Smart tools for antimicrobial peptides expression and application

Colomina-Alfaro, Laura; Marchesan, Silvia; Stamboulis, Artemis; Bandiera, Antonella

DOI:
10.1002/bit.28283

License:
Other (please specify with Rights Statement)

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Colomina-Alfaro, L, Marchesan, S, Stamboulis, A & Bandiera, A 2023, 'Smart tools for antimicrobial peptides expression and application: the elastic perspective', *Biotechnology and Bioengineering*, vol. 120, no. 2, pp. 323-332. https://doi.org/10.1002/bit.28283

MINIREVIEW

Smart tools for antimicrobial peptides expression and application: the elastic perspective

Laura Colomina-Alfaro[1], Silvia Marchesan[2], Artemis Stamboulis[3], and Antonella Bandiera[1*]

[1]Department of Life Sciences, University of Trieste, 34127 Trieste, Italy

[2]Department of Chemical and Pharmaceutical Sciences, University of Trieste, 34127 Trieste, Italy

[3]School of Metallurgy and Materials, Biomaterials Research Group, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[*]Correspondence:

Antonella Bandiera, Department of Life Sciences, University of Trieste, Via Licio Giorgieri 1, I-34127, Trieste, Italy.

Email: abandiera@units.it

https://orcid.org/0000-0002-0376-9291

ABSTRACT

In recent years, antimicrobial peptides (AMPs) have become a promising alternative to the use of conventional and chemically synthesized antibiotics, especially after the emergence of multidrug-resistant organisms. Thus, this review aims to provide an updated overview of the state-of-the-art for producing antimicrobial peptides fused or conjugated with the elastin-like (ELP) peculiar carriers, and that are mostly intended for biomedical application. The elastin-like biopolymers are thermosensitive proteins with unique properties. Due to the flexibility of their modular structure, their features can be tuned and customized to improve the production of the antimicrobial domain while reducing their toxic effects on the host cells. Both fields of research faced a huge rise in interest in the last decade, as witnessed by the increasing number of publications on these topics, and several recombinant fusion proteins made of these two domains have been already described but they still present a limited variability. Here in, the approaches described to recombinantly fuse and chemically conjugate diverse AMPs with ELPs are reviewed, and the nature of the AMPs and the ELPs used, as well as the main features of the expression and production systems are summarized.

KEYWORDS

Antimicrobial peptides

Elastin-like polypeptides

Recombinant expression

Conjugation

1. INTRODUCTION

In the last decades, the sudden rise of bacterial resistance against conventional chemically synthesized antibiotics has boosted the seek for novel molecules and strategies to fight infections. This defensive response of bacteria underpins the occurrence of the so-called superbugs that cause infections that are hard to treat and eradicate, mainly due to the emergence of new resistance mechanisms that quickly spread globally. Alternative treatments based on innovative antimicrobial mechanisms are urgently required to withstand the threat of multi-drug resistant (MDR) bacterial and nosocomial infections.

Antimicrobial peptides (AMPs) naturally occur as a component of innate immunity, and are widely produced by many diverse organisms. They represent the earliest physiological response of the living entities (animals and plants) and were evolved by them in the fight for survival (Magana et al., 2020). For this reason, they seem to be less prone to give rise to bacterial resistance. Thus, using these peptides instead of chemically synthesized antibiotics is considered a powerful tool to counteract the phenomenon of bacterial resistance.

There is a general agreement that AMP's multiple site-targeting mechanisms of action, together with the rapid microorganism killing capacity, hinder the development of resistance, in contrast to what happens for conventional antibiotics. In addition, some AMPs exhibit other favorable properties, such as anti-inflammatory, regenerative and anticancer capacities that extend their opportunity to be employed in many other clinical applications (Rai et al., 2022; Ramazi et al., 2022).

However, there are still several concerns that hinder the introduction of AMPs into the market soon, such as the risk of toxicity and adverse reactions, often linked to the issue of finding an adequate delivery route to the infection site as well as the high costs for their production on a commercial scale (Wibowo & Zhao, 2019). The feasibility of AMP employment as antibiotics is strictly connected with the peptide availability in an appropriate amount, and in a cost-effective manner (Li, 2011). Moreover, to optimize the therapeutic use of AMPs, the conjugation and functionalization with other polymers or macromolecules have been proven as effective strategies able to fully exploit their antimicrobial activity, the mode of action, the route of delivery as well as the half-life (Bellotto et al., 2022) minimizing their systemic toxicity (Cui et al., 2021). Many AMP conjugation strategies based on chemical and biotechnological approaches have been described and were recently reviewed (Silva et al., 2022).

