X)

Based on secondary structures assumed by AMPs in the presence of other biological membranes, they are categorized into different conformations, such as α-helix, β-sheet, extended helix and loop (***Figure 2***) [25–27].

Circular Dichroism (CD), X-ray crystallography and Nuclear Magnetic Resonance (NMR) studies are commonly used to determine the secondary structure of these peptides [30, 31].

The **α-helical AMPs**, including cecropin and pexiganan, tend to form amphipathic helices in certain organic solvents, such as trifluoroethanol. These α-helical AMPs disturb the bacterial membrane by employing various mechanisms of action, including the formation of barrel-like bundles (barrel-stave model), carpet-like clusters (carpet model) and toroidal pores (toroidal pore model) into the membrane.

AMPs with **β-sheet structure**, such as α-, β-defensins and protegrin, form β-hairpin structures stabilized by disulfide bridges. Most of the β-sheet AMPs have a rigid structure and the bacterial membrane is disturbed by a perpendicular insertion into the lipid bilayer and the formation of toroidal pores.

Mixed structure (αβ-peptides) AMPs, such as bactenecin, adopt a loop formation with one disulfide bridge [32, 33].

The **extended AMPs**, which are rich in specific amino acids, have irregular secondary structures. Many of these peptides show antimicrobial activity only after interacting with the membrane and undergoing consequent conformational changes. Indolicidin with 13 amino acids, a member of this group of AMPs, contains five tryptophan and three proline residues.

The peptide adopts a poly-L-II helical structure in the presence of liposomes, and the high content of tryptophan residues is responsible for their interaction with lipid membranes.

2.1.2.2 Charge

Many of the antimicrobial peptides display a net positive charge, ranging from 2 to 9, and may contain highly defined cationic domains. Cationicity is essential for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms [34-38]. However, this relationship is not fully linear. Within a certain range, increasing peptide cationicity is generally associated with increasing antimicrobial strength. For instance, studies with magainin 2 analogs show that increasing the charge from 3 to 5 results in increasing antibacterial activities against Gram-negative and Gram-positive pathogens. However, a net charge from 6 to 7 leads to an increase in the hemolytic propensity and to a loss of antimicrobial activity. Therefore, it can be concluded that there is a risk beyond which increasing the positive charge no longer increased the activity of AMP [32]. Although in a wide majority of cases AMPs are cationic, anionic AMPs (AAMPs) have also been described as an integral and important part of the innate immune system and increasingly identified in vertebrates, invertebrates and plants over the last decade [39]. While cationic peptides are rich in arginine and lysine, AAMPs are small peptides rich in glutamic and aspartic acids. AAMPs that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge [40].

2.1.2.3 Amphipathicity (A) and hydrophobicity (H)

In research by *Mihajlovic et al.*, the amphipathicity of antimicrobial peptides plays a crucial role in pore formation and can also contribute to a better understanding of the mode of action in antimicrobial peptides [41, 42]. *Kondejewski et al.* have reported that the antimicrobial activity and toxicity of peptides are notably enhanced with amphipathicity. However, an extremely amphipathic nature is not desirable in cyclic AMPs since it decreased the specificity and increased interactions with outer membrane components [43].

Hydrophobicity is a main feature for the effective membrane permeabilization of AMPs as it determines the extent to which a peptide can partition into the lipid bilayer. However, an

increase in the levels of hydrophobicity is strongly related to mammalian cell toxicity and loss of antimicrobial specificity. Therefore, moderate hydrophobicity is needed against the bacterial membrane [32]. A research by *Wood et al*. on a linear cysteine-deleted tachyplesin (CDT), examined the effect of hydrophobicity on antimicrobial activity. Analogs with hydrophobic isoleucine residues placed throughout the sequence of CDT showed comparable antimicrobial activity to CDT but lower hemolysis [44].

AMPs with moderate features (charge, hydrophobicity, amphipathicity) and a good balance between these characteristics showed higher antimicrobial activity and lower cytotoxicity and hemolysis in mammalian cells [36, 45].

2.1.3 Therapeutic aim

As shown in ***Figure 2*** and ***Table 1,*** AMPs can be categorized based on their therapeutic aim. 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 [46, 47]. According to the results of a research project, the sustained release of drugs at the site of action presented excellent results in the treatment of chronic wounds [48]. In the table below, the association between some of the most common AMPs with different conditions, including infectious and inflammatory diseases, is listed and classified into two groups of acute and chronic diseases. Based on literature review, AMPs which are used to treat chronic diseases should provide controlled and sustained release by choosing the proper administration route, while an immediate release formulation of AMPs is effective for acute diseases. Thus, the therapeutic aim of AMPs has significant influence on formulation parameters in the delivery of these peptides, and depending on the medical application of AMPs, the drug release profile and therefore the administration route are different [49, 50].

Table 1. Some of the most recent researches showing various AMPs and their administration routes effective in the treatment of acute or chronic diseases

Peptide	Disease	Chronic/Acute	Administration Route	Ref.
Rhesus theta defensin-1 (RTD-1)	Acute lung injury (ALI)	Acute	Parenteral	[51]
Cathelicidin LL-37	Acute thrombosis			[52]
Catestatin (CST)	Acute and chronic pain			[53]
Human beta defensins 1 (HBD1)	Acute HIV-1 infection			[54]
Human cathelicidin (hCAP18/LL-37)	Chronic obstructive pulmonary disease (COPD)	Chronic	Inhalation	[49]
HBD1, HBD2, HBD5 and HBD6	Crohn's disease (CD)		Oral	[50]
hBD-3	Wound		Dermal	[54]
β defensin	Chronic rhinosinusitis (CRS)		Nasal	[55]

2.1.4 Advantages and limitations of AMPS

In relation to small molecule drugs, 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. Generally, even synthetic peptides are less immunogenic than recombinant proteins and antibodies. Among different peptides, AMPs emerged as essential tools with a broad-spectrum of activity and a low rate of resistance development [34, 51]. Besides the mentioned advantages, AMPs have limitations, such as low metabolic stability and low permeability across biological barriers, high costs and poor relevance of antimicrobial activity of AMPs *in vivo* and *in vitro*, cytotoxicity, and difficulty in reaching targeted sites at active concentration due to degradation. In the next section, it is shown that these initial barriers are being increasingly overcome with new chemical modification strategies for the development of stable, more cost-effective and potent broad-spectrum synthetic peptides [56].

2.1.5 Modification strategies of AMPS

Strategies such as N- and C-modifications, incorporation of non-natural or D-amino acids, cyclization and the attachment of the polyethylene glycol polymer to peptides (PEGylation) allowed several researchers to enhance the bioavailability of AMPs and improve their bio-distribution and rate of clearance. The proteolytic degradation of peptides can be decreased by protecting their C- and N-terminus with acetylation or amidation. Also, modifying the sequences of peptides by substitution of natural L-amino acids for their D enantiomers, α/β-substituted α-amino acids or even β-amino acids are other similar approaches that result in overcoming peptide hydrolysis. D-amino acid substitution in a peptide may influence not only the peptide's stability but also its secondary structure and therefore its ability to incorporate into membranes [9, 11].

For PEGylation at specific sites of the peptide, we have to use alternative side-chain protecting groups for the selected lysine residues which can be removed before the cleavage of the peptide. Selective removal of these protecting groups such as methyltrityl allows the coupling of the PEG chain at the selected position, followed by the removal of the N-terminal protecting group and the cleavage of the peptide. PEG-Linker-Drug strategy is another possibility to increase the half-life of PGLa. By using a linker which is degraded by the bacteria itself, the risk of decreased antimicrobial activity by the PEGylation can be circumvented and there is no need to site-specific modifications. Bacterial enzymes which play roles in antibiotic resistance (such as β-lactamase) are the most promising candidates, as the overexpression of the enzymes would facilitate the release of the drug. Solid-phase FMOC /tBu strategy is a common strategy designed and developed by using protected amino acids as building blocks [57, 58]. The controlled synthesis of peptides and formation of amide bonds requires the use of reversible ion of the amino group. Common amino protecting groups are: tert-Butoxycarbonyl (tBoc), 9-Fluorenylmethyloxycarbonyl (Fmoc) and N-Allyloxycarbonyl (Alloc). It is also necessary to reversibly mask reactive side chain functional groups. The peptide remains anchored to an insoluble solid resin support. Resins commonly used are composed of polystyrene. The excess reagents and soluble byproducts will be removed after each reaction cycle.

As can be seen in ***Figure 3***, in this approach the first protected amino acid is attached to the resin through its carboxyl group (coupling; the addition of activating agent). Then the protecting group is removed (deprotection) under a mildly basic condition. This exposes a

free α-amino group to react with the next incoming protected amino acid. Then again deprotection step is repeated. (To confirm that the protecting groups are removed, a Kaiser-test is performed). The process is repeated through a cycle of deprotection, coupling and washing until the peptide is completely synthesized. The synthesized peptide is usually cleaved from the resin by trifluoroacetic acid (TFA), which removes the side chain protection groups at the same time. The purification steps usually include the precipitation from the cleavage reaction mixture by ice-cold diethyl ether. Further purification can be achieved by gel-filtration, ion exchange chromatography and reversed-phase HPLC [59].

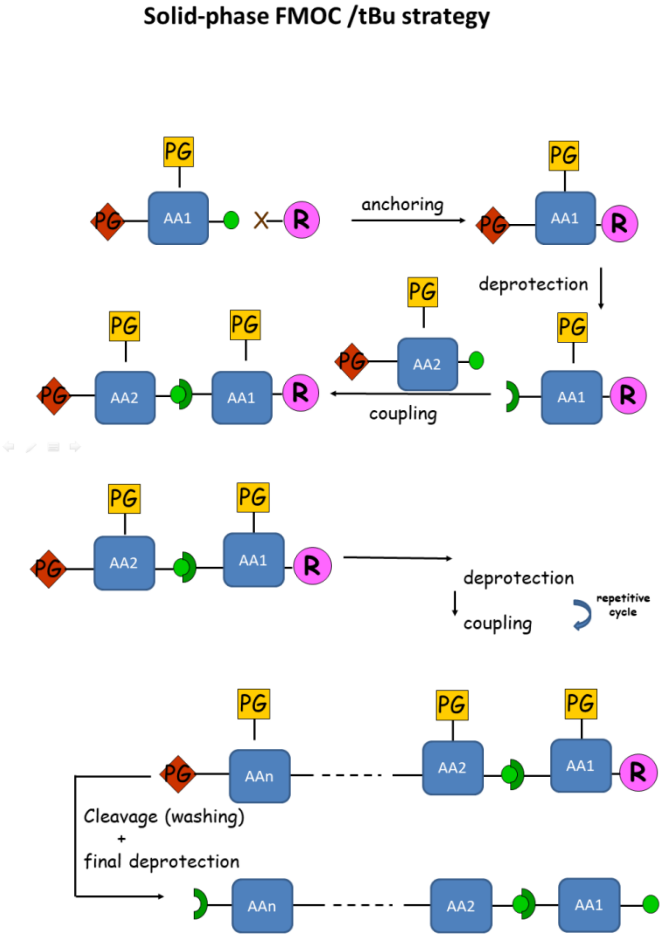

Figure 3. Schematic representation of solid phase peptide synthesis

2.2. The Quality by Design method (QbD)

The Quality by Design (QbD) approach is a holistic, systematic, knowledge and risk based methodology of pharmaceutical developments, which focuses on the profound preliminary

design [60] considering all of the influencing parameters from the industry, the regulatory body and from the user (e.g., patient, doctor). The application of the QbD method in the industrial development and manufacturing is forced by the regulatory authorities but it has also many benefits in the early phase of the developments [57, 58]. As it brings scientific results closer to the practical requirements and has a facilitating effect on industrial scale-up and product transfer to the market. The QbD has several steps, described in the guidelines of the International Council for Harmonization (ICH Q8 (R2), ICH Q9, ICH Q10) [61, 62].

The main steps are:

(1) The definition of the Quality Target Product Profile (QTPP)

(2) The identification of the quality attributes and the selection of the Critical Quality Attributes (CQAs) related to the target product

(3) The prior selection of the production method and the identification of the Critical Process Parameters (CPPs) as well as the Critical Material Attributes (CMAs)

(4) Performing of the initial Risk Assessment (RA). RA is a systematic process of organizing information to support a risk decision and is the key activity in this model. The results of the RA will be the ranking of the CQAs and CPPs according to their calculated risk severity. RA results help to aim attention on the most critical influencing factors and avoid profitless efforts in later phases of the development process.

The following steps of the QbD approach are:

(5) The Design of the Experiments (DoE) which namely means the planning of the practical tasks by the RA results (6) The performing of the experiments in practice and establishment of the Design Space (DS). These are followed by the (7) Compilation of the Control Strategy which is the monitoring of the factors with highly risk potential in the process. The whole QbD guided process should be designed and performed by considering the possibilities of the (8) Continuous Improvement. In this thinking, generally the RA is the most accentual element which it is especially advantageous in the case of complex and sensitive drugs like peptides.

2.3. Optimization of layering technique

Nanotechnology has been performed to improve the drug delivery performance, basically by improving the bioavailability through the administration of the drug entity in nanoscale particles (NPs) or molecules which can overcome the biological barriers, targeting the absorption site, enhancing the stability and solubility by increasing the surface area. Therefore, various nanotechnology-based drug formulations have been introduced into the market for treating and controlling numerous diseases such as cancer, central nervous system diseases and infections management [63-67]. Moreover, the delivery of the drug in NPs form protects the natural products such as proteins from the degrading enzymes, as well as controls the release of the incorporated bioactive molecules [68].

The intensively used nano-carrier systems for proteins delivery are based on synthetic polymer, liposomes and metal; they have been replaced as a result of many limitations like instability and low loading capacity of liposomes, whereas metal-based nano-carriers have disadvantages of low clearance rate and hence enhanced toxicity, and that made from synthetic polymers has a limitation of the aggregation of the encapsulated proteins in their inner core [69, 70]. Accordingly, proteins NPs and their conjugate have been substituted the nano-carrier systems and having advantages of nano-sized structure with good biopharmaceutical characteristics, also, their production is a cost-effective, and easy to tailor to fit the specific requirements [71-73]. In contrast to nano-carriers based on metal, LYZ encapsulated in the Selenium NPs and their nano-hybrids were prepared by precipitation method by using sodium phosphate at various pH, showed a synergistic antibacterial effect, and meanwhile, their interaction retains the native enzyme activity and conformation [74].

The precipitation technique (bottom-up approach) represents the most applicable method for NPs production for both small scale and bulk production, this owing to its simplicity, low energy input, low generated temperature and cost-effective compared to the other top-up methods[75, 76]. Xanthan/LYZ NPs have been produced by precipitation in the alkali-coupled thermal condition, the NPs showed favourable size distribution and stability [77]. Whereas LYZ loaded in chitosan (CS) NPs have been prepared through ionic gelation of chitosan and tripolyphosphate (TPP), it was reported the CS molecular weight and content, TPP content, and initial LYZ have effects on the encapsulation efficiency (EE), release performance and activity of the LYZ [78]. Moreover, LYZ complexed with different concentrations of sodium alginate showed two stages of aggregates formed with the loss of the activity based on the alginate content, the antimicrobial activity was recovered upon the

addition of calcium chloride [79]. Similarly, LYZ encapsulated in a novel cationic polymethacrylate/alginate NPs as a polyelectrolyte carrier system, the NPs showed a high capacity to encapsulate the enzyme, with acceptable polydispersity, biodegradable, high stability and sustaining the release of LYZ. The *in vitro* cytotoxicity of the complex was found a dose-dependent [80]. Also, polyelectrolyte core-shell of bovine serum albumin nanoparticles (BSA-NPs) developed through the layer-by-layer (LBL) technique utilized as a carrier system to control the release of ibuprofen, the inner layer is anionically made from poly (sodium-4-styrene) sulphonate, and the cationic outer layer made from CS which enables the interaction with a negatively charged cell membrane and facilitates the cell up-take [81]. Accordingly, the disposition of oppositely charged polyelectrolyte polymers through the LBL approach represents a promising technique for fabrication of micro/nanoparticles and enables to modify their physiochemical and morphological properties by controlling the ionic strength, polymerization degree, and the ratio of the polymers [82-85]. Most importantly, the technique is usually conducted at the normal experimental conditions and mainly in an aqueous solution and hence, it is suitable to encapsulate protein in and polypeptide drugs [86], and recently the application of three layers of the polyelectrolyte polymers on the anticancer NPs demonstrated a cancer-cells targeting with efficient internalization [87]. Also, three polyelectrolyte polymer layers were prepared on the surface of human serum albumin nanoparticles containing interferon-α with layering technique, and the core-shell nanoparticles has sustained release [88].

For this study, LYZ is a good model protein because of its detailed structural and enzymatic properties and the straightforward assessment of its biological activity [89-91].

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-NH_2) is 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 [60].

3.2. Methods (Part A)

3.2.1. Knowledge space development

The collection and systemic organization of the related scientific literature and experience from the previous studies means the "knowledge space development" [58]. After the analysis of the relevant scientific literature, the data collected were structured and visualized. Ishikawa diagram was prepared for categorization of the influencing factors (causes) [92]; flow charts were prepared for PEGylation process description [93].

3.2.2. Definition of QTPP

The QTPP forms the basis of product development design. The QTPP is always unique depending on the target. Generally, it should include patient-relevant product performance and characteristics related to the aimed therapeutic or clinical use. Considerations for QTPP selection are described in the ICH Q8 (R2) guideline [61], e.g., the route of administration, dosage form, etc. 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.

3.2.3. Determination of the CQAs

CQAs are those 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 [61]. The selection of CQAs is based on a holistic view of the formulation development and is based on previous knowledge and experience.

3.2.4. Determination of the CMAs and CPPs

CMAs are critical material attributes such as physical, chemical, biological, or microbiological properties or characteristics of 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 [61]. CMA and CPP selection was based on prior knowledge resulting from the knowledge space development phase of the study.

3.2.5. Initial risk assessment

The initial RA was performed by means of the Lean QbD Software® (QbDWorks LLC., Fremont. CA, USA, qbdworks.com). 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 was 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. 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 an amino protecting group, and solid-phase strategy was selected for the design.

3.3. Materials (Part B)

Lyophilized LYZ (CAT. HY-B2237/CS-7671, MedChemExpress, Hungary), stored at freeze

(-20°C) was used as a model protein, lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) used as a Gram-positive bacteria for layered NPs activity investigation, sodium sulphate (Molar Chemicals Kft., Hungary) was used as precipitating agent, Alginic acid sodium salt (AppliChem GmbH. An ITW Company, Germany) was utilized as layering polymer, sodium hydroxide and hydrochloric acid (Ph. Eur.) were used as pH ad justifiers and the other all reagents were of analytical grades [63].

3.4. Methods (Part B)

3.4.1. Experimental design

Factorial design (DOE) 2^3

Table 2. The levels of factors

Sample	c (alg.)%	pH	Mixing time (h)
1	0.004 (-1)	6 (-1)	1 (-1)
2	0.004 (-1)	10 (+1)	1 (-1)
3	0.006 (+1)	6 (-1)	1 (-1)
4	0.006 (+1)	10 (+1)	1 (-1)
5	0.004 (-1)	6 (-1)	2 (+1)
6	0.004 (-1)	10 (+1)	2 (+1)
7	0.006 (+1)	6 (-1)	2 (+1)
8	0.006 (+1)	10 (+1)	2 (+1)

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.

3.4.2. Preparation of LYZ NPs

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 (G-force 3640) for 15 minutes by using Hermle Z323K high performance refrigerated centrifuge (Hermle AG, Gossheim, Germany).

3.4.3. Precipitation and encapsulation efficiency

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 spectrometer (ThermoScientific-Genesys 10 S UV-Vis Spectrometer, USA) at lambda max 281nm. Based on the absorbance, the concentration of unprecipitated NPs enzymes was 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.

3.4.4. Particle size and zeta potential measurement

The precipitated NPs were adequately diluted, and the particle size of the sample was measured with a Malvern Mastersizer (Malvern Instruments, Malvern, UK). The Zeta potential of the same sample was measured with a Malvern Zetasizer apparatus with three parallel measurements (Malvern Instruments, Malvern, UK). 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 high shear mixer (Ultra-Turrax, Germany) for 15 seconds, then followed by re-centrifugation with same parameters as mentioned before.

3.4.5. The Morphological Study

The structure and the morphology of the precipitants NPs (after layering) were described with transmission electron microscopy (TEM). The TEM images were made with the FEI TecnaiTM G2 X-Twin HRTEM microscope (FEI Company, Hillsboro, Oregon, US) with

accelerating voltage of 200 kV in bright field mode. Sample were suspended in water and dropped onto a carbon film-coated copper grid.

3.4.6. The Enzyme Activity of Layered NPs

The activity of the prepared layered nanoparticle samples was carried out by measuring the degradation of lyophilized *Micrococcus lysodeikticus* by using UV spectrometer (ThermoScientific-Genesys 10 S UV-Vis Spectrometer, USA). 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 shaken for 20 seconds in a 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.

3.4.7. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra for the prepared samples and the other excipients were obtained by using FT-IR (Avatar 330 FT-IR ThermoScientific, USA) 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 (version 1.2) software was used for the second derivation of spectra. For deconvolution of second derivatives spectra was used the Fityk software [94]. After assigning of peaks, the area was calculated. From these data the α-helix content was determined.