The recombinant approach is considered an efficient alternative for peptide production on a large scale, offering several advantages with respect to the conventional methods, such as the labor-intensive isolation from natural sources and the costly chemical synthesis (Li, 2011). Most of the described recombinant systems are based on expression in *Escherichia coli* and AMPs are often expressed as fusion proteins, with several advantages ranging from masking the potentially lethal effects on the host microorganism to protecting the peptides from proteolytic degradation (Li, 2009). However, an ideal platform for AMP functionalization and large-scale production is not established yet. Among those described, the recombinant elastin-like polypeptides (ELPs) fusion technology still appears the least exploited. ELPs are macromolecules modeled after elastin first described by Urry (Urry, 1988).

ELPs primary structure is characterized by the presence of repeated motifs, typically the pentapeptidic VPGVG sequence found in the bovine elastin homologue, where the fourth position can be replaced by any "guest" aminoacid except proline (Luan et al., 1992). They retain several biophysical properties peculiar of the native tropoelastin, mainly the lower critical solution temperature (LCST) phase behavior. Above their LCST, also known as the inverse transition temperature (Tt), these polypeptides coalesce, forming insoluble, aggregates that result in a coacervate phase (McDaniel et al., 2013). The Tt is a function of several intrinsic and extrinsic factors that can be controlled, like the amino acid composition of the "guest" residue position, the chain length as well as the polypeptide concentration, and the concentration of other solutes in the buffer. This feature allowed to set up a procedure designated as the inverse transition cycle (ITC) that is a time saving and cost-effective way of purifying the recombinant ELP based proteins (Figure 1). The unique ELPs properties, including their minimal immunogenicity, make them ideal candidates for a variety of biomedical applications, since they benefit from recombinant synthesis and genetically encoded design that enable control over their size, sequence and, consequently, thermos-responsive behavior (Varanko et al., 2020).

Currently, ELPs are considered a strategic fusion partner for components of biological origin (Yeboah et al., 2016).

This review aims to provide an updated overview of the described AMP fusion proteins that use the ELP carriers. All the constructs reported were analyzed, keeping into account the structure, the features of the expression systems, the antimicrobial activity, and the potential applications of this approach. This analysis will be functional to define the state-of-the-art in this cutting-edge technology and, thus, identify its possible future development directions.

2. AMP SEQUENCES SELECTED FOR RECOMBINANT FUSION WITH ELPs

Antimicrobial peptide research is currently one of the most active fields of investigation, as witnessed by the huge number of reviews on this topic in the last decade. It is reported that approximately six thousands of AMPs have been either isolated from natural sources or chemically synthesized. Less than one hundred peptide drugs have reached the market, but several hundreds of novel therapeutic peptides have undergone the route for drug development (Boparai & Sharma, 2020). However, according to the FDA database, less than ten are currently approved for clinical applications (Rai et al., 2022).

Many approaches were described for biotechnological AMP production routes, such as the fusions with many different carriers (reviewed in (Li, 2009), expressed mainly in the *E. coli* bacterial system (Li, 2011). The first report of a recombinantly AMP expressed as an ELP fusion dates back to 2008, and from 2010 to the present twenty constructs of this kind have been successfully produced mainly using bacterial recombinant systems, whereas five of them were produced in a plant expression system (see Table 1).

An analysis of the AMP sequences that were selected as ELP fusion partners reveals that their size ranges from 12 to 69 amino acids, and with respect to the total fusion protein, they represent from about 1/70 to 1/3 of the whole macromolecule mass (Table 1). The majority of the selected AMP sequences were cationic as most of the AMPs, with the net charge ranging from +1 to +7. Among them, about half presented the amphipathic α-helical structure and the others are cathelicidin and defensin-like peptides (Table 1).

All the anionic AMPs described as ELP fusion partners are characterized by the presence of intra-chain disulfide bonds, showing a cysteine knot, which is typical for defensin-like structures. Only one was expressed as an ELP fusion in a bacterial recombinant system (Table 1, #5), whereas the other negatively charged AMPs were successfully expressed as ELP fusions in a plant system, (Table 1, #14 to #18) according to the finding that, in general, AMPs of plant origin are significantly less cationic than the others (Ghidey et al., 2020).