3.4.8. Circular dichroism spectroscopy (CD)

To determine the α-helix content of the initial LYZ and the synthesized NPs, circular dichroism (CD) spectra were recorded on an ABL&E-JASCO J-1100 CD 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 by the following equations [95]:

$$MRE_{208} = \frac{[\Theta]}{n} \text{ and} \qquad \textit{Eq. 1}$$

$$\alpha - helix\ \% = \frac{-MRE_{208} - 4000}{33000 - 4000} * 100, \qquad \textit{Eq.2}$$

where the [Θ] is the molar ellipticity at ca. 208 nm, the n is the number of the amino acids, the 33000 is the pure α-helix content at 208 nm, while the 4000 is the amount of the β-sheet and random coil.

4. RESULTS AND DISCUSSION

4.1. Results (Part A)

The basis for initial RA was an evaluation of the present knowledge in the different limitations of PEGylation and how these barriers can lead to risks and how these risks can be overcome by novel opportunities offered by chemistry or biochemistry for achieving desirable bioactive AMP (***Table 3***).

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 (***Figure 4***), where the different factors and possible associated risks in selection, modification and formulation of in a suitable delivery system are highlighted [96].

Table 3. 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

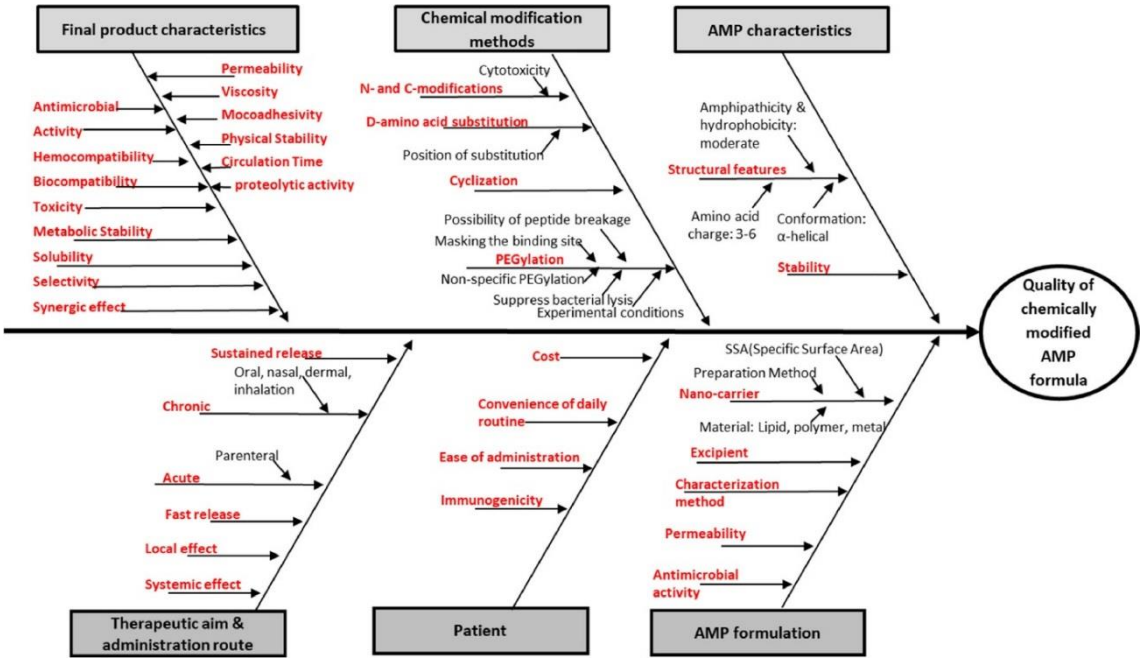

Figure 4. Ishikawa diagram including all the parameters

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 ***Figure 5***. These graphical representations (***Figure 4 and 5***) aimed the selection of the CQAs that could critically affect the desired QTPP and also helped in the 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 of the PEGylated PGLa were defined.

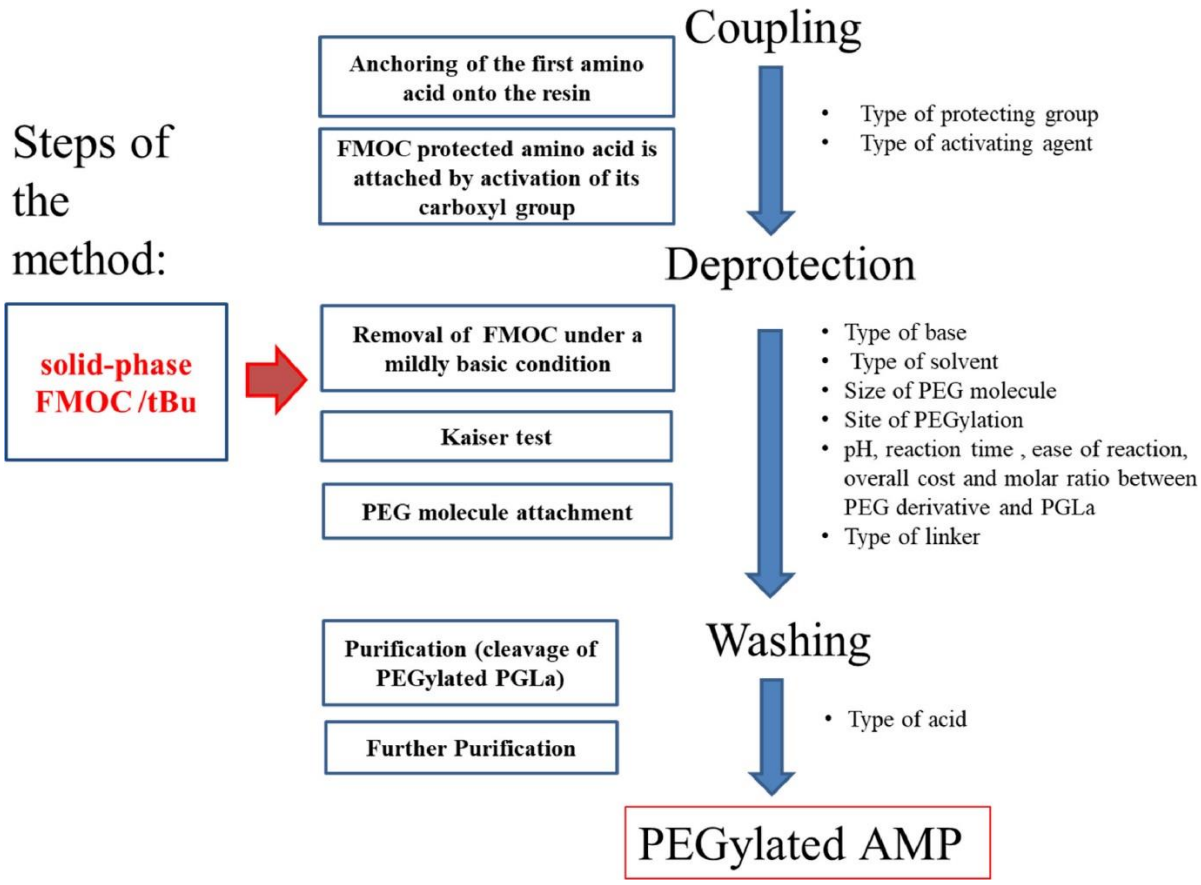

Figure 5. Flow-chart of solid-phase PEGylation process of the selected AMP (PGLa)

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 ***Figure 6***. 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 (***Figure 6A***). 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.

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				
CQA \ CPP/CMA		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 6. Interdependence rating results among the QTPP elements and CQAs (Part A), as well as among the CPPs and CQAs (Part B) and the results of the occurrence rating as steps of the RA.

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 ***Figure 6B***. 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 ***Figure 6C.*** Its interpretation is, that the size of the PEG molecule and PEGylation site are greater risk factors and have the highest occurrence potentials compared to other parameters. Both the size of PEG and the site of PEGylations are highly related to specificity and conjugate rate of PEG.

Figure 7 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. 7***).

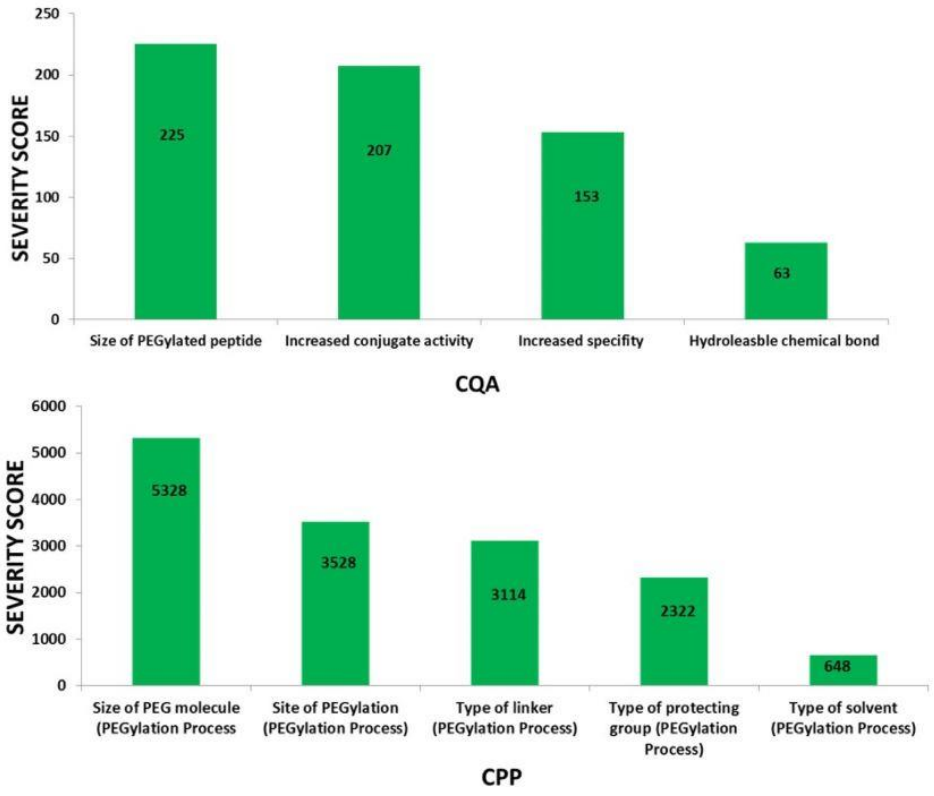

Figure 7. Pareto charts presenting the ranking of the selected CQAs and the CPPs as results of the RA.

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. The type of the protecting group and solvent have a lower effect. In ***Figure 8*** the most potential process factors, as CPPs with the highest estimated or relative occurrence and relative severity rate can be seen. ***Figure 8*** 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.

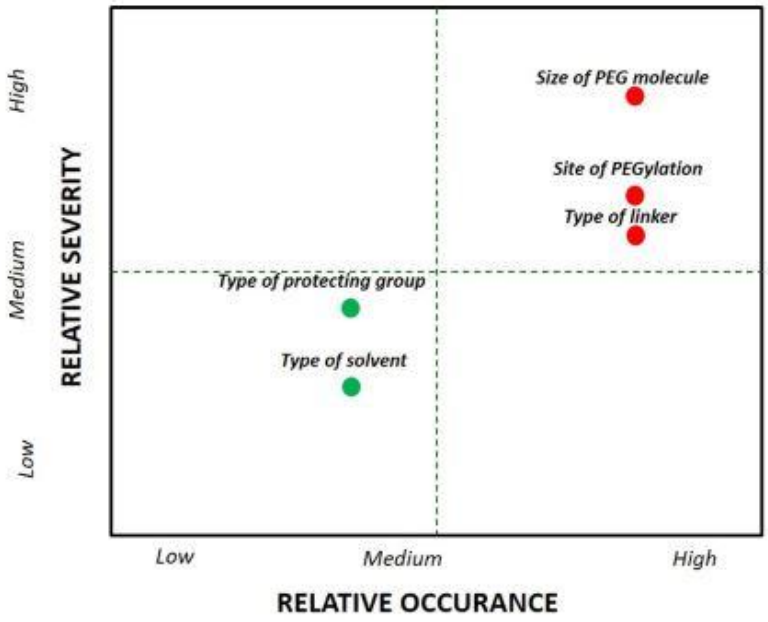

Figure 8. The relative occurrence and relative severity diagram of the CPPs.

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 PGLa 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

The enzyme activity was measured according the speed coefficient of degrading of *Micrococcus lysodeicticus* cell wall. In ***Table 4*** the enzyme activity results can be seen in the case of the all samples prepared according to factorial design. In all cases the enzyme activity was between 12.1 and 65.2 %. The highest value was at 10 pH (+1 level), 0.006% alginate concentration (+1 level), and with 1 h mixing time (-1 level).

Table 4. The enzyme activity results

Sample	Enzyme activity (%)
1	12.10
2	19.18
3	30.49
4	65.20
5	41.60
6	27.14
7	19.77
8	47.99

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 9***), 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:

$$y=32.92^{*}+7.94x_1+6.96x_2+1.20x_3+8.78x_1x_2-8.19x_1x_3-3.51x_2x_3 \qquad \textit{Eq. 3}$$

R^2=0.9838; MS Residual: 28.69

* statistically significant ($p<0.05$)

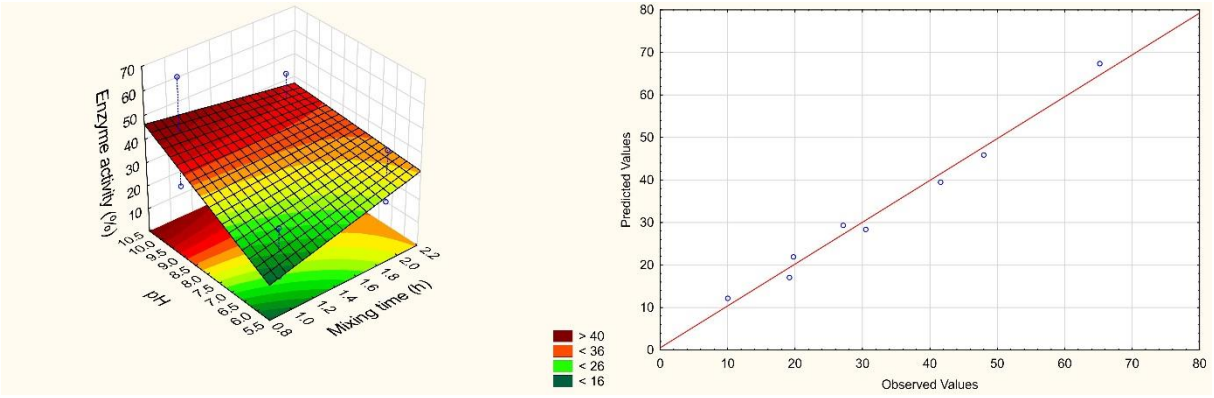

Figure 9. The response surface (alginate concentration on zero level) and the predicted values of enzyme activity

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.3*). 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 9***. 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 (***Table 5***). 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.

Table 5. The encapsulation efficiency results

Sample	Encapsulation efficiency (%)
1	65.17
2	65.76
3	65.65
4	65.87
5	62.98
6	64.21
7	63.64
8	66.35

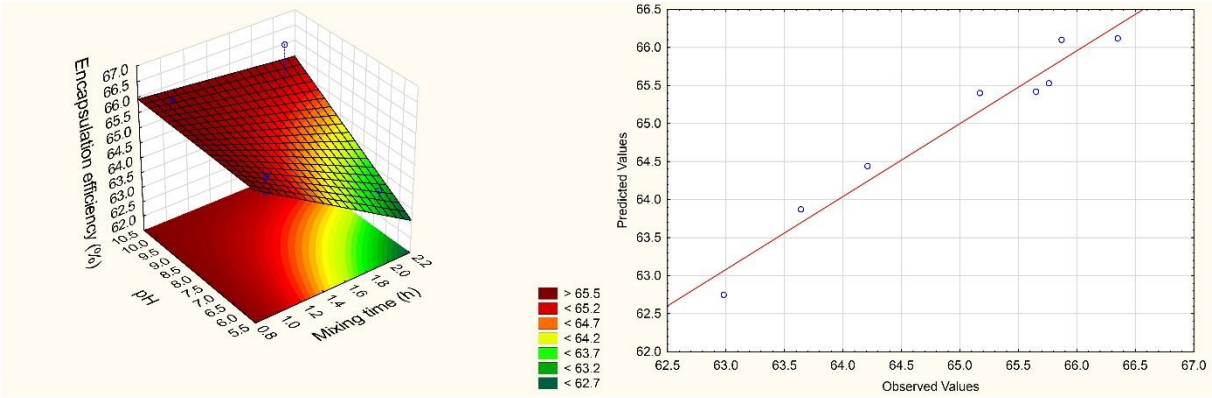

Figure 10. The response surface and the predicted values of encapsulation efficiency

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 10***). 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. 4*). 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.

The predicted and the observed values can be seen in ***Figure 10***. 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.

$$y=64.95^{*}+0.85x_1+1.19x_2-1.32x_3+0.28x_1x_2+0.55x_1x_3+0.78x_2x_3 \qquad Eq.\ 4$$

R^2=0.9867; MS Residual: 0.7875

4.3.3. Results of particle size and Zeta potential

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 (***Table 6***). 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.

Table 6. The particle size results

Sample	Particle size (nm)
1	185
2	170
3	184
4	207
5	164
6	168
7	165
8	177

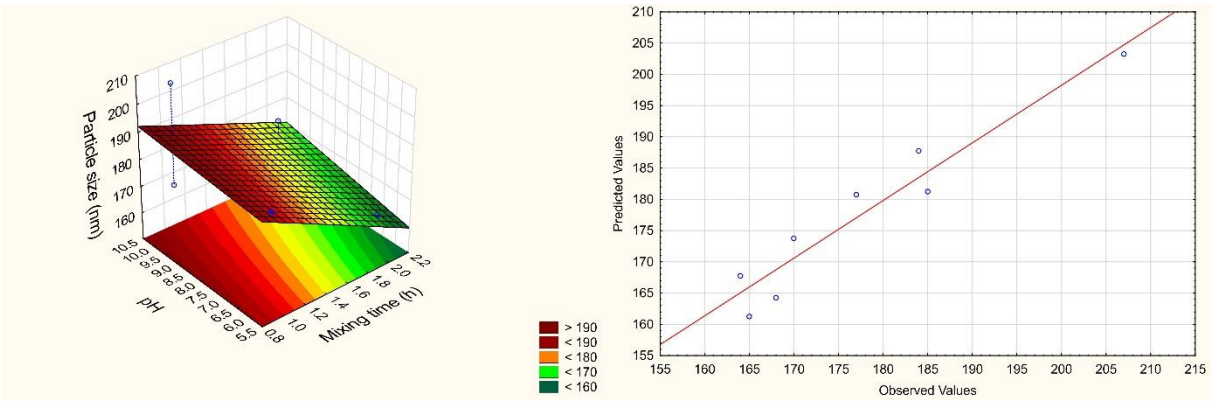

Figure 11. The response surface and the predicted values of particle size

It can be seen in ***Figure 11*** 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. 5*), 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. 5*) 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.

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 11*** 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.

$$y=177.5^{*}+5.75x_1+3.0x_2-9x_3+5.75x_1x_2-3.25x_1x_3+1.0x_2x_3 \qquad Eq.\ 5$$

$R^2=0.9226$; MS Residual: 112.5

The alginate layer on the surface of the precipitated LYS can be observed well (***Figure 12***). 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.

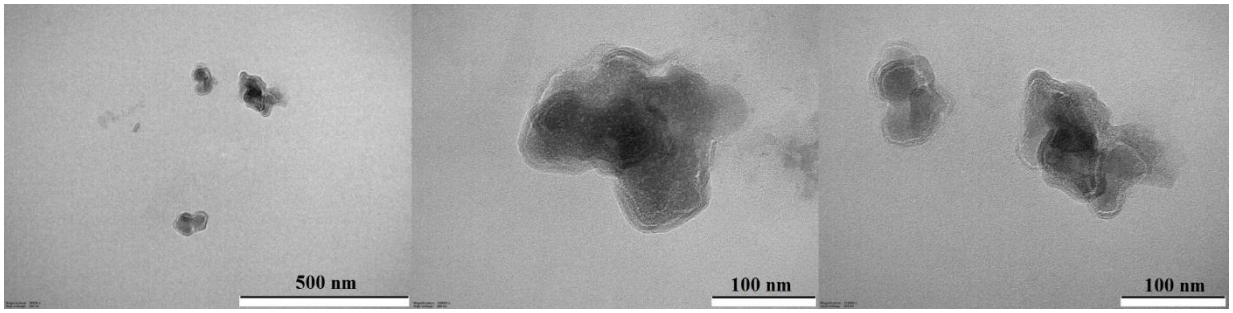

Figure 12. The TEM pictures of alginate layered NPs

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 13***). The amide I region can be found between 1700-1615 cm^{-1} [30].

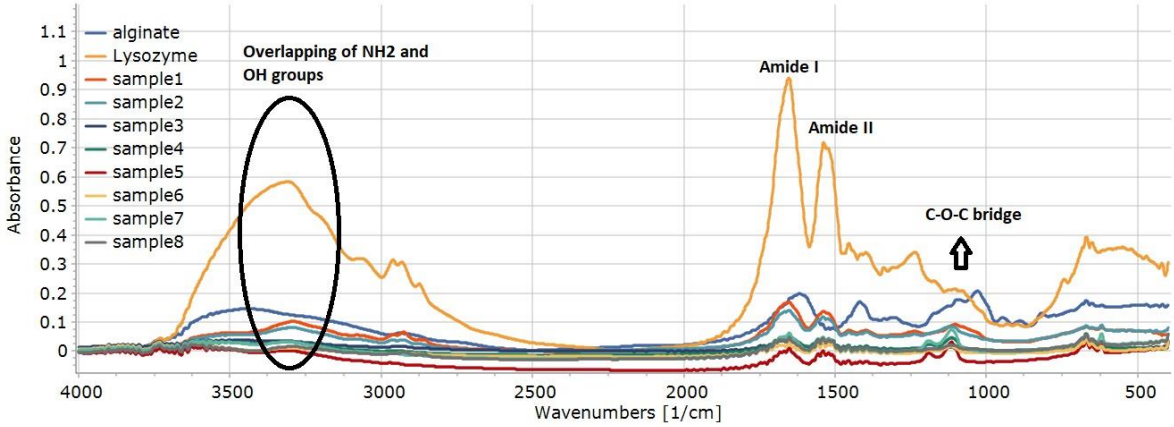

Figure 13. The results of FTIR spectroscopy measurements

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 14***. 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 ***Table 7*** 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% [97]; 34% in phosphate buffer pH 5.1 [98]; 40% in D_2O solution [99]; 30% in water [100]). 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 7)***. 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 15***).

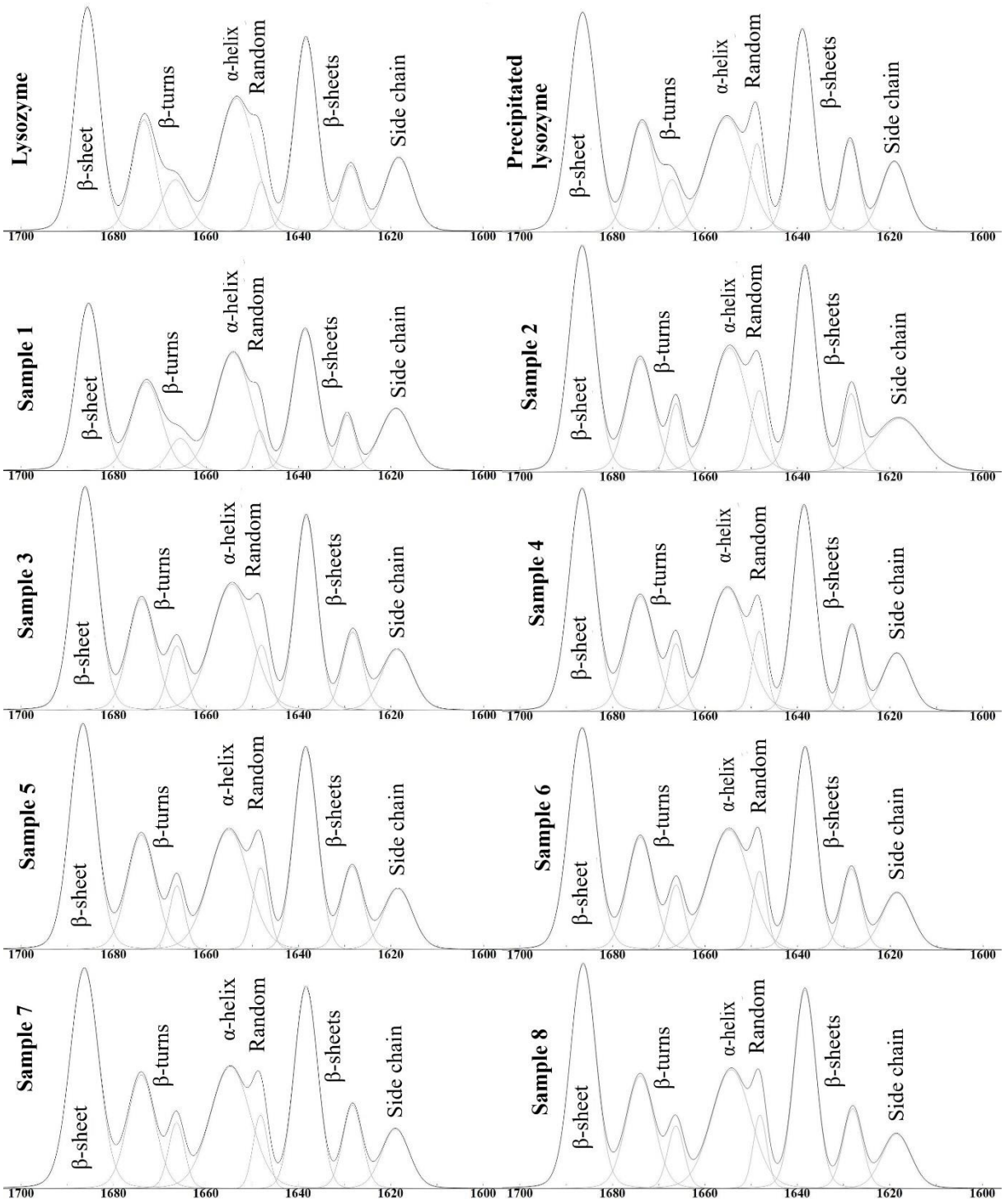

Figure 14. Deconvolution of infrared spectrum of LYZ, precipitated LYZ and the samples

Table 7. The α-helix content of the samples

Sample	Content of α-helix (%)
LYZ	22.69
Precipitated LYZ	19.66
1	13.76
2	22.61
3	20.37
4	22.05
5	19.13
6	21.16
7	20.94
8	21.25

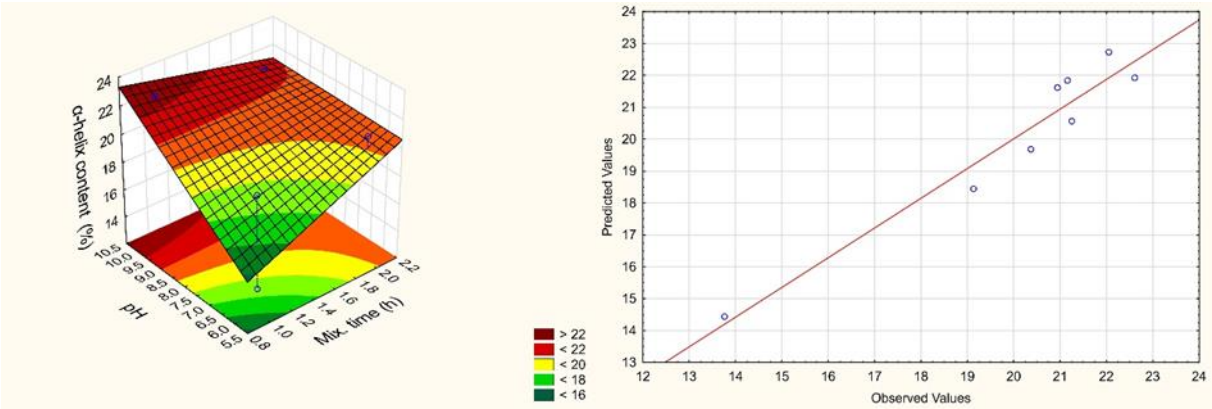

Figure 15. The response surface of α-helix content (alginate concentration on zero level) and the predicted value

This tendency correlates very well with the enzyme activity results (***Figure 9***). 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. 6), 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.

$$y=20.16^{*}+0.99x_1+1.61x_2+0.46x_3-1.11x_1x_2-0.52x_1x_3+1.02x_2x_3 \qquad \textit{Eq. 6}$$

4.3.5. CD spectroscopy

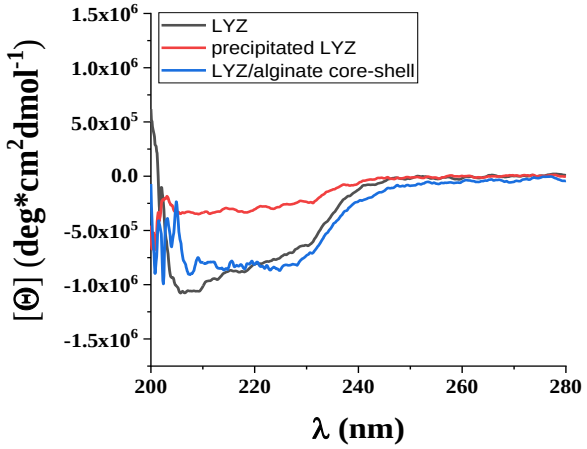

Figure 16. The results of CD spectroscopy

As shown in ***Figure 16***, the spectra of LYS NPs and alginate-LYS core-shell nanostructures consist of more disordered secondary structures than the initial LYS. 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 □-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

Many researches have been done with the aim of overcoming AMPs challenges. Different modification strategies as well as different delivery development ideas were offered by these researches. However, 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-shell 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 nanoparticles were analyzed with transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), and circular dichroism (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. NOVELTY AND 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. Therefore, we started to investigate the risk factors that influence the PEGylation process of PGLa by the application of the Quality by Design and after it we started another phase of our project; using LYZ as a good model protein and preparing NPs containing LYZ in a novel formulation strategy as layer-by-layer polyelectrolyte core-shell NPs.

- The integration of the Quality by Design (QbD) approach in the early pharmaceutical developments supports researchers in optimizing the targeted product by a risk-based manner. In the first phase of our work we presented a Quality by Design based antimicrobial peptide modification and formulation design.
    - ✓ 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 pI 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.

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APPENDIX

Related articles

I.

Recent insight into strategies for the design of antimicrobial peptides (AMPs)

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Abstract: With the increasing development of antibiotic resistance among key bacterial pathogens, there is an urgent need to discover novel classes of antibiotics. Although antimicrobial peptides (AMP) with their specific mode of action are considered major candidates for next-generation antibiotics, several challenges limit the use of these peptides for therapeutic applications.

In a large body of research, the focus is given to different approaches to the chemical modification of AMPs and how these modifications may improve the stability, antibiotic activity, proteolytic activity and prevent the cytotoxicity and side effects of AMPs. On the other hand, another group of research investigates the delivery of AMPs via nanocarrier systems as strategies used to enhance stability, control the release of peptides and reduce adverse peptide-related side effects, as well as improve their anti-microbial activities.

In the present article, we surveyed most recently published researches that provide us with good knowledge on structural features, mechanism of action, therapeutic aim, advantages and limitations, chemical modification approaches and carrying strategies of AMPs. Finally, according to Quality by Design, the most important potential effective factor and potential risk were mentioned in the development of AMP delivery systems.

Keywords: Antibiotic resistance, Antimicrobial peptides, Post-translational modification, Nanocarrier system, Quality by design

1 Introduction

Antibiotics are substances that treat infections by affecting bacteria through two main mechanisms: a bactericidal or a bacteriostatic one. Bactericidal antibiotics kill bacteria directly, while bacteriostatic antibiotics prevent their growth by inducing them into a stationary phase of growth [1,2]. *In vivo* as well as *in vitro* effectiveness, lack of toxicity and reasonable cost are vital features that antimicrobial agents must possess to provide an effective therapy [3]. The major concern about antibiotics is the ability of bacteria to develop resistance to them. This became one of the greatest challenges in the global health sector [4–6]. In the late 1960s and early 1970s, the significant success of antimicrobial drugs created a misleading belief that infectious diseases had been defeated. However, in the 2010s infectious diseases remained the second leading cause of death globally. Moreover, the emergence of antibiotic multi-resistance is increasing in different parts of the globe, thus creating a major concern as there are few or no treatments available for infections with certain microorganisms [7–9]. There are many factors that affect bacterial insensitivity to an antibiotic, including the spread of resistance genes and the over-prescription, overconsumption or misuse of antibiotics [10–12].

Since the global public healthcare system has been threatened by antibiotic-resistant bacteria, numerous researchers proposed to obtain new alternative agents which are called antimicrobial peptides (AMPs) and display a low rate of resistance development [13–15].

2 Antimicrobial peptides

AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation methods. A schematic representation of different classifications of AMPs is shown in *Figure 1.* According to our present knowledge, this is a new overview of various AMP classifications.

2.1 Definition, history, source, target and mechanism of action

The relatively small size of antimicrobial peptides (<10kDa) made their isolation possible only in the 1980s. This was initially achieved in frogs, insects and granules of human and rabbit granulocytes. Since then, a large number of additional antimicrobial peptides has been found virtually everywhere in nature, amounting to over 2500 peptides known at present [16,17]. Antimicrobial peptides seem to have effector functions in innate immunity and can upregulate the expression of multiple genes in eukaryotic cells [18]. They represent a wide range of short, cationic or anionic, geneencoded peptide antibiotics. Despite sharing a few common features (such as cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups have been proposed to incorporate the diversity of the observed AMP conformation. As shown in *Figure 1*, AMPs are produced by bacteria and eukaryotes, such as protozoa, fungi, plants, insects and several types of vertebrate and invertebrate animals. They show a variety of targets, including Gram-positive and Gram-negative bacteria, parasites, fungi and some viruses [19–23]. AMP genes are present in the genetic material of a number of mammals. The expression of these genes has been detected in different cells, including neutrophils, monocytes, macrophages, epithelial cells, keratinocytes and mast cells. AMPs are synthesized as pre-pro-peptides and a post-translational process allows their final maturation into active peptides [24,25].

An insight into the mechanism of action of AMPs is essential for the further development and design of optimized AMPs that could be efficiently used as therapeutic drugs. Thus a broad range of researches are assigned to study the mechanism of action of AMPs [26–28]. According to these researches, AMPs are divided into two main groups based on their mode of action: membrane disruptive AMPs and non-membrane disruptive AMPs [29].

2.2 Structural and physicochemical features

The antimicrobial activity and selective toxicity of AMPs are significantly influenced by their structural and physicochemical features. Furthermore, studying different structural parameters of AMPs is a vital part of the design and development of novel antimicrobial agents with enhanced antimicrobial activity [30].

2.2.1 Conformation (X)

Based on secondary structures assumed by AMPs in the presence of other biological membranes, they are categorized into different conformations, such as α-helix, β-sheet, extended helix and loop (*Figure 1 and 2*) [25–27].

Circular Dichroism (CD), X-ray crystallography and Nuclear Magnetic Resonance (NMR) studies are commonly used to determine the secondary structure of these peptides [30,31] The α-helical AMPs, including cecropin and pexiganan, tend to form amphipathic helices in certain organic solvents, such as trifluoroethanol. These α-helical AMPs disturb the bacterial membrane by employing various mechanisms of action, including the formation of barrel-like bundles (barrel-stave model), carpet-like clusters (carpet model) and toroidal pores (toroidal pore model) into the membrane.

AMPs with **β-sheet structure**, such as α-, β-defensins and protegrin, form β-hairpin structures stabilized by disulfide bridges. Most of the β-sheet AMPs have a rigid structure and the bacterial membrane is disturbed by a perpendicular insertion into the lipid bilayer and the formation of toroidal pores.

Mixed structure ($\alpha\beta$-peptides) AMPs, such as bactenecin, adopt a loop formation with one disulfide bridge [32,33].

The **extended AMPs**, which are rich in specific amino acids, have irregular secondary structures. Many of these peptides show antimicrobial activity only after interacting with the membrane and undergoing consequent conformational changes. Indolicidin with 13 amino acids, a member of this group of AMPs, contains five tryptophan and three proline residues. The peptide adopts a poly-L-II helical structure in the presence of liposomes, and the high content of tryptophan residues is responsible for their interaction with lipid membranes.

2.2.2 Charge

Many of the antimicrobial peptides display a net positive charge, ranging from 2 to 9, and may contain highly defined cationic domains. Cationicity is essential for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms [30,34–38]. However, this relationship is not fully linear. Within a certain range,

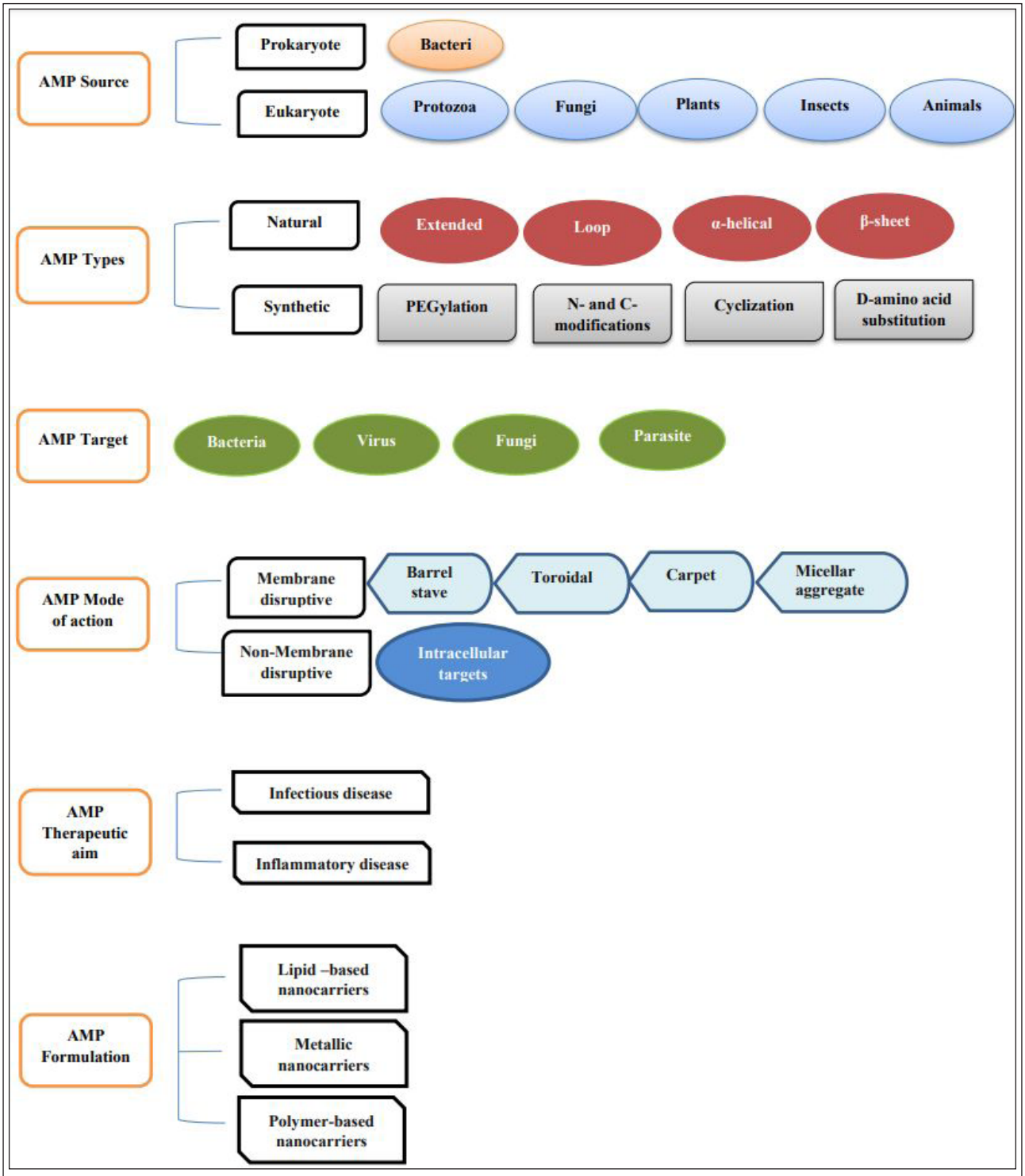

Figure 1 Schematic representation of different classifications of antimicrobial peptides. AMPs can be categorized based on their source, target, structure, mechanism of action, therapeutic aim, modification and formulation type

increasing peptide cationicity is generally associated with increasing antimicrobial strength. For instance, studies with magainin 2 analogs show that increasing the charge from 3 to 5 results in increasing antibacterial activities against Gram-negative and Gram-positive pathogens. However, a net charge from 6 to 7 leads to an increase in the hemolytic propensity and to a loss of antimicrobial activity. Therefore, it can be concluded that there is a risk beyond which increasing the positive charge no longer increased the activity of AMP [32]. Although in a wide majority of cases

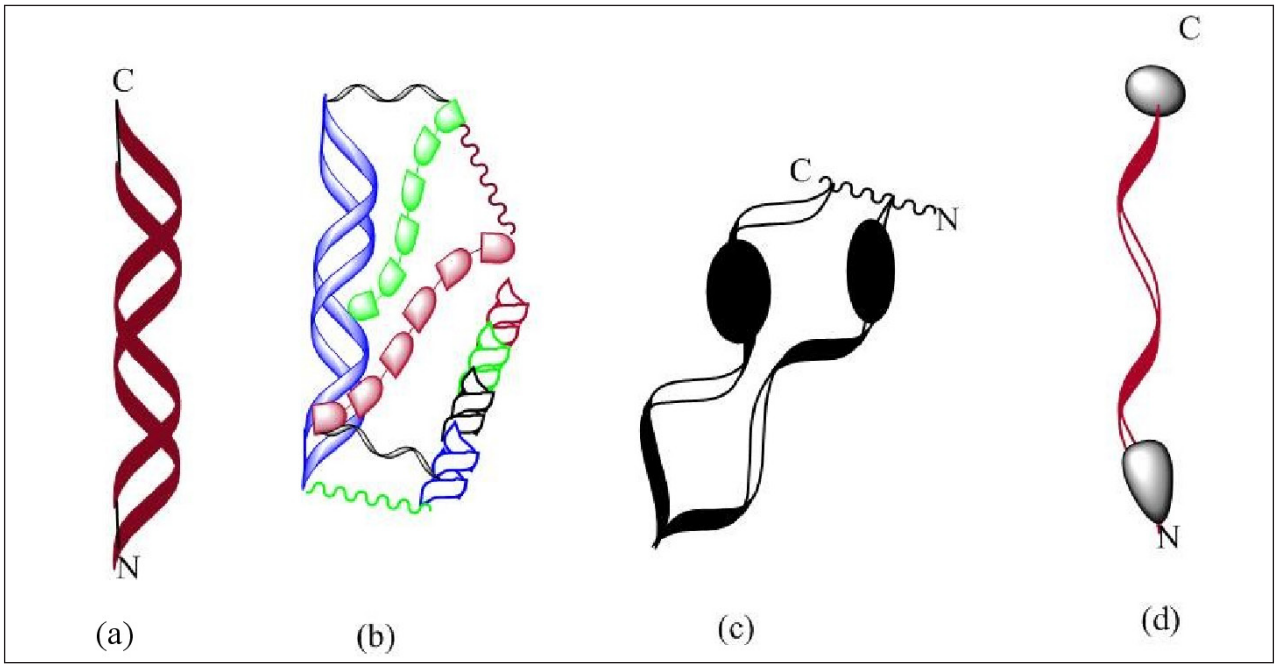

Figure 2 The four structure classes of AMPs. (a) α-helical (LL-37), (b) β-sheet or loop or turn (Protegrin-1), (c) $\alpha\beta$-peptides (mixed-structure) (Microcin J25) and (d) extended (non-$\alpha\beta$-peptides) (Indolicidin)

AMPs are cationic, anionic AMPs (AAMPs) have also been described as an integral and important part of the innate immune system and increasingly identified in vertebrates, invertebrates and plants over the last decade [39]. While cationic peptides are rich in arginine and lysine, AAMPs are small peptides rich in glutamic and aspartic acids. AAMPs that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge [40].

2.2.3 Amphipathicity (A) and hydrophobicity (H)

In a research by Mihajlovic *et al*., the amphipathicity of antimicrobial peptides plays a crucial role in pore formation and can also contribute to a better understanding of the mode of action in antimicrobial peptides [41,42]. Kondejewski *et al*. have reported that the antimicrobial activity and toxicity of peptides are notably enhanced with amphipathicity. However, an extremely amphipathic nature is not desirable in cyclic AMPs since it decreased the specificity and increased interactions with outer membrane components [43].

Hydrophobicity is a main feature for the effective membrane permeabilization of AMPs as it determines the extent to which a peptide can partition into the lipid bilayer. However, an increase in the levels of hydrophobicity is strongly related to mammalian cell toxicity and loss of antimicrobial specificity. Therefore, moderate hydrophobicity is needed against the bacterial membrane [32]. A research by Wood *et al*. on a linear cysteine-deleted tachyplesin (CDT), examined the effect of hydrophobicity on antimicrobial activity. Analogs with hydrophobic isoleucine residues placed throughout the sequence of CDT showed comparable antimicrobial activity to CDT but lower hemolysis [44].

AMPs with moderate features (charge, hydrophobicity, amphipathicity) and a good balance between these characteristics showed higher antimicrobial activity and lower cytotoxicity and hemolysis in mammalian cells [36,45].

2.3 Therapeutic aim

As shown in *Figure 1* and *Table I*, AMPs can be categorized based on their therapeutic aim. 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 [46,47]. According to the results of a research project, the sustained release of drugs at the site of action presented excellent results in the treatment of chronic wounds [48]. In the table below, the association between some of the most common AMPs with different conditions, including infectious and inflammatory diseases, is listed and classified

Table I *Some of the most recent researches showing various AMPs and their administration routes effective in the treatment of acute or chronic diseases*

Peptide	Disease	Chronic/Acute	Administration Route	Ref.
Rhesus theta defensin-1 (RTD-1)	Acute lung injury (ALI)	Acute	Parenteral	[51].
Cathelicidin LL-37	Acute thrombosis			[52]
Catestatin (CST)	Acute and chronic pain			[53]
Human beta defensins 1 (HBD1)	Acute HIV-1 infection			[54]
Human cathelicidin (hCAP18/LL-37)	Chronic obstructive pulmonary disease (COPD)	Chronic	Inhalation	[49]
HBD1, HBD2, HBD5 and HBD6	Crohn's disease (CD)		Oral	[50]
hBD-3	Wound		Dermal	[55]
β defensin	Chronic rhinosinusitis (CRS)		Nasal	[56]

into two groups of acute and chronic diseases. Based on literature review, AMPs which are used to treat chronic diseases should provide controlled and sustained release by choosing the proper administration route, while an immediate release formulation of AMPs is effective for acute diseases. Thus the therapeutic aim of AMPs has significant influence on formulation parameters in the delivery of these peptides, and depending on the medical application of AMPs, the drug release profile and therefore the administration route are different [49,50].

3 Advantages and limitations of AMPs

In relation to small molecule drugs, 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. Generally, even synthetic peptides are less immunogenic than recombinant proteins and antibodies. Among different peptides, AMPs emerged as essential tools with a broad-spectrum of activity and a low rate of resistance development [34,57]. Besides the mentioned advantages, AMPs have limitations, such as low metabolic stability and low permeability across biological barriers, high costs and poor relevance of antimicrobial activity of AMPs *in vivo* and *in vitro*, cytotoxicity and difficulty in reaching targeted sites at active concentration due to degradation. In the next chapter, it is shown that these initial barriers are being increasingly overcome with new chemical modification strategies for the development of stable, more cost-effective and potent broad-spectrum synthetic peptides [58]. The following figure shows the critical points to consider during formulation to obtain a good quality product (Figure 3).

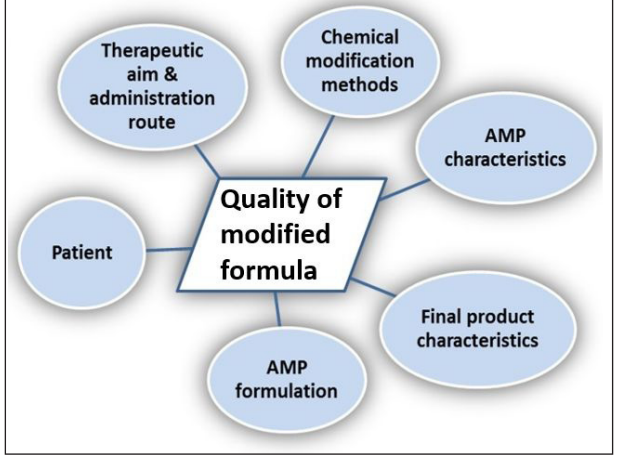

Figure 3 Critical points to consider during formulation

4 Post-translational /chemical modifications of AMPs

Strategies such as N- and C-modifications, incorporation of non-natural or D-amino acids, cyclization and attachment of the polyethylene glycol polymer to peptides (PEGylation) allowed several researchers to enhance the bioavailability of AMPs and improve both their bio-distribution and rate of clearance. Polyethylene glycol (PEG) protects peptides from serum proteases and releases them in a traceless fashion with full bioactivity. PEGylated peptides display a longer circulation time, in which water solubility, stability, resistance, biocompatibility, minimal toxicity and immunogenicity of the peptide are improved [59]. The proteolytic degradation of peptides can be decreased by protecting their C- and N-terminus with acetylation or amidation. Also, modifying

Table II *Some of the most recent researches showing various AMPs, their target, their administration route, different carrier systems for loading them and their advantages, risks and perspectives of further development*

Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Esculentin-1a, Esc(1-21)	*P. aeruginosa* (treatment of epithelial infections and healing of the injured tissue)	Soluble AuNPs covalently conjugated to AMP ***via*** a poly(ethylene glycol) linker	- Highly enhanced antipseudomonal activity - Preserved mode of action of the free peptide, without being toxic to human cells - Accelerated recovery of an injured skin layer - Resistant to proteolytic digestion - Biocidal against a wide range of microbial pathogens - Ease of AuNPs synthesis - Biocompatibility	- Possibility of inefficient delivery of AMPs to the target infectious site - Lack of information on the basic rules governing molecular interactions between such coated-AuNPs and cells or complex tissues	[82]
Ubiquicidin 29–41 (UBI)	*E. coli,* *P. aeruginosa*	Engineered multivalent silver nanoparticles functionalized with UBI	- Enhanced antimicrobial activity - High affinity and selectivity towards bacterial infection - Synergic effects against microorganisms	- Microbicidal effects are limited to certain types of microorganisms - Lack of understanding of the structure of multivalent nanoparticles and stabilization mechanisms	[83]
LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*E. coli,* *S. aureus*	Peptide-loaded mesoporous silica nanoparticle	- Antimicrobial effects can be controlled in peptide-loaded mesoporous silica nanoparticle systems	- In the case of positive charge, mesoporous silica nanoparticles can cause toxicity against the human erythrocytes	[85]
LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*E. coli,* *S. aureus*	Mesoporous silica containing LL-37	- Potential as an implantable material or surface coating - Controlling implant-related infections, e.g., for multiresistant *S. aureus*	- Low toxicity	[86]
HHC-36 (KRWWKWWRR)	*S. aureus*	HHC-36 loaded self-organized, vertically oriented titanium TiO_2 nanotube	- Slow release profile from 4 hours up to 7 days - It can be applied on the surface of implants as locally delivered antimicrobial agent for peri-implant infections	-	[87]
Indolicidin		Carbon nanotube-indolicidin conjugate	- It can improve the efficacy of indolicidin at 1000-fold less concentration than the free indolicidin	- In the future it will be tested in animal model	[88]
TP359, TP226 and TP557	*S. aureus*	AMP-functionalized silver-coated carbon nanotubes	- Non-toxic - Help reduce the infection on the skin model	- Further evaluating the antibacterial potential of AMP-functionalized silver-coated carbon nanotubes in a time-dependent manner (longer incubation times)	[89]

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Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Cationic peptides	*S. aureus*	Fullero-peptide containing cationic AMP with solid-phase synthesis	- Fullero-peptides could be easily purified and tested for their biological activity	- The resins have shown a strong tendency to retain the fullerene-based peptides, it can be a problem during the removal step	[90]
Leucine-rich antimicrobial peptide (FALALKALKKALKKLKKALKKAL)	*E. coli*, *S. aureus*, *MRSA*	Paramagnetic nanoparticles encapsulated in cationic liposomes	- Enhanced hemocompatibility and antimicrobial activity - Liposomal envelope protects the cargo against unwanted interactions with the environment resulting in the prolonged persistence of the cargo in the body	- Toxicity risks and adverse effect of advanced nanomaterials on the therapeutic index of peptides	[93]
Nisin Z	*S. aureus*, *S. epidermidis*	Nanostructured lipid carriers (NLCs)	- Enhanced stability, solubility, antimicrobial activity, biodegradability and selectivity of peptide towards bacterial cells - Synergism was observed for the combination of nisin Z with conventional antibiotics - No toxicity in mammalian cells	- No activity against Gram-negative bacteria. However, the activity towards Gram-negative bacteria can be enhanced by using the chelating agent ethylenediaminetetraacetic acid (EDTA). Therefore more studies are required on incorporating nisin Z and EDTA in NLCs simultaneously and testing the effectiveness *ex vivo* and *in vitro* for topical application	[94]
Human cathelicidin LL-37	Infection, immunity and wound repair	pH-tunable nanocarriers named nanobiointerfaces (OA/LL-37 self-assemblies)	- Enhanced solubility and antimicrobial activity - Protection of the peptide from degradation by partitioning into the hydrophobic or the hydrophilic sections of the self-assemblies, or by localization at their water-lipid interfaces - Directing the antimicrobial activity to the affected tissues, while minimizing toxicity	- Limitations in controlling and triggering self-assembly	[95]
GIBIM-P5S9K	*E. coli*, *MRSA*, *P. aeruginosa* (infectious diseases caused by resistant microorganism)	PLA and PLGA NPs	- Enhanced antimicrobial activity - Protection of peptide against degradation - Slow release - Biodegradability - Biocompatibility - No toxicity - Hemocompatibility	- Limitations of natural polymer NPs, such as risk of purity variation - Further modification of PLGA-NPs can control the structure, encapsulation degree, administration route, drug release and degradation rate	[97]
S16 and S32	*ESKAPE* group *MDR* and *CMDR*	Star-shaped peptide polymer nanoparticles synthesized via ring-opening polymerization	- Enhanced antimicrobial activity - No resistance acquisition by CMDR bacteria - Low toxicity - Low-cost - Selectivity of the peptides towards pathogens over mammalian cells - Applications in nanomedicine, particularly in the fields of gene therapy	- Lack of information on the exact mechanism of membrane disruption	[98]

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Peptide name	Target /disease	Nanocarrier system	Advantages	Risks and future perspective	Ref.
Colistin	*P. aeruginosa*	PLGA nano-embedded microparticles containing colistin	- It displays prolonged efficacy in biofilm eradication compared to the free colistin	- It can be a novel antimicrobial formulation for *P. aeruginosa* lung infection in cystic fibrosis patients	[99]
Nisin	*E. coli, Listeriamonocytogenes*	Nisin/g-PLG nanoparticle	- The release of nisin from the nanoparticles was pH-dependent	- It could be a promising food preservative	[100]
APO (All peptides optimized, name of designer antimicrobial peptide), colistin	*Acinetobacter baumannii*	APO monomer-impregnated nanofiber dressing	- It resulted in significantly reduced wound size and wound bacterial load	- It can be developed as an economical first-line treatment option to skin injuries in general	[101]
ε-poly(L-lysine) (ε-PL)	*S. aureus, S. epidermidis, E. coli*	ε-PL functionalized poly(acrylic acid) /poly(vinyl alcohol electrospun nanofibers	- It did not display cytotoxicity to human corneal epithelial cells - EC10 and EC50 in the order *S. epidermidis* > *S. aureus* > *E. coli*	- In the future it can be used in biocompatible nanofibrous dressings with durable antibacterial and antifouling efficiency, and potential application as wound dressings or other medical uses	[102]
Novicidin	*E. coli, S. aureus*	Octenyl succinic anhydride-modified hyaluronic acid nanogels loaded novicidin	- It is colloidally stable in a physiological ionic strength buffer - It displays sustained release over 12 days - Reduced cytotoxic effects - Relatively high drug load	- In the future other amphipathic AMPs may also be successfully applied with this method	[104]
LLKKK18	*M. avium, M. tuberculosis*	Self-assembling hyaluronic acid nanogels loaded LLKKK18	- Intratracheal administration of nanogel significantly reduced infection levels in mice after just 5 or 10 every other day administrations	- It may hold great potential as an alternative approach to control tuberculosis and other mycobacterioses	[105]
Poly(Lys-Ala) polypeptides	*E. coli, S. aureus* (wound healing)	Cell-adhesive hydrogel (formed by cross-linking of poly$(Lys)_{60}$-$(Ala)_{40}$ and 6-arm PEG-ASG)	**-** Enhanced cell adhesion and proliferation accelerating wound healing - Enhanced antimicrobial activity - Ease of the hydrogel synthesis - Inherent antibacterial activity of the hydrogel - Low cost	- Lack of kinetic studies on release profile - Further studies are in progress to enhance cell adhesion through the incorporation of additional biological moieties	[106]
Tet213	*S. aureus*	RADA16-AMP self-assembling peptide hydrogel	- The synthetic process was simple without the limitation of time or temperature - It could be used conveniently and easily for patients	- It could be used as a promising material for bone infection and osteomyelitis treatment	[108]

the sequences of peptides by the substitution of natural L-amino acids for their D enantiomers, α/β-substituted α-amino acids or even β-amino acids are other similar approaches that result in overcoming peptide hydrolysis. D-amino acid substitution in a peptide may influence not only the stability of the peptide but also its secondary structure and therefore its ability to incorporate into membranes [60–63]. It is worth mentioning that in addition to the modification strategies described in this review article, the use of other types of AMP modification, such as computer-assisted methods, has been increasing significantly[64].

4.1 N- and C-modifications

In a new finding by Kuzmin *et al.*, N-terminal acetylation and C-terminal amidation significantly increased the stability and hemolytic activity of the modified AMP in human serum. In addition, the hemolytic activity and specific and non-specific cytotoxicity of the peptide increased [65]. In another research, different series of branched tetramers of a proline-rich antimicrobial peptide (PrAMP) named Chex1-Arg20 was studied against a number of Gram-negative nosocomial pathogens. C-terminal PrAMP hydrazidation together with its tetramerization resulted in both broad-spectrum antibacterial selectivity and potency of PrAMP action [66]. The relationship between AMP property and chemical modification indicates peptide engineering. Depending on the aim of our design, peptides could be modified with various methods so that the desired features can be enhanced whereas side effects can be reduced [67].

4.2 Cyclization

Cyclization of the linear peptide $HAfp^{1\text{-}23}$_KK resulted in a cyclic peptide with considerably improved antibacterial activity and minimum inhibitory concentration (MIC) value against multidrug-resistant *Pseudomonas aeruginosa* **(***P. aeruginosa***)** and methicillin-resistant *Staphylococcus aureus* (*S. aureus*). The designed $cHAfp^{1\text{-}23}$_KK also exhibited very low cytotoxicity with respect to its MIC values determined against different bacteria [68]. In another new study, C-MPI-1 and C-MPI-2, cyclic analogs of a natural AMP named Polybia-MPI (MPI), were synthesized by the click chemistry approach. As a result, C-MPI-1 showed improved stability against trypsin in comparison with the parent peptide. Moreover, MPI displayed sustained antimicrobial activity while C-MPI-2 displayed no antimicrobial activity [35]. Cyclization can show significant effects on enhancement of antibacterial activity, MIC value, stability and cytotoxicity of AMPs. However, there is a risk of losing the activity of the peptide after the cyclization of AMPs. With the application of cyclisation, Chan *et al.* could improve stability and enhance cell toxicity against a cancer cell line without toxicity on a noncancer cell line and they could improve the antimalarial effect of gomesin [69].

4.3 D-amino acid substitution

D-amino acid substitutions can result in antimicrobial peptides resistant to proteolysis [63,70]. A recent study shows that a proline-rich antimicrobial peptide, A3-APO (a discontinuous dimer of the peptide Chex1-Arg20), underwent degradation to small fragments at positions Pro6-Arg7 and Val19-Arg20. To minimize this degradation, a series of Chex1-Arg20 analogs were prepared via Fmoc/tBu solid phase peptide synthesis with D-arginine substitution at these sites. The activity of the peptides decreased against Klebsiella pneumonia by the replacement of arginine at position 7, while substitution at position 20 did not greatly affect the activity. Moreover, none of these peptides showed any cytotoxicity to mammalian cells. These findings can result in the development of more effective and stable peptide analogs with further substitution at position 20 [71]. Thus depending on the position of substitution, D-amino acid substitution can result in the sustained or decreased antibacterial activity of AMPs. Moreover, this type of modification is an effective means for decreasing the cytotoxicity of AMPs. Jia *et al.* applied D-amino acid substitution to improve the stability of polybia-CP. The results demonstrated that all D-amino acid derivatives and partial D-lysine substitution derivatives have improved stability against trypsin and chymotrypsin[72].

4.4 PEGylation

A proline-rich AMP named Bac7(1e35) (which protects mice against *Salmonella typhimurium* infection) was linked to a PEG molecule via a cleavable ester bond or via a non-hydrolysable amide bond. Both PEGylated derivatives exhibited the same mode of actions. However, the antimicrobial activity of the releasable conjugate is higher than that of the stably linked one. Moreover, both derivatives exhibited a lower clearance rate and wider distribution in mice compared to the unmodified peptide [73]. Although the PEGylation of peptide drugs prolongs their circulating lifetimes in plasma, it can mask the binding site in peptides and therefore extremely reduce the activity of the peptide (PEGylation of biopharmaceuticals). Thus, in order to circumvent the conflicting effects of PEGylation, different strategies such as changing the size and the location of the PEG molecule were offered in several researches. For instance, an anti-

microbial synthesized peptide named MA was modified with low molar mass PEG chains. The PEGylated peptides self-assembled in aqueous solution into micelles with a PEG shell and a peptide core, resulting in the increased photolytic stability of the AMP due to the shielding protection of the core peptide by the PEG shell, leading to the increased antimicrobial activity and the decreased hemolytic activity of AMP [74]. In another recent research project, by using the PEG-Linker-Drug strategy, the linker sequence can be optimized for a given therapeutic peptide named Onc112 providing release rates from <1 h to >40 h [75]. It can be concluded that PEGylation significantly increases the half-life of AMPs, with potential improvement in bioavailability and distribution but without adversely affecting the binding and the activity of peptides. Factors such as the molecular weight of PEG molecules and the type of linkage for the attachment of PEG to AMPs improve the performance of PEGylated AMPs and overcome limitations such as masking the binding sites of peptides by PEG molecules. Thus the advantages of PEGylation outweigh its limitations and make it a promising method of modification. Most PEGylated proteins are designed with a covalent bond between the PEG molecule and the protein or peptide. In many cases the PEGylation method can cause decreased functional activity. Therefore the releasable PEGylation method was developed. In this case, the PEG molecule can be released over time in the circulation and the therapeutic protein can keep its activity [76]. Gong *et al.* prepared releasable PEGylated arginine-rich AMPs. These conjugates were insensitive to serum proteases and the AMP could be released with fully functional activity [77].

5 Strategies for AMP delivery

The diversity of novel formulations within the limits of nanotechnology may also provide novel applications going beyond antimicrobial activity [78,79]. In the following, some of these researches using different nanocarrier systems are discussed. More evaluations on the advantages and risks related to each approach are listed in *Table II*.

5.1. Inorganic nanoparticles

5.1.1 Metallic nanoparticles

AMP delivery strategies can be implemented with inorganic materials, one of which is gold nanoparticles (NPs) (Figure 4). Gold nanoparticles can be functionalized (Figure 5) by beta-amyloid peptides or pentapeptide fragments [80]. On the other hand, metallic NPs by themselves are known to have antimicrobial activities and thus disrupt the bacterial cell membrane and cause cell penetration or react with intracellular targets and cause toxicity. The immobilization of AMPs to metallic nanoparticles might therefore represent an alternative solution in the fight against antibiotic resistant pathogens and could also improve the antimicrobial activity of both components. Moreover, immobilization to nanoparticles could also help to overcome some limitations of AMPs, such as susceptibility to proteases

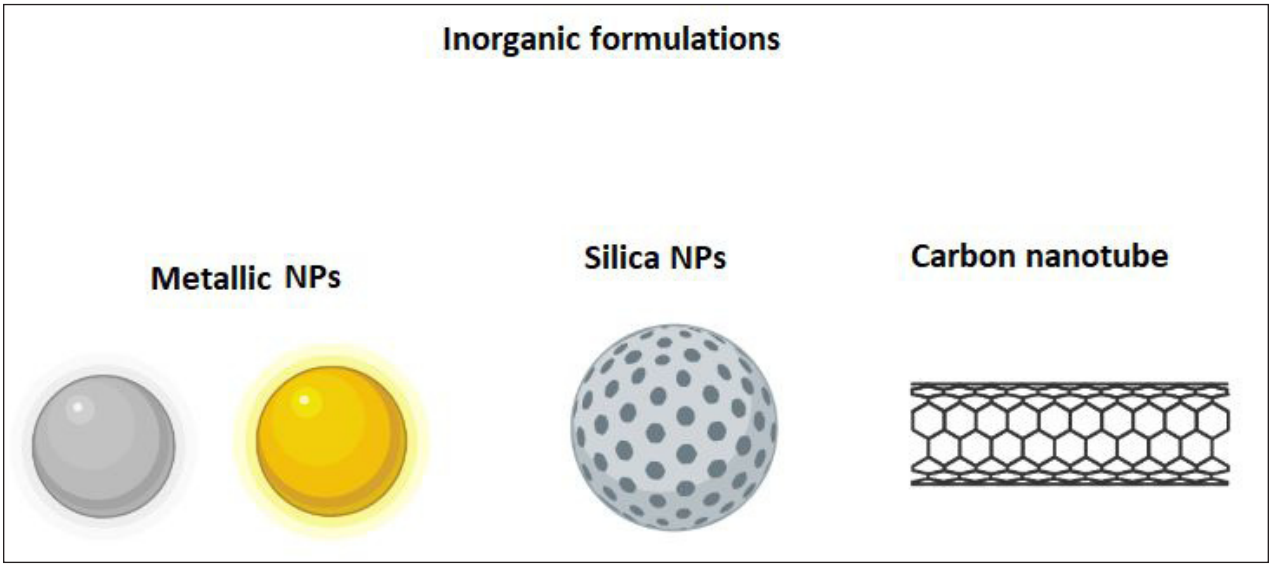

Figure 4 Inorganic formulations (Created with BioRender.com)

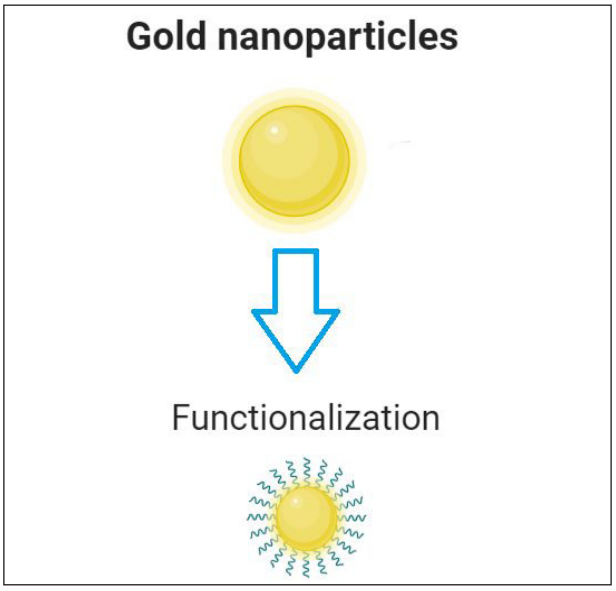

Figure 5 Functionalization of gold nanoparticle (Created with BioRender.com)

and poor permeability across biological barriers [81,82]. A derivative of the frog skin AMP esculentin-1a, Esc(1-21), covalently conjugated to soluble AuNPs *via* a poly(ethylene glycol) linker, results in a significant rise in the activity of AMP against the motile and sessile forms of *P. aeruginosa* without being toxic to human keratinocytes. Moreover, the peptide displayed more resistance to proteolytic digestion and disintegrated the bacterial membrane at a very low concentration. Wound healing activity on a keratinocyte monolayer is another advantage of engineered AuNPs [82]. In a recent research, an increase in antibacterial activity against Gram-negative bacteria appeared when silver nanoparticles were capped with cationic antimicrobial peptide ubiquicidin 29–41 (UBI). This probably results from the multimeric or polyvalent arrangement of ligands distributed on the metallic NP surface [83].

5.1.2 Silica nanoparticles

Silica NPs can be appropriate carriers of AMPs because they have well-defined mesopores in nm range, are chemically stable and relatively biocompatible [84]. Braun *et al.* found that the surface charge of mesoporous silica NPs strongly influence the loading of the LL-37 AMP into mesoporous silica NPs and the release onto the surface [85]. Izquierdo-Barba *et al.* developed a novel one-pot evaporation induced self-assembly method for the preparation of mesoporous silica reservoir, which can be applied for encapsulating antimicrobial agents and AMP (LL-37) [86].

5.1.3 TiO_2 nanotube, carbon nanotube

Ma *et al.* established that TiO_2 nanotubes can be used as potential carriers of AMPs. The AMP (HHC-36) was loaded into the TiO_2 nanotubes with vacuum-assisted physical adsorption. The AMP-loaded TiO_2 decreased the adhesion of *S. aureus* on the surface and could kill bacteria significantly [87]. Sur *et al.* prepared carbon nanotube-indolicidin and gold nanoparticles-indolicidin conjugates and these conjugates were found to be able to improve the efficacy of indolicidin at 1000-fold less concentration than the free indolicidin [88]. Chaudhary *et al.* prepared silver-coated carbon nanotube functionalized with AMP (TP359, TP226 and TP557). They investigated toxicity, morphology with scanning electron microscopy and antibacterial activity against *S. aureus*. The results showed that the silver coated carbon nanotube functionalized with antimicrobial peptides was non-toxic and helped reduce the infections [89]. Pantarotto *et al.* successfully performed the solid-phase synthesis of fullero-peptides containing cationic AMP. They have specific activity against Gram-positive bacteria and can therefore be anti-infective agents [90].

5.2 Organic materials

5.2.1 Lipid structures

MP delivery strategies can also be implemented using organic materials. One group of them is lipid systems (Figure 6). Liposomes reduce toxicity, extend drug half-life, possess biocompatibility and biodegradability [91]. They have also proved to be capable of improving the delivery of bioactive molecules by functioning as circulating micro-reservoirs for sustained release [92]. A delivery system with paramagnetic nanoparticles encapsulated in cationic liposomes tested with the leucine-rich antimicrobial peptide (FALALKALKKALKKLKKALKKAL) results in better hemocompatibility (7.5%) and antimicrobial activity of the entire complex against *Escherichia coli (E. coli), S. aureus* and methicillin-resistant *S. aureus* (MRSA) compared to conventional penicillin antibiotics. However, further in vivo experiments are required to specify the real effect of advanced nanomaterials on the therapeutic index of peptides [93] (*Table II*).

In another study, nanostructured lipid carriers (NLCs) were investigated as a delivery system for

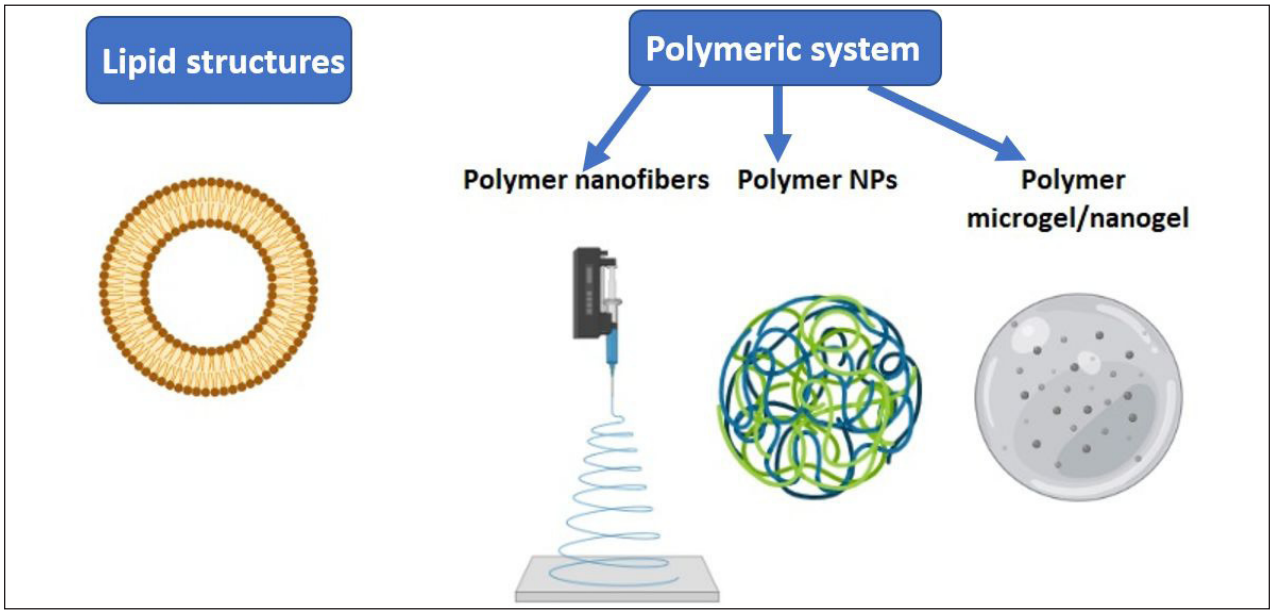

Figure 6 AMP formulations containing organic materials (Created with BioRender.com)

an antimicrobial peptide named nisin Z against two Gram-positive species found on the skin surface, *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) at physiological pH. The results proved the effectiveness of NLCs as promising biodegradable delivery systems for the enhancement of the stability, solubility and antimicrobial activity of AMP and also as promising potential systems for dermal applications [94].

In addition to liposomes, micelles also show great potential as vectors for drug delivery, especially for poorly water-soluble drugs because of their size and their ability to solubilize hydrophobic drugs and to achieve target or site-based drug delivery. For instance, the potential delivery of human cathelicidin LL-37, and its protection from degradation was investigated by Gontsarik *et al.* Nanocarriers named nano-biointerfaces were prepared through the self-assembly of oleic acid (OA) with human cathelicidin LL-37 in an excessive amount of water. According to the results, hydrophobic and electrostatic interactions between OA and the peptide molecules drove the detected structural transformations (from normal emulsions via micellar cubosomes and hexosomes to vesicles) with both composition and pH. These structural changes are interesting for the further development of pH-driven nanocarriers for the targeted delivery of poorly water-soluble AMPs as an alternative to conventional antibiotics. They may also be valuable for the further understanding of the mechanism behind the AMP-driven destruction of the bacterial membrane [95].

5.2.2 Polymeric systems

5.2.2.1 Polymeric nanoparticles

Polymeric NPs are often used as carriers for protein and peptides [96] that can be prepared by different techniques such as emulsion/solvent evaporation, double emulsion, solvent spread, coacervation, nanoprecipitation, ionotropic gelation and salt precipitation [97]. A new AMP named GIBIM-P5S9K was loaded into polylactic acid (PLA) and poly (lactic-co-glycolic) acid (PLGA) NPs via the double-emulsion solvent evaporation method resulting in a release of around 50% of the peptide from the NPs during the first 8 hours. These peptide-loaded NPs presented higher antibacterial activity than the free peptide against *E. coli*, MRSA. Other advantages listed in *Table II* suggested these synthesized NPs as a promising candidate for AMP delivery and a protection system against enzymatic peptide degradation [97]. A new class of antimicrobial agents, termed 'structurally nano-engineered antimicrobial peptide polymers' (SNAPPs), was synthesized in the form of 16- and 32-arm star peptide polymer nanoparticles (S16 and S32) and showed sub-μM activity against Gram-negative bacteria, including a group of pathogens responsible for the majority of hospital-acquired infections (referred to 'ESKAPE' pathogens) and colistin-resistant and multidrug-resistant (CMDR) pathogens, while demonstrating low toxicity [98]. Angelo *et al.* prepared PLGA nano-embedded microparticles containing cationic AMP (colistin) as a lung delivery system. It is a promis-

ing formula because it can help diffusion through the mucus and the bacterial biofilm. These particles can be appropriate for the treatment of lungs infected with *P. aeruginosa* in cystic fibrosis [99]. The aim of another research group was to improve the antimicrobial efficiency of nisin with encapsulation in a poly-g-glutamic acid (g-PGA) and chitosan nanoparticle using the self-assembly method. The dissolution of nisin from these formulations was pH-dependent. It was found that the g-PGA/chitosan nanoparticle containing nisin had higher antimicrobial efficiency than the g-PGA nanoparticle containing nisin [100].

5.2.2.2 Polymer nanofibers

Sebe *et al.* formulated polyvinyl alcohol nanofiber-loaded AMP (proline-rich peptide dimer A3 APO) and it was polymerized into a solid patch dressing. It was tested in wounds of mice infected with multidrug resistant *A. baumannii* and the results revealed that the patch containing APO improved the wound appearance significantly more than the patch without APO. When compared with the patch containing colistin, the patch containing APO displayed accelerated wound healing and significantly reduced wound size [101]. Andreu *et al.* also investigated the nanofibers containing AMP. It can be appropriate for wound treatment in wound dressing [102]. Amariei *et al.* designed and prepared poly (acryl acid) and poly (vinyl alcohol) nanofiber containing e-polylysine as an AMP. They determined that the antimicrobial efficiency of these nanofibers with minimum inhibition concentration was in the following order: *S. epidermidis* > *S. aureus* > *E. coli* [103].

5.2.2.3 Polymer microgels, nanogels and hydrogels

Water *et al.* demonstrated that octenyl succinic anhydride-modified hyaluronic acid nanogels can apply as AMP (novicidin) in drug delivery systems. The maximum peptide loading of nanogels was 36±4%. The nanogels containing novicidin had reduced cytotoxicity, relatively high drug load, colloidal stability and showed the sustained release of drug over twelve days [104]. Silva *et al.* formulated hyaluronic acid nanogels containing AMP (LKKK18) and they demonstrated these nanogels can be applied in high therapeutic doses of the drug and display improved proteolytic stability [105].

Features such as high hydrophilicity, unique three-dimensional network, fine biocompatibility and cell adhesion make them suitable biomaterials for drug delivery in antimicrobial areas [106]. Various materials have been used in different researches for hydrogel preparation with AMP to target different organisms. Hydrogel formulations allow the sustained release of drugs, therefore, the incorporation of AMPs into these systems would offer prolonged AMP release at target sites and retain high AMP concentration in the nearby tissues [91]. In a research by Song *et al.*, an easily synthesized cell-adhesive hydrogel with inherent antibacterial activity was prepared as a potential scaffold for dermal wound healing based on chemical cross-linking between poly (Lys-Ala) polypeptides and 6-arm PEG-amide succinimidyl glutarate (ASG). As demonstrated in *Table II*, this hydrogel displayed significant antibacterial activity against *E. coli* and *S. aureus* [107]. Yang *et al.* prepared RADA-AMP (Tet213) self-assembling hydrogel, which can be appropriate for the treatment of bone infection and osteomyelitis [108].

5.3 Quality by Design based development

After the selection of the proper AMP, the extended Quality by Design (QbD) based development [109] is suggested (Figure 7). After the definition of the Quality Target Product Profile (QTTP), the next step is the initial risk assessment due to the complex interdependency of different factors and a number of possible risks [110, 111]. By means of proper quality management tools such as the Ishikawa diagram, the control of the Critical Quality Attributes (CQAs) and the Critical Process Parameters (CPPs) during modification and formulation of AMPs can be tested. The parameters can be divided into six groups: AMP characteristics, chemical modification method, final product characteristics, AMP formulation, therapeutic aim and administration rate, patient and AMP formulation. The application of Ishikawa diagram can high-

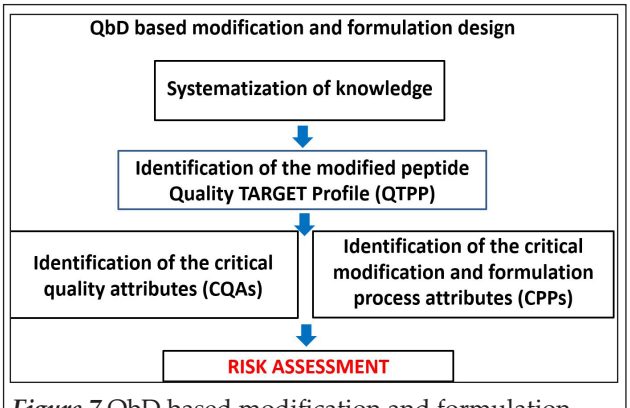

Figure 7 QbD based modification and formulation

light how identifying different factors that can affect the desired product quality, and thus the predefined AMP quality, including high metabolic stability, high cost efficiency, biocompatibility, low toxicity, low immunogenicity, retained antimicrobial activity and feasibility in reaching target sites at active concentration, is achievable.

6 Conclusions

As can be seen in *Table II*, it can be concluded that among different carrier systems, lipid-based nanocarriers offered a number of potential advantages as a delivery system for dermal or parenteral administration. The advantages include improved bioavailability of poorly soluble AMPs, enhanced hemocompatibility, high antimicrobial activity, high biodegradability and high selectivity of the peptide towards bacterial cells, protecting the cargo against unwanted interactions with the environment and providing a synergic effect. Besides this, these delivery systems have drawbacks, such as generation of undesired side products and limitations of controlling and triggering self-assemblies in lipid structure systems. On the other hand, recent scientific studies showed that polymer-based nanocarriers have several promising advantages, including enhanced antimicrobial activities of AMPs, low toxicity, low costs and selectivity of peptides for the target. Polymeric nanocarriers also showed disadvantages, such as possibility of inefficient delivery of AMPs to the target infectious site due to degradation. However, among different polymeric nanocarriers, hydrogels possess inherent antimicrobial activity and offer ease of synthesis and great potential to avoid secondary infections. Therefore it seems that among the above-mentioned delivery systems, lipid-based nanocarriers and polymeric hydrogels not only offer ease of synthesis, but their various advantages also outweigh their limitations and make them preferred nanocarriers in dermal and parenteral delivery systems of AMPs.

In this work, collecting and evaluating the results of various published researches led to achieving specifications in AMP delivery development. The knowledge of the physicochemical and structural features of AMPs facilitates the selection of a peptide with optimal features, such as: α-helical structure, amino acid charge between 3 to 6 and moderate amphipathicity and hydrophobicity, resulting in low toxicity and high antimicrobial activity.

Overall, in this review article different factors and possible associated risks in selection, modification and formulation of AMPs in a suitable delivery system were highlighted.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Pegylation and formulation strategy of Anti-Microbial Peptide (AMP) according to the quality by design approach

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ABSTRACT

Antimicrobial resistance is one of the main global threats according to the World Health Organization's (WHO) report (World Health Organization 2014), therefore there is a need for the development of other agents, such as antimicrobial peptides (AMPs). Although AMPs are considered as major candidates for next-generation antibiotics, several challenges including low bioavailability, high manufacturing cost and toxicity are still to be solved for their practical use in therapeutic applications. Novel chemical modification approaches as well as strategies for their delivery offer several opportunities to overcome these barriers and develop more stable and cost-effective synthetic peptides with efficient delivery to the target site.

The integration of the Quality by Design (QbD) approach in the early pharmaceutical developments supports researchers in optimizing the targeted product by a risk based manner. 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 give 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.

This study presents a Quality by Design based antimicrobial peptide modification and formulation design. Analyses the potential risks in the AMP PEGylation process through the example of PGLa. The QbD based initial RA screened and evaluated the risk factors in this AMP modification procedure. The critical quality and process related factors were defined and their ranking was performed due to their estimated critical effect on the PEGylated AMP. This pre-formulation design study highlights the critical risk factors as decision points for the further steps.

1. Introduction

1.1. Antimicrobial peptides and their potential in antibiotic therapy

Increased development of antimicrobial resistance to many available antibiotics is one of the biggest challenges in the global health sector together with advancements in biotechnology, genetic engineering and synthetic chemistry lead scientist to focus on alternative substitutes 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 (Boge et al., 2019; Ghosh et al., 2019, World Health Organization, 2014). 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 (Zaiou, 2007; Roby and Di Nardo, 2013). Beside the previously mentioned advantages, AMPs have also limitations such as low bioavailability, high manufacturing cost and toxicity which still need to be faced in order to be able to use these peptides in therapeutic applications (Marr et al., 2006).

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 polymer to peptides (PEGylation).

Abbreviations: alloc, n-allyloxycarbonyl; AMP, antimicrobial peptide; CMAS, critical material attributes; CPPS, critical process parameters; CQAS, critical quality attributes; DOE, design of experiments; FDA, food and drug administration; FMOC, 9-fluorenylmethyl carbonate; MW, molecular weight; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; QBD, quality by design; QTPP, quality target product profile; RA, risk assessment; tBOC, tert-Butoxycarbonyl; TFA, Trifluoroacetic acid; WHO, World Health Organization's report

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These approaches allowed several researchers to enhance the bioavailability of AMPs and improve their bio-distribution and rate of clearance. The proteolytic degradation of peptides can be decreased by protecting their C- and N-terminus with acetylation or amidation. Also, modifying the sequences of peptides by substitution of natural L-amino acids for their D enantiomers, α/β-substituted α-amino acids or even β-amino acids are other similar approaches that result in overcoming peptide hydrolysis. D-amino acid substitution in a peptide may influence not only the peptide's stability but also its secondary structure and therefore its ability to incorporate into membranes (Gomes et al., 2018; Castro et al., 2017; Sun et al., 2017; Hamamoto et al., 2002). The attachment of PEG molecules to proteins and peptides provide steric interference and thus protects peptides from proteolysis and offers several functional advantages for AMPs such as prolonged plasma half-lives, improved water solubility, stability, resistance, biocompatibility, minimal toxicity and immunogenicity (Hamley, 2014). After approval of the first PEGylated protein drug product by the FDA in 1990, several PEGylated protein drug products have become part of the pharmaceutical market (Pinholt et al., 2011). However non-specific covalent attachment of a large PEG molecule to AMP is a risky factor which can change AMP structure and thus its antimicrobial activity (Christian et al., 2009); complexity of the peptide structure itself may lead to decreased reproducibility (Wei et al., 2012). Moreover, PEG molecules may mask the binding (active) site of AMP and therefore cause loss of antimicrobial activity of AMP. Too low molecular weight (Mw) of PEG can reduce specificity and conjugate activity while too high Mw can sterically shield the bioactive domain of the peptide and reduce its biological activity (Turecek et al., 2016). The experimental conditions of PEGylation reaction (i.e. pH, temperature, reaction time, overall cost of the process and molar ratio between PEG derivative and peptide) also have an impact on the stability of the final PEGylated AMP (González-Valdez et al., 2012). For AMPs with membrane disruptive mechanism of action another important risk of PEGylation is the possibility of reducing membrane affinity and interference of PEG molecule with the mechanism of action of AMPs; the attachment of PEG molecule cause AMP to lose its positive charges and thus suppress bacterial lysis resulting in reduction of biological activity (Singh et al., 2014). To overcome the abovementioned barriers, different strategies such as changing Mw of PEG, site of PEGylation and type of linkage of PEG molecule were offered by several researchers (Zhang et al., 2008; Obuobi et al., 2018). This research article mostly describes the risks that may occur during PEGylation process of AMPs. Nevertheless, risk related to PEGylated therapeutics (risks that may occur after PEGylation) must also be considered. One of the common risks reported by numerous research groups is the production of antibodies by immune system. These antibodies specifically bind PEG and thus cause “accelerated blood clearance” of PEGylated therapeutics (Yang and Lai, 2015). An improved understanding of the mechanisms of anti-PEG immunity, monitoring patients before and during PEGylated drug treatment and less immunogenic delivery approaches are needed as strategies to overcome the challenge of PEG-specific immunity (Zhang et al., 2016).

PGLa as a model AMP in this study is an 21-residue amphipathic antimicrobial peptide-amide (GMASKAGAIAGKIAKVALKAL-NH_2), isolated from the African clawed frog *Xenopus leavis*, that can destroy bacteria by interacting with their lipid membrane (Bechinger et al., 1998; Hartmann et al., 2010). The folded structure of PGLa displays the positively charged lysine sidechains on one side and hydrophobic residues on the opposite side (Strandberg et al., 2006). It is shown to be helical between residues 6 and 21 when associated with detergent micelles by multidimensional solution nuclear magnetic resonance (NMR) spectroscopy and the helix axis is parallel to the plane of the bilayers. NMR spectroscopy indicates that the amino-terminal residues are highly mobile and that the fluctuations of backbone sites decrease from Ala6 toward the carboxyl terminus (Bechinger et al., 1998). PGLa is known to decrease the antibiotic resistance level of resistant bacteria when co-administered as an adjuvant (Lázár et al., 2018). The exact mechanism of action is not known, but it is believed to form pores upon interacting with the bacterial membrane, and induces membrane permeability even at sub-MIC conditions (Hartmann et al., 2010).

There are two possible ways of its PEGylation: PEGylation in solvent phase or during the solid-phase synthesis, both carried out through the amino groups. Solvent-phase reactions are quite common in protein ligation techniques where only limited chemical modifications are possible during the synthesis. PEGylation can be carried out easily in the solvent phase, but either all amino groups are PEGylated with a high excess of reagent, or the PEG groups are randomly distributed when using sub-equimolar amounts. In case of AMPs however, PEGylation during solid-phase synthesis is possible, which allows selective modifications at desired sites. N-terminal PEGylation can be achieved after removing the final amino protecting group, and coupling an amine-reactive PEG derivative before cleaving the peptide from the resin.

For PEGylation at specific sites of the peptide, we have to use alternative sidechain protecting groups for the selected lysine residues which can be removed before the cleavage of the peptide. Selective removal of these protecting groups such as methyltrityl allows the coupling of the PEG chain at the selected position, followed by the removal of the N-terminal protecting group and the cleavage of the peptide.

PEG-Linker-Drug strategy is another possibility to increase the half-life of PGLa. By using a linker which is degraded by the bacteria itself, the risk of decreased antimicrobial activity by the PEGylation can be circumvented and there is no need to site-specific modifications. Bacterial enzymes which play roles in antibiotic resistance (such as β-lactamase) are the most promising candidates, as the overexpression of the enzymes would facilitate the release of the drug.

Solid-phase FMOC /tBu strategy is a common strategy designed and developed by using protected amino acids as building blocks (Tsubery et al., 2004; Lu et al., 2009). The controlled synthesis of peptides and formation of amide bonds requires the use of reversible ion of the amino group. Common amino protecting groups are: tert-Butoxycarbonyl (tBoc), 9-Fluorenylmethyloxycarbonyl (Fmoc) and N-Allyloxycarbonyl (Alloc). It is also necessary to reversibly mask reactive side chain functional groups. The peptide remains anchored to an insoluble solid resin support. Resins commonly used are composed of polystyrene. The excess reagents and soluble byproducts will be removed after each reaction cycle.

As shown in Fig. 1, in this approach the first protected amino acid is attached to the resin through its carboxyl group (Coupling) (the addition of activating agent). Then the protecting group is removed (deprotection) under a mildly basic condition. This exposes a free α-amino group to react with the next incoming protected amino acid. Then again deprotection step is repeated (To confirm that the protecting groups are removed, a Kaiser-test is performed). The process is repeated through a cycle of deprotection, coupling and washing until the peptide is completely synthesized. The synthesized peptide is usually cleaved from the resin by trifluoroacetic acid (TFA), which removes the side chain protection groups at the same time. The purification steps usually includes the precipitation from the cleavage reaction mixture by ice-cold diethyl ether. Further purification can be achieved by gel-filtration, ion exchange chromatography and reversed-phase HPLC (Lu et al., 2009).

1.2. The quality by design method

The Quality by Design (QbD) approach is a holistic, systematic, knowledge and risk based methodology of pharmaceutical developments, which focuses on the profound preliminary design (Soravia et al., 1988) considering all of the influencing parameters from the industry, the regulatory body and from the user (eg. patient, doctor). The application of the QbD method in the industrial development and manufacturing is forced by the regulatory authorities but it

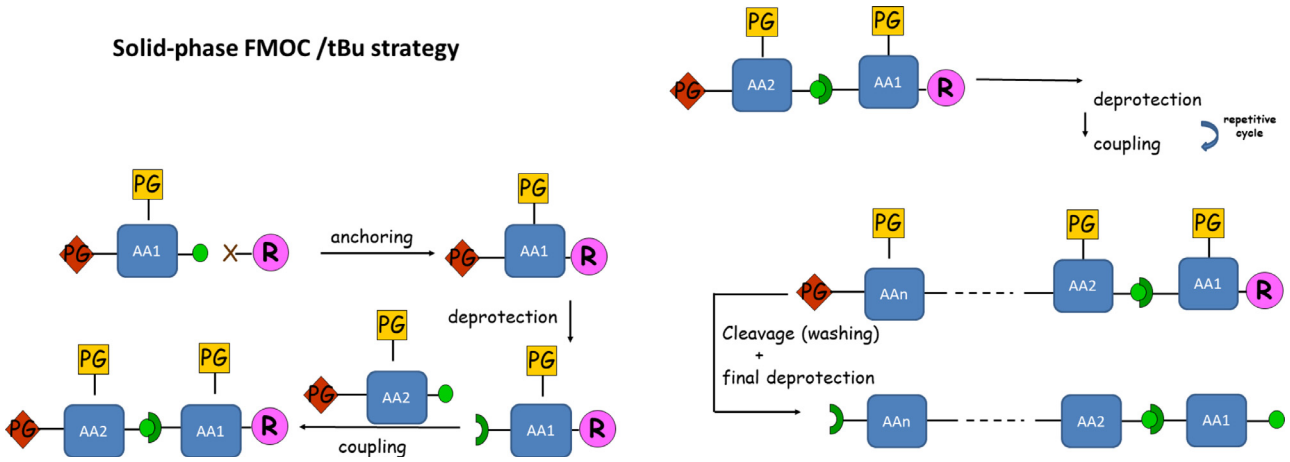


Fig. 1. Schematic representation of solid phase peptide synthesis.

has also many benefits in the early phase of the developments (Pallagi et al., 2018; Zerweck et al., 2017), as brings scientific results closer to the practical requirements and has a facilitating effect on industrial scale up and product transfer to the market.

The QbD has several steps, described in the guidelines of the International Council of harmonization (ICH Q8 (R2), ICH Q9, ICH Q10) (Werle and Bernkop-Schnürch, 2006; Bahar and Ren, 2013; Santos et al., 2018). The main steps are: (1) the definition of the Quality Target Product Profile (QTPP), (2) the identification of the quality attributes and the selection of the Critical Quality Attributes (CQAs) related to the target product, (3) the prior selection of the production method and the identification of the Critical Process Parameters (CPPs) as well as the Critical Material Attributes (CMAs), (4) performing of the initial Risk Assessment (RA). RA is a systematic process of organizing information to support a risk decision and is the key activity in this model. The results of the RA will be the ranking of the CQAs and CPPs according to their calculated risk severity. RA results help to aim attention on the most critical influencing factors and avoid profitless efforts in later phases of the development process. The following steps of the QbD approach are: (5) the Design of the Experiments (DoE) which namely means the planning of the practical tasks by the RA results, (6) the performing of the experiments in practice and establishment of the Design Space (DS). These are followed by the (7) compilation of the Control Strategy which is the monitoring of the factors with highly risk potential in the process. The whole QbD guided process should be designed and performed by considering the possibilities of the (8) Continuous Improvement. In this thinking, generally the RA is the most accentual element which it is especially advantageous in the case of complex and sensitive drugs like peptides.

This paper aims to take steps in the enhancement of AMPs' properties for pharmaceutical use and evaluate the risks of AMP PEGylation such as loosing antimicrobial activity of peptides, used PGLa as a model AMP. This study analyses the potential risks in the antimicrobial peptide PEGylation process by means of the RA method within the QbD approach of early pharmaceutical development.

2. Materials and methods

2.1. Materials

PGLa (H-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-NH_2) is 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 (Soravia et al., 1988).

2.2. Methods

2.2.1. Knowledge space development

The collection and systemic organization of the related scientific literature and experience from the previous studies means the "knowledge space development" (Zerweck et al., 2017). After the analysis of the relevant scientific literature, the data collected were structured and visualized. Ishikawa diagram was prepared for categorization of the influencing factors (causes) (Rao et al., 2017), flow charts were prepared for PEGylation process description (Dozier and Distefano, 2015).

2.2.2. Definition of QTPP

The QTPP forms the basis of product development design. The QTPP is always unique depending on the target. Generally, it should include patient-relevant product performance and characteristics related to the aimed therapeutic or clinical use. Considerations for QTPP selection are described in the ICH Q8 (R2) guideline (Werle and Bernkop-Schnürch, 2006), e.g. the route of administration, dosage form, etc. 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.

2.2.3. Determination of the CQAs

CQAs are those 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 (Werle and Bernkop-Schnürch, 2006). The selection of CQAs is based on a holistic view of the formulation development and is based on previous knowledge and experience.

2.2.4. Determination of the CMAs and CPPs

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 (Werle and Bernkop-Schnürch, 2006). CMA and CPP selection was based on prior knowledge resulting from the knowledge space development phase of the study.

2.2.5. Initial risk assessment

The initial RA was performed by means of the Lean QbD Software® (QbDWorks LLC., Fremont. CA, USA, qbdworks.com). 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. Results

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

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. 2), where the different factors and possible associated risks in selection, modification and formulation of AMP in a suitable delivery system are highlighted (Zhao et al., 2006). 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. 3.

These graphical representations (Figs. 2 and 3) 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. All of them are listed in Table 2 with their selected targets and their proper justification.

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. 4. 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. 4A). 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. 4B. 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. 4C. 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.

The Fig. 5 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. 5). 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. 6 the most potential process factors, as CPPs with the highest estimated or relative occurrence and relative severity rate can be seen. Fig. 6 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

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, hydrolysis by water	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

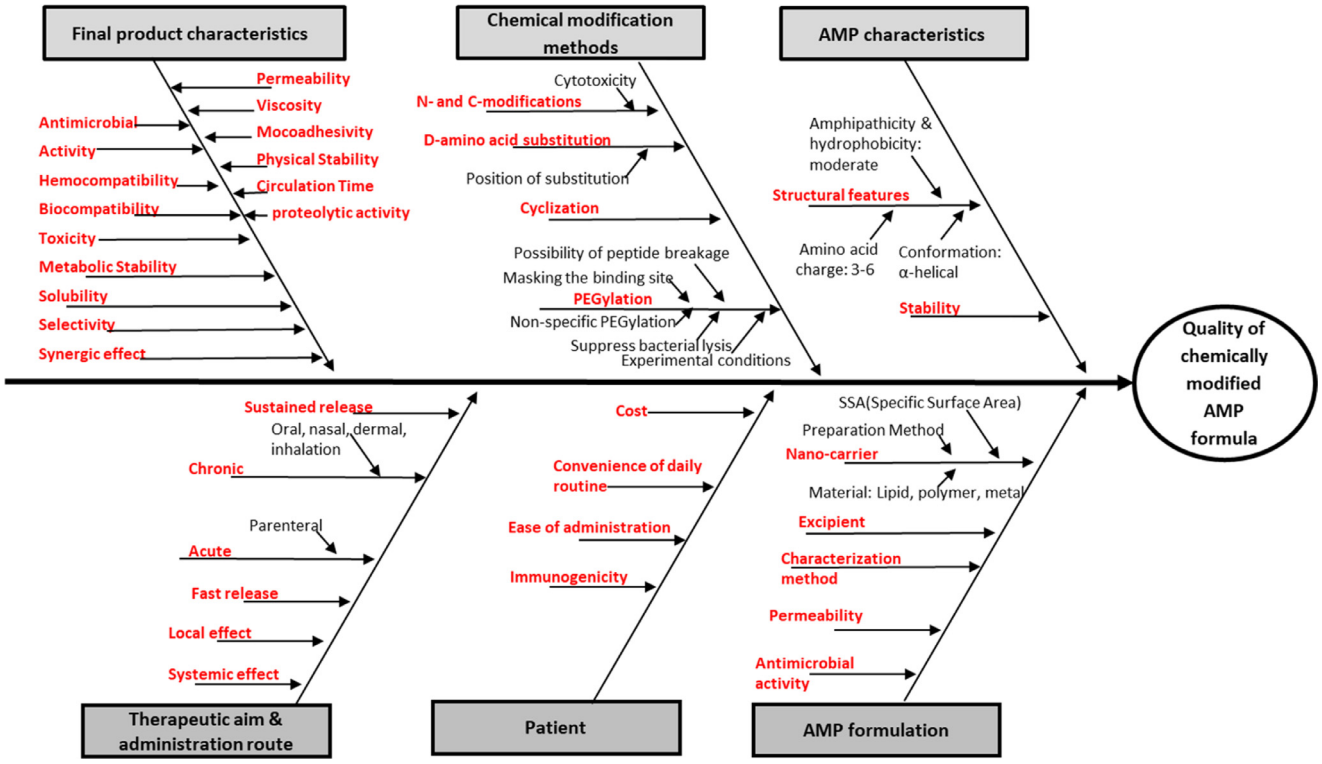


Fig. 2. Ishikawa diagram including all the parameters influencing the desired chemically modified AMP formula.

in the research executed in practice.

4. Discussion

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

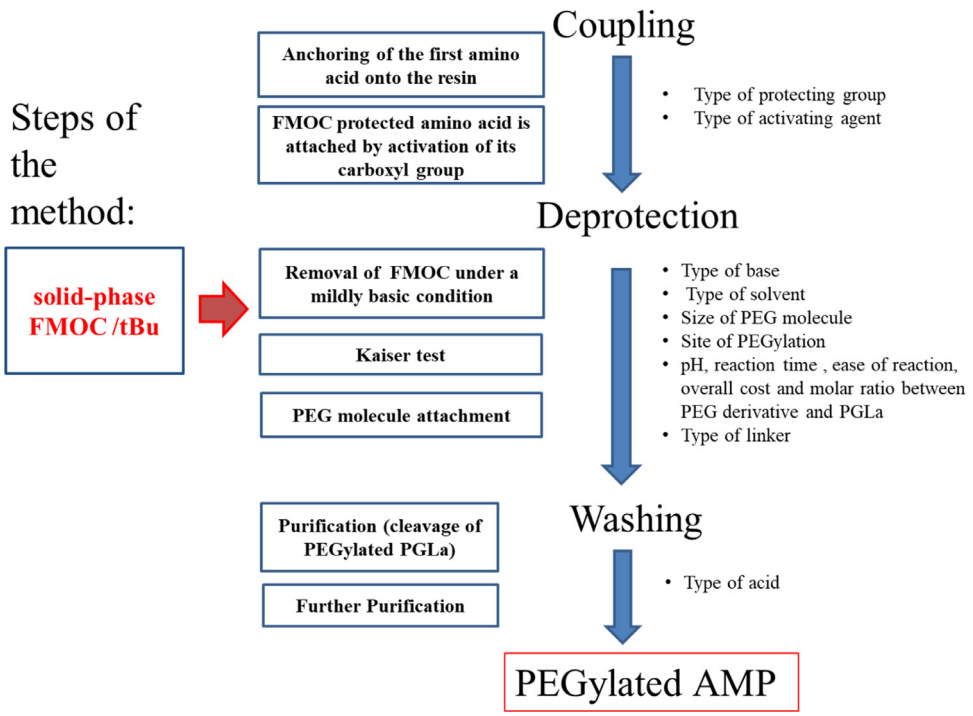
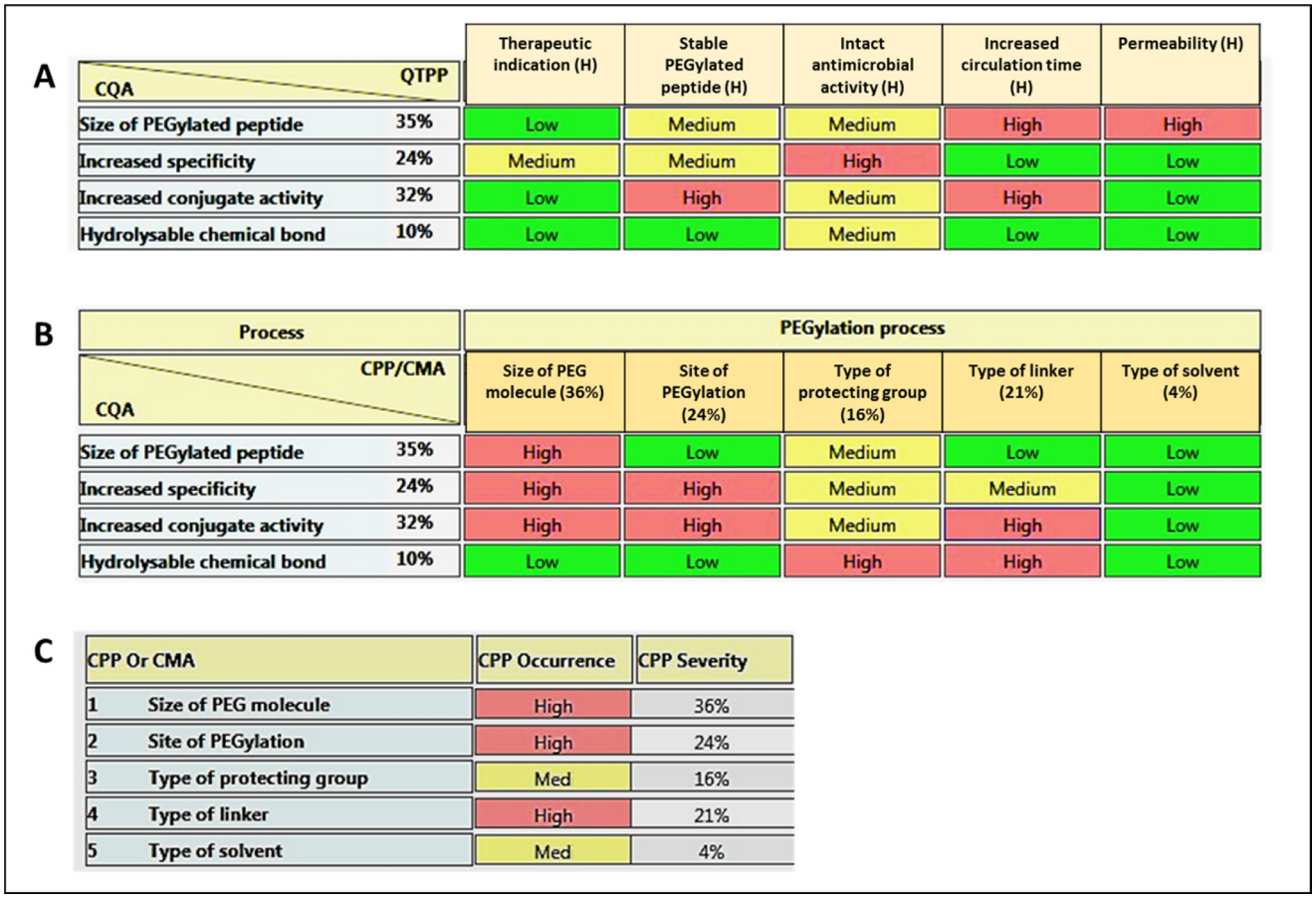


Fig. 3. Flow-chart of solid-phase PEGylation process of the selected AMP (PGLa).

Table 2
Selected QTPPs, CQAs and CPPs/CMAs of a PEGylated PGLa formula.

QTPPs	Target	Justification
Therapeutic indication	Infectious diseases (antimicrobial therapy required)	Infectious diseases are disorders caused by organisms such as bacteria, viruses, fungi or parasites. with the increased development of antimicrobial resistance to many available antibiotics, AMPs as essential alternatives show specific mode of action and low resistance development. PGLa is an AMP originally isolated from frog. It has been shown to have antibacterial, antifungal, and antiviral, activities (Csóka et al., 2018).
Circulation time	Increased	Because of the fast renal clearance of peptides in systemic circulation, prolonging plasma half life is imprtant and enhance the therapeutic efficacy (Werle and Bernkop-Schnürch, 2006).
Antimicrobial activity	Intact	It is easy to change characteristics of an AMP with different types of modifications, However the results of these changes is still challenging and can cuase AMP to loose its activity. Thus Intact antimicrobial activity is strongly important and effect the therapeutic efficacy (Bahar and Ren, 2013).
PEGylated peptide	Stable, No aggreagtion, No degradation	The PEGylated Peptide should provide a good protection toward proteolytic enzyme for enhanced efficacy, quality and safety [40].
Permeability	No interference of PEG molecule with the mechanism of action of AMPs	For PGLa with its membrane distruptive mechanism of action there is a possibility of reducing membrane affinity and interference of PEG molecule with the mechanism of action of PGLa. Development of new reagents and methodologies with no disturbing effect on biological activity of the peptide is vital (Singh et al., 2014).
CQAs	Target	Justification
Size of the PEGylated peptide	Nano-sized	Proper for nasal administration to reach systemic effect. Considering low patient acceptance in parenteral administration routes, the nasal route of administration is more desirable for AMP delivery. Besides, Intranasal administration offers a rapid onset of therapeutic effects, avoids the first- pass effect or gastrointestinal degradation of drugs [41].
Specificity	Increased	Specific PEGylation targeting peptide to specific site result in higher half-life and prevent loosing antimicrobial activity, minimizing side effects and thus requiring less frequent dosing [42].
Chemical bond between PEG and peptide	Hydrolysable or slowly degradable bonds	A covalent stable linkage between the PEG molecule and the peptideb in many cases is leading to peptid es with very low or reduced functional activity. However degradable linkages between the peptide and the PEG make PEGylated peptide to act as prodrug and the PEG polymer is released over time in the circulation and the activity of the therapeutic peptide can be recovered. It is better to design linkage in an optimal level, which is not too stable and not quickly degradable (Turecek et al., 2016).
Conjugate activity	Optimal	It influences the stability of the linkage between PEG and peptide (Turecek et al., 2016). (It is better to be moderate as describe for chemical bond between PEG and peptide)
CPPs/CMAs	Target	Justification
Size of PEG molecule	Optimal for attended application (in this case nano-sized)	Influence the antimicrobial activity, degradability, specificity of the peptide (Turecek et al., 2016).
Site of PEGylation	Site specific PEGylation	Antimicrobial activity of PGLa can be reduced by random PEGylation. Also it results in higher half-life and minimizing side effects and thus requiring less frequent dosing (Dozier and Distefano, 2015).
Type of protecting group	Cleavable and stable	In solid phase synthesis strategy the protecting group should be select as a reagent that is cleaved in deprotection step easily under very basic conditions, but also it remains stable under acidic conditions. The selection of the right type of protecting group can reduce the time and the costs of the reaction and causes less impurities (Lu et al., 2009).
Type of linker	Increased	The selection of the right linker displays suitable characteristics for applications in formulation of therapeutic proteins. It increases the half-life of the peptide and also improves antimicrobial activity. It has potential to greatly enhance the use of peptide as therapeutics [43].
Type of solvent	Appropriate for solid phase peptide synthesis strategy	The selection of the right solvent minimizes side reactions and influence the cost and time of the reaction (Lu et al., 2009).



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				
CQA \ CPP/CMA		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%

Fig. 4. Interdependence rating results among the QTPP elements and CQAs (Part A), as well as among the CPPs and CQAs (Part B) and the results of the occurrence rating (Part C) as steps of the RA.

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.

5. Conclusion

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 [44,45]. 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.

Authors Contributions

The University of Szeged, Faculty of Pharmacy, Doctoral School of Pharmaceutical sciences and the Institute of Pharmaceutical Technology and Regulatory Affairs made possible the scientific work.

Data availability: The datasets generated and analysed during the present study are available from the corresponding author on reasonable request.

Declaration of competing interest

None

Acknowledgement

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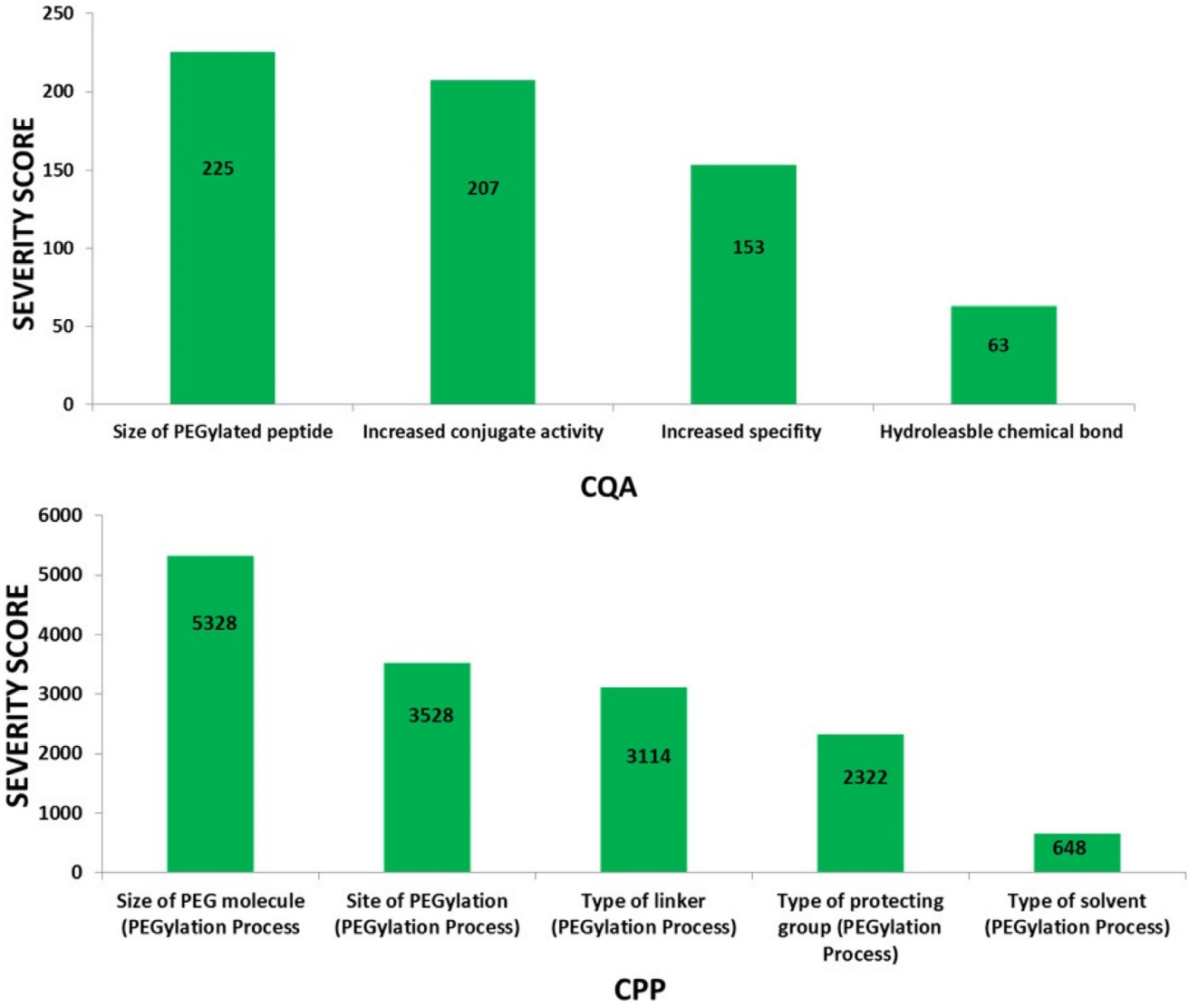

Fig. 5. Pareto charts presenting the ranking of the selected CQAs and the CPPs as results of the RA.

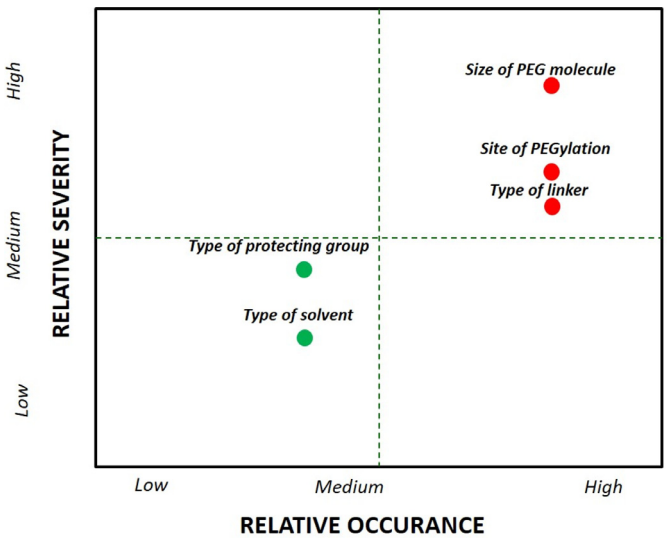

Fig. 6. The relative occurrence and relative severity diagram of the CPPs.

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III.

PLOS ONE

RESEARCH ARTICLE

Optimization of layering technique and secondary structure analysis during the formulation of nanoparticles containing lysozyme by quality by design approach

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Abstract

In our study, 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 Quality by Design (QbD) approach. The factors were the concentration of lysozyme and sodium alginate, and pH. 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 nanoparticles were analysed with transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR) and circular dichroism (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 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 protein and other optimization parameters can be controlled in the future during development of nanoparticle based on the process parameters.

Introduction

Recently, the use of proteins in the biomedical field has become more extensive, and they have been investigated intensively as potential biopharmaceutical drugs [1]. They have well-known high specificity, complexity and low toxicity compared to small drug entities, but at the same time a number of barriers need to be overcome for the development of stably absorbable delivery systems [2].

Lysozyme (LYS) is a harmless natural antimicrobial enzyme protein that can be derived from plants, animals and microorganisms as a single chain polypeptide. It has a globular shape constructed of 129 amino acids with an approximate molecular weight of 14 kDa, with a quite alkaline nature of 11 isoelectric points, its main physiological role is to perform the host's natural immune defence effect [3, 4]. Hen-egg white is a common source in LYS separation studies, which is mainly performed by the precipitation method of egg-white proteins upon the addition of salts, solvents or the reduction of ionic strength [5]. The diversity of the source renders LYS more affordable and cost-effective protein for the investigations [6]. The antimicrobial activity of lysozyme is attributed to the destruction and lysation of the cell wall of Gram-positive bacteria and some fungi [7]. Moreover, *in vitro* studies prove the activity of LYS against many Gram-negative bacteria including Pseudomonas aeruginosa [8].

Pharmaceutically, nanotechnology has been performed to improve drug delivery performance, basically by improving bioavailability through the administration of the drug entity in nanoscale particles (NPs) or molecules (in the range of 1-1000nm) which can overcome the biological barriers, targeting the absorption site, enhancing stability and solubility by increasing the surface area [9]. Therefore, various nanotechnology-based drug formulations have been introduced to the market for treating and controlling numerous diseases, such as cancer, central nervous system diseases and infections [10]. Moreover, the delivery of the drug in the form of NPs protects the natural products, such as proteins, from degrading enzymes, as well as controls the release of the incorporated bioactive molecules [11].

Nanocarrier systems used extensively for protein delivery based on synthetic polymers, liposomes and metal have been replaced as a result of many limitations, e.g. the instability and low loading capacity of liposomes, the disadvantages of low clearance rate and hence enhanced toxicity of metal-based nanocarriers, while those made from synthetic polymers have a limitation of the aggregation of the encapsulated proteins in their inner core [12]. Accordingly, protein NPs and their conjugates have replaced nanocarrier systems, offering the advantages of nanosized structure with good biopharmaceutical characteristics. Also, their production is cost-effective and easy to tailor to meet the specific requirements [13].

The precipitation technique (bottom-up approach) represents the most applicable method for the production of NPs for both small-scale and bulk production, owing to its simplicity, low energy input, low generated temperature and cost-effectiveness compared to the other top-up methods [14]. Zhang et al. prepared a carboxymethyl starch (CMS) microgel system for the control of up taking and releasing proteins (lysozyme) with high pH and high salt-concentration. The microgel particle size was between 25 μm and 45 μm [15]. Xanthan/LYS NPs were produced by precipitation in alkali-coupled thermal condition, and the NPs showed favourable size distribution and stability [16]. On the other hand, when LYS loaded in chitosan (CS) NPs were prepared by the ionic gelation of chitosan and tripolyphosphate (TPP), the CS molecular weight and content, TPP content and initial LYS were reported to have effects on the encapsulation efficiency (EE), release performance and activity of LYS [17]. Moreover, LYS complexed with different concentrations of sodium alginate showed two stages of aggregates with loss of activity based on the alginate content, but antimicrobial activity was recovered upon the addition of calcium chloride [18]. Similarly, LYS was encapsulated in novel cationic

polymethacrylate/alginate NPs as a polyelectrolyte carrier system, and the NPs showed high capacity to encapsulate the enzyme, with acceptable polydispersity, biodegradability, high stability and sustained release of LYS. The *in vitro* cytotoxicity of the complex was found to be dose-dependent [19]. Polyelectrolyte multilayers are well-defined nanostructure with some potential applications, such as biomaterial coatings [20]. Also, polyelectrolyte core-shells of bovine serum albumin nanoparticles (BSA-NPs) developed through the layer-by-layer (LBL) technique were used as a carrier system to control the release of ibuprofen. The inner layer was anionically made from poly (sodium-4-styrene) sulphonate, and the cationic outer layer from CS, which enabled interaction with the negatively charged cell membrane and facilitated cell up-take [21].

Accordingly, the arrangement of oppositely charged polyelectrolyte polymers through the LBL approach represents a promising technique for fabrication of micro/nanoparticles and allows their physiochemical and morphological properties to be modified by controlling ionic strength, polymerization degree and the ratio of the polymers [22, 23]. Most importantly, the technique is usually conducted under normal experimental conditions and mainly in an aqueous solution, hence it is suitable to encapsulate proteins and polypeptide drugs [24], and recently the application of three layers of polyelectrolyte polymers in anticancer NPs demonstrated cancer-cell targeting with efficient internalization [25]. In our previous work NPs containing human interferon-α were formulated with the LBL approach by applying chitosan and polystyrene sulphonate [26]. The aim of our present work is to formulate, analyse and optimize core-shell type NPs containing LYS, as well as to write mathematical relationships between process parameters and product parameters.

Materials and methods

Materials

Lyophilized lysozyme (MedChemExpress, Hungary), stored frozen (-20˚C), was used as a model protein, lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) as Gram-positive bacteria to investigate the activity of layered NPs, sodium sulphate (Molar Chemicals Ltd., Hungary) as a precipitating agent, Alginic acid sodium salt (AppliChem GmbH—An ITW Company, Germany) as a layering polymer, sodium hydroxide and hydrochloric acid (Ph. Eur.) as pH adjusters, and all the other reagent s were of analytical grade.

Methods

Preparation of LYS NPs. The preparation of LYS NPs was made according to 2^3 full factorial design; therefore 8 samples were prepared. Namely, 0.6 g of lyophilized enzyme was dissolved in purified water to obtain 20 g of homogenous aqueous solution, and then each sample was mixed with 4ml of 2M Na_2SO_4 solution by using a magnetic stirrer for 1 hour. The 8 samples were centrifuged at 5000 rpm for 15 minutes by using a Hermle Z323K high performance refrigerated centrifuge (Hermle AG, Gossheim, Germany). Upon redispersion the total amount of precipitated LYS aqueous alginate solutions (25 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 were mixed with a high shear mixer (Ultra-Turrax, Germany) for 15 seconds, followed by mixing for 1 h and 2 h. After the mixing time, the samples were centrifuged again, the supernatant was separated and finally lyophilized at −25˚C for 24 h under a pressure of 1.3 Pa, and then kept at 25˚C for 24 h for secondary drying to obtain lyophilized powders by using a Scanvac Coolsafe laboratory freeze-dryer (LaboGene, Denmark). The samples were pre-frozen at -10 ±2˚C before lyophilization. The samples were stored at -10±2˚C until further investigations.

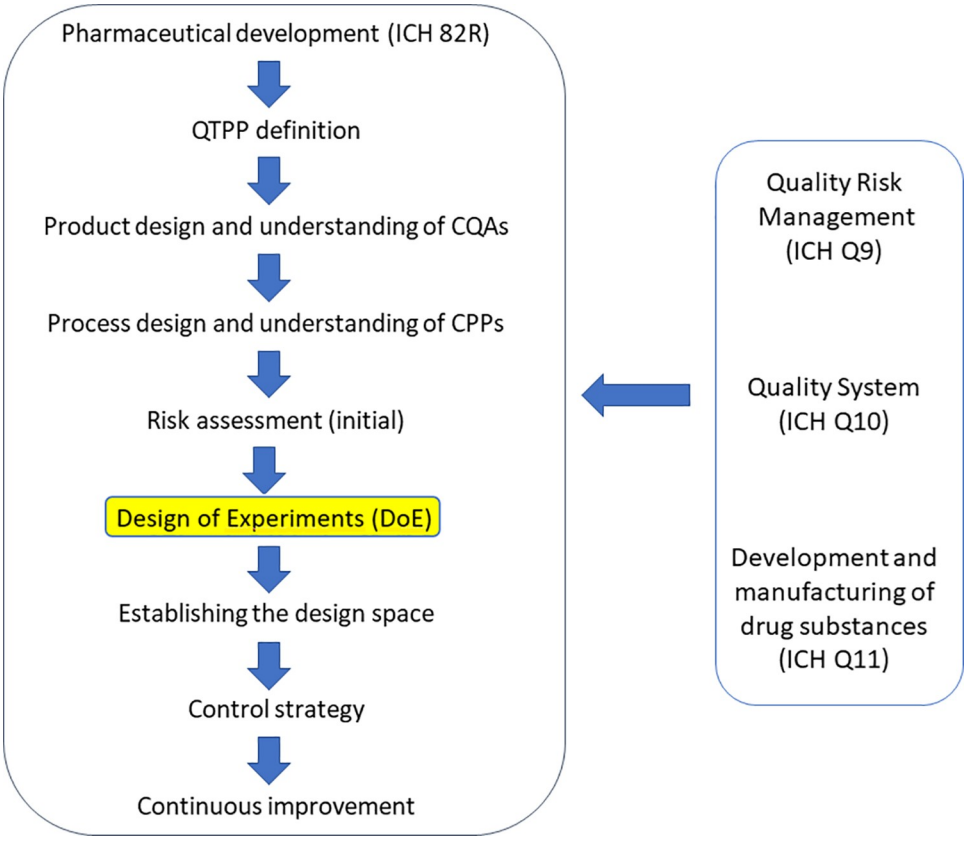

Fig 1. Steps of the extended QbD method.

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Experimental design. Following the guidelines of QbD, the steps shown in Fig 1 were performed [27]. Based on the results of the risk analysis, the next step was to select the factors for the factorial experimental design. The factors selected were the concentration of alginate, pH and mixing time, which were used on 2 levels in DOE (Table 1).

The experiments were conducted according to 2^3 full factorial design, 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. Enzyme activity, particle size, encapsulation efficiency and amount of α-helix were optimization parameters.

The 2^3 full factorial design (DOE) was applied by Tibco Statistica v13.4.0.14 (Statsoft, Tulsa, OK, USA) software for the evaluation of the effect of factors on the optimization parameters.

Table 1. Factor values for samples according to 2^3 full factorial design.

Sample	c (alg.)%	pH	Mixing time (h)
1	0.004 (-1)	6 (-1)	1 (-1)
2	0.004 (-1)	10 (+1)	1 (-1)
3	0.006 (+1)	6 (-1)	1 (-1)
4	0.006 (+1)	10 (+1)	1 (-1)
5	0.004 (-1)	6 (-1)	2 (+1)
6	0.004 (-1)	10 (+1)	2 (+1)
7	0.006 (+1)	6 (-1)	2 (+1)
8	0.006 (+1)	10 (+1)	2 (+1)

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Precipitation and encapsulation efficiency. 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 a UV spectrometer (ThermoScientific-Genesys 10 S UV-Vis Spectrometer, USA) at lambda max 281 nm. Based on the absorbance, the concentration of unprecipitated NP enzymes was used to calculate precipitation efficiency.

The obtained supernatants were carefully separated from the encapsulated NPs and absorption was measured at 281 nm for each sample based on a pre-recorded calibration line. The concentration of free enzyme NPs was measured for all samples, from which encapsulation efficiency was determined. Encapsulation efficiency (EE%) resulting from the adsorption of alginate on the LYS composite was calculated according to the following equation:

$$EE\% = \frac{(total\ amount\ of\ LYS\ (mg) - amount\ of\ LYZ\ in\ the\ supernatant\ sol.(mg))}{total\ amount\ of\ LYS\ (mg)} * 100 \text{ Eq 1}$$

Particle size and Zeta potential measurement. The precipitated NPs were adequately diluted, and the particle size of the sample was measured with a Malvern Mastersizer (Malvern Instruments, Malvern, UK). The Zeta potential of the same sample was measured with a Malvern Zetasizer apparatus with three parallel measurements (Malvern Instruments, Malvern, UK).

Morphological study. The structure and the morphology of the NPs (after layering) were described with transmission electron microscopy (TEM). The TEM images were made with a FEI TecnaiTM G2 X-Twin HRTEM microscope (FEI Company, Hillsboro, Oregon, US) with accelerating voltage of 200 kV in bright field mode. The samples were suspended in ethanol and dropped onto a carbon film-coated copper grid.

Enzyme activity of layered NPs. The activity of the prepared layered nanoparticle samples was recorded by measuring the degradation of lyophilized *Micrococcus lysodeikticus* by using a UV spectrometer (ThermoScientific-Genesys 10 S UV-Vis Spectrometer, USA). 25 mg of lyophilized bacterial cells was dispersed in 100 ml of phosphate buffer (pH 6.8); the basic absorption at 450 nm was around 0.7. The absorptions of the bacterial suspension were measured for 5 minutes before each test to reduce the error arising from bacterial sedimentation. 10 mg of the layered NPs or 10 mg of crude LYS were dissolved in 25 ml of phosphate buffer. 0.1 ml of layered NPs/or crude enzyme solution was added to 2.5 ml of bacterial suspension and shaken for 20 seconds in a quartz cuvette, then the change in bacterial absorption was measured for 5 minutes. LYS activity was calculated from the percentage degradation of the bacterial cells relative to crude LYS activity as a reference.

Fourier-transform infrared spectroscopy (FT-IR). The infrared spectra of the prepared samples and the other excipients were obtained with a FT-IR (Avatar 330 FT-IR ThermoScientific, USA) apparatus, by using the potassium bromide disc method, scanning was run in the wavelength range of 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 (version 1.2.14.; Dr. Friedrich Menges Software-Entwicklung, Germany) software was used for the second derivation of spectra. For the deconvolution of the second derivatives, the Fityk software was used [28]. After assigning the peaks, the area under the curve was calculated. From these data the α-helix content was determined.

Circular Dichroism spectroscopy (CD). To determine the α-helix content of the initial LYS and the synthesized NPs, circular dichroism (CD) spectra were recorded on an ABL&E--JASCO J-1100 CD spectrometer between 250–190 nm. For the measurements, a 4-opened quartz cuvette with an optical length of 1 cm was used, and the solid samples were dissolved in

PBS buffer applying a protein concentration of 0.04 mg/mL. The spectra were corrected with the PBS buffer background. The α-helix content was calculated by using the following equations [29]:

$$[\![MRE]\!]_{208} = [\Theta]/n \text{ and} \quad \text{Eq 2}$$

$$\alpha - helix\ \% = ([\![MRE]\!]_{208} - 4000)/(33000 - 4000) * 100, \quad \text{Eq 3}$$

where [Θ] is molar ellipticity at ca. 208 nm, n is the number of the amino acids, 33000 is the pure -helix content at 208 nm, while 4000 is the amount of the β-sheet and random coil.

Results and discussion

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 (Table 2). 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.

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 (Fig 2). 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 4*). Fig 2 reveals that this factor had an effect on EE only in the lower pH range. In the higher pH range dissolution did

Table 2. Encapsulation efficiency results.

Sample	Encapsulation efficiency (%)
1	65.17
2	65.76
3	65.65
4	65.87
5	62.98
6	64.21
7	63.64
8	66.35

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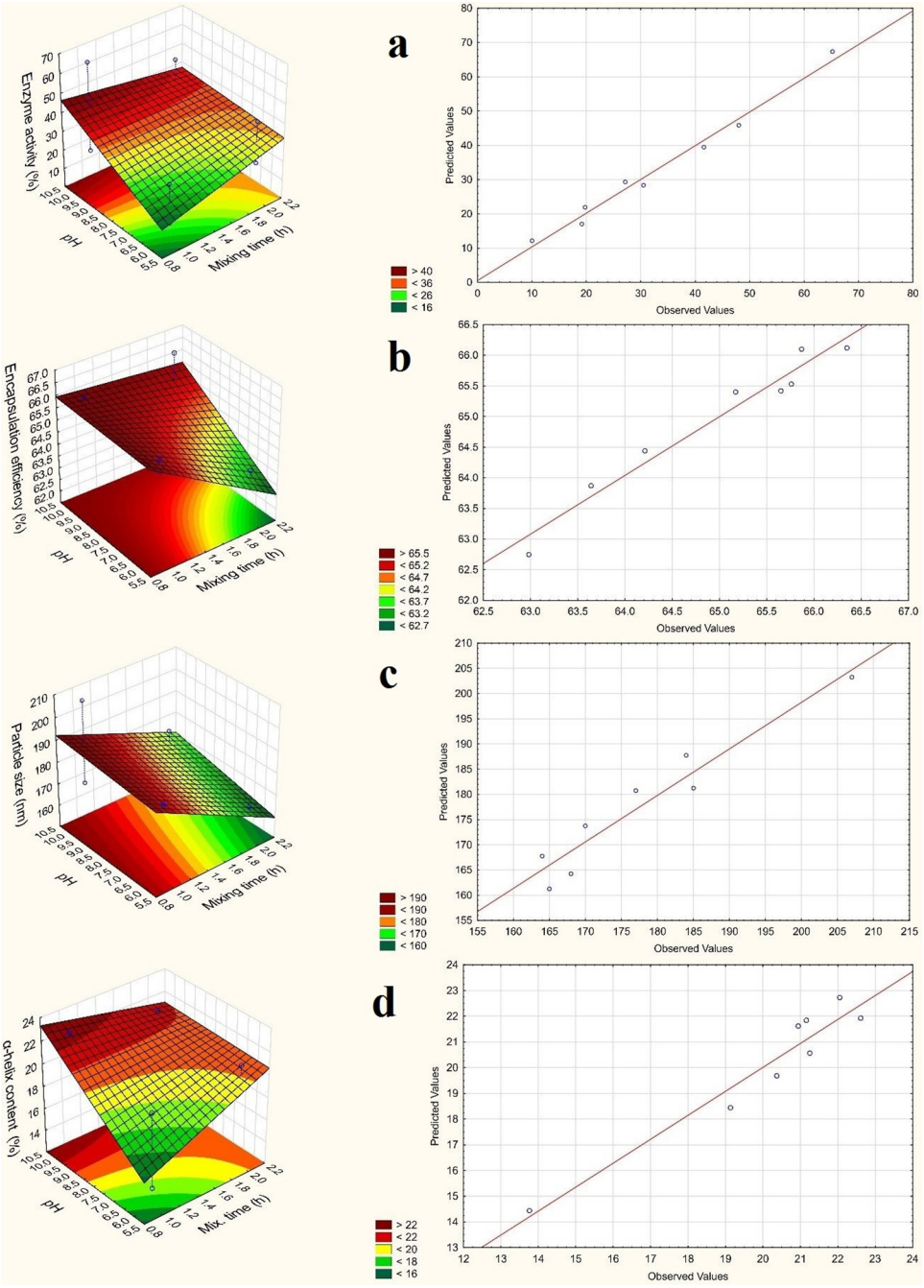

Fig 2. The response surfaces (alginate concentration on zero level) and the predicted values of (a) enzyme activity; (b) EE%; (c) particle size; (d) α-helix content.

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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.

Table 3. Particle size and zeta potential results.

Sample	Particle size (nm)	Zeta potential (mV)
1	185±1	-18.2
2	170±6	-19.8
3	184±2	-17.6
4	207±3	-18.6
5	164±1	-17.9
6	168±2	-17.5
7	165±1	-18.2
8	177±2	-18.1

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The predicted and the observed values can be seen in Fig 2. 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.

$$y = 64.95^{*} + 0.85x_1 + 1.19x_2 - 1.32x_3 + 0.28x_1x_2 + 0.55x_1x_3 + 0.78x_2x_3 \quad \text{Eq 4}$$

R^2 = 0.9867; MS Residual: 0.7875

* statistically significant ($p<0.05$)

Results of particle size and Zeta potential

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 (Table 3). 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.

It can be seen in Fig 2 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 5*), 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 5*) 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.

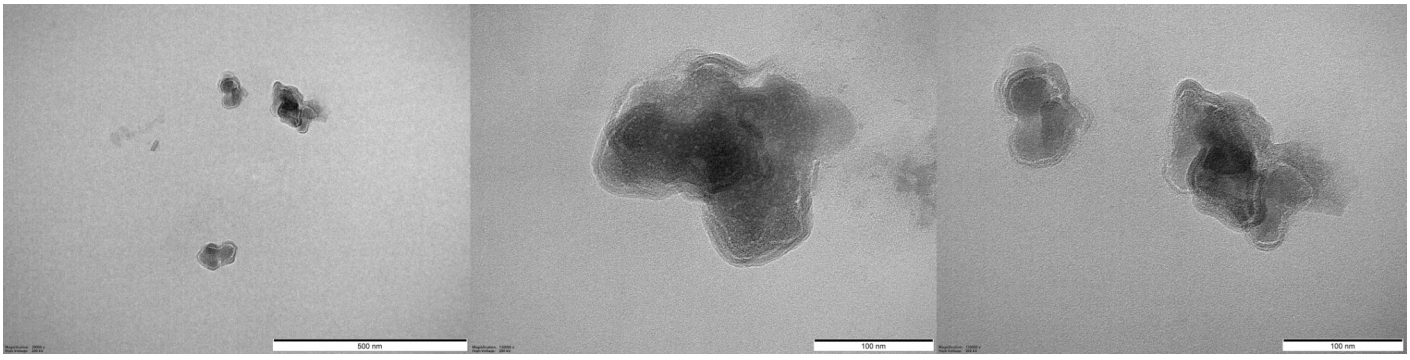

Fig 3. Representative TEM pictures of alginate layered NPs (Sample 2).

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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. Fig 2 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.

$$y = 177.5^{*} + 5.75x_1 + 3.0x_2 - 9x_3 + 5.75x_1x_2 - 3.25x_1x_3 + 1.0x_2x_3 \quad \text{Eq 5}$$

R^2 = 0.9226; MS Residual: 112.5

* statistically significant ($p<0.05$)

The alginate layer on the surface of the precipitated LYS can be observed well (Fig 3). 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.

Enzyme activity

Enzyme activity was measured according to the speed coefficient of the degradation of *Micrococcus lysodeicticus* cell wall. Table 4 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 (Fig 2), 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:

$$y = 32.92^{*} + 7.94x_1 + 6.96x_2 + 1.20x_3 + 8.78x_1x_2 - 8.19x_1x_3 - 3.51x_2x_3 \quad \text{Eq 6}$$

R^2 = 0.9838; MS Residual: 28.69

* statistically significant ($p<0.05$)

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 6*). 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 Fig 2. It can show the accuracy of the calculated

Table 4. The determined enzyme activity (%) values of the different samples.

Sample	Enzyme activity (%)
1	12.10
2	19.18
3	30.49
4	65.20
5	41.60
6	27.14
7	19.77
8	47.99

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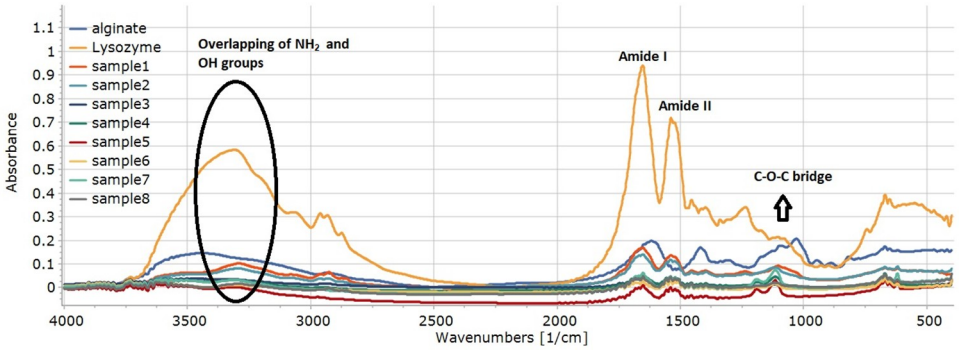

Fig 4. FT-IR spectra of the different samples.

https://doi.org/10.1371/journal.pone.0260603.g004

mathematical model for enzyme activity. This means that enzyme activity can predict well in this range with the application of this mathematical model.

FTIR and 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 (Fig 4). The amide I region can be found between 1700–1615 cm^{-1} [30].

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 Fig 5. 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 Table 4 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% [31]; 34% in phosphate buffer pH 5.1 [32]; 40% in D_2O solution [33]; 30% in water [34]). 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 (Fig 2). However, Szigeti et al. concluded that pH changes in the 6.0–8.7 range cannot influence the secondary structure of the protein significantly, but in this study only the horseradish peroxidase was investigated [35].

This tendency correlates very well with the enzyme activity results (Fig 2). 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 7*), 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

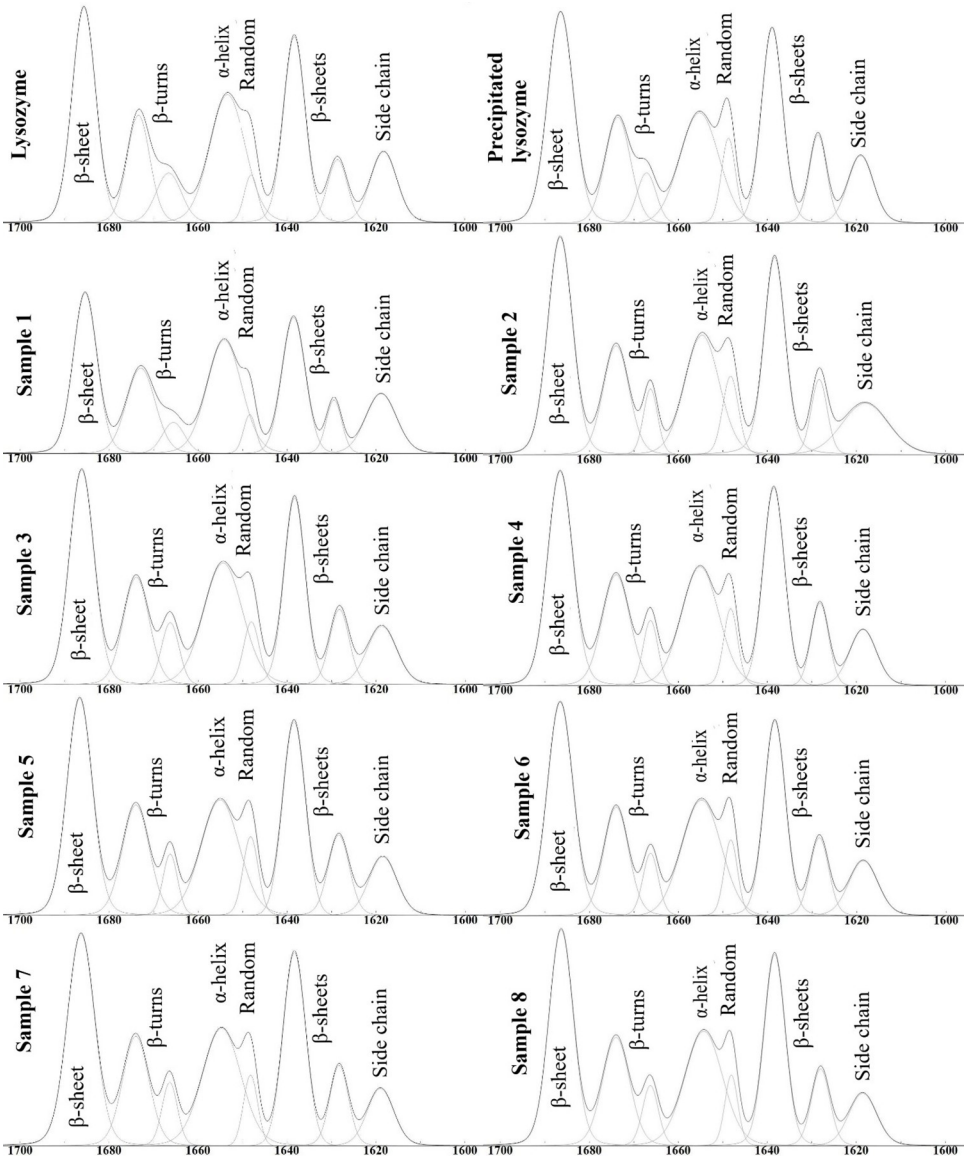

Fig 5. Deconvolution of FT-IR spectrum of LYS, precipitated LYS and the samples.

https://doi.org/10.1371/journal.pone.0260603.g005

α-helix increases slightly with increasing alginate concentration and mixing time.

$$y = 20.16^{*} + 0.99x_1 + 1.61x_2 + 0.46x_3 - 1.11x_1x_2 - 0.52x_1x_3 + 1.02x_2x_3 \quad \text{Eq 7}$$

$R^2 = 0.9318$; MS Residual: 3.71

* statistically significant ($p<0.05$)

CD spectroscopy

As shown in Fig 6, the spectra of LYS NPs and alginate-LYS core-shell nanostructures consist of more disordered secondary structures than the initial LYS. 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,

Table 5. The α-helix content of the samples.

Sample	Content of α-helix (%)
LYS	22.69
Precipitated LYS	19.66
1	13.76
2	22.61
3	20.37
4	22.05
5	19.13
6	21.16
7	20.94
8	21.25

https://doi.org/10.1371/journal.pone.0260603.t005

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.

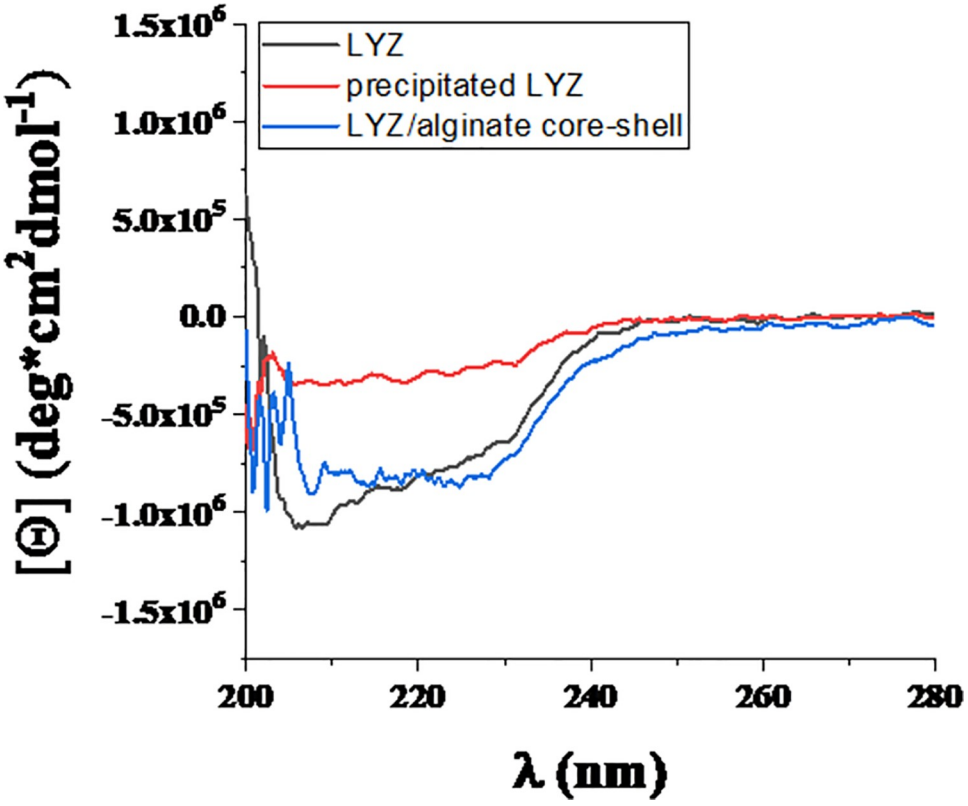

Fig 6. Results of CD spectroscopy for LYS, precipitated LYS and core-shell NPs.

https://doi.org/10.1371/journal.pone.0260603.g006

Conclusions

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.

Supporting information

S1 File.
(PDF)

Author Contributions

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Funding acquisition: Ildikó Csóka.

Investigation: Reihaneh Manteghi, Yousif H-E. Y. Ibrahim, Ditta Ungor, Edit Csapó, Dániel Berkesi, Zoltán Kónya.

Methodology: Katalin Kristó.

Supervision: Ildikó Csóka.

Visualization: Dániel Berkesi, Zoltán Kónya.

Writing – original draft: Katalin Kristó, Reihaneh Manteghi.

Writing – review & editing: Ildikó Csóka.

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