3. STRATEGIES EMPLOYED FOR ELP FUSION AND ELP FUNCTIONALIZATION WITH AMP

Three main approaches for positioning the AMP domain within the ELP fusion construct were described (Fig. 2A-C).

Seven AMPs were placed at the C-terminus of the ELP region (ELP-AMP). Five of them were placed after an intein domain to trigger their release from the expressed construct by self-

splicing of the intein (Fig. 2A, Table 1, #1). Inteins are indeed widely used as auto-processable tools for protein splicing so that adjacent domains are post-translationally linked together with the extrusion of the intein domain (Shah & Muir, 2014). The other two were preceded by the enterokinase proteolytic domain, to be enzymatically released without additional amino acids at their N-terminus (Fig. 2A).

Several ELP fusion constructs carrying the AMP at the N-terminal end (AMP-ELP) were described (Fig. 2B). Three of them were placed before a chemical cleavage site to release the AMP (Table 1, #2a, #8 and #9) whereas two were fused to functionalize the ELP moiety for the realization of biomaterial endowed with antimicrobial properties (Table 1, #10 and #11). The last four fusion constructs were based on a modified ELP domain containing alternate blocks of silk-derived repeats (Table 1, #2b, #9a to 11a). In addition, other N-terminal AMP-ELP fusion constructs produced in a plant expression system were described (Table 1, #14 to #18).

In the third approach, the AMP domain was embedded in the middle of the ELP moiety (ELP-AMP-ELP). In this configuration, the N-terminal ELP domain is intended as a protective "sacrificial block" which is subsequently cleaved by CNBr to release an N-terminal AMP fusion with ELP (Fig. 2C and Table 1, #12, #13 and #13a).

In addition, ELP conjugation with AMPs was reported (Fig. 2D). Two different chemical methods were employed. In one of them, a synthesized D-AMP enantiomer was covalently bonded to the expressed ELP by "click chemistry" (Table 1, #19) and in the other ELP was functionalized with the AMP by the EDC/NHS chemistry (Table 1, #20).

Overall, it emerges that AMPs belonging to different classes and spanning the entire range of length were successfully expressed as N-terminal and C-terminal ELP fusions, as well as in the middle of the ELP moiety (Fig. 2). Most of the described constructs were aimed to obtain the AMP without any modification as an alternative route to the chemical synthesis. The maximum yield reported for purified AMPs released from the ELP fusion construct was almost 100 mg/L (Table 1, #4).

Interestingly, in some cases, the whole ELP fusion construct showed to possess the antimicrobial activity conferred by the AMP domain, (Table 1, #5, #14 to #18). On the other hand, in several cases, the ELP fusion protein was designed with the aim of obtaining a bioactive component endowed with antimicrobial properties for the realization of biomaterials, matrices, and surfaces (Table 1, #10, #11, #13, #13a),.

The yield of the AMPs that were released by different means and purified ranged from 0.5 mg/L to above 100 mg/L under optimized expression conditions (Table 1). However, it should be noted that all of the reported recombinant fusions (except those expressed in plants) were produced by the T7 expression system using vectors of the pET series. From this point of view, there is likely room for further improvement in production.

4. ELP SEQUENCES EMPLOYED AS FUSION PARTNERS

The interest that AMPs rise as an alternative approach to antibiotics of chemical synthesis is linked to their potential application as novel antimicrobial therapeutics. However, this implies that they should be produced cost-effectively. The recombinant expression is still considered one of the most appealing routes to meet the needs for large-scale peptide manufacturing. Although the AMPs recombinant expression is described, the strategy of the antimicrobial domain fusion with a carrier protein has been largely adopted to circumvent toxicity towards the bacterial host and to prevent proteolytic degradation of the peptides themselves (Li, 2009). In this regard, ELPs with their peculiar properties, represent a still underexploited fusion partner for the AMPs. Among the reported ELP constructs with AMPs, it is interesting to analyze the elastin-like sequences that were employed.

The five ELP-AMP fusion constructs based on intein as the system to release the bioactive domain were constituted by an N-terminal ELP region of the VPGXG pentapeptidic repeats ranging from 300 to 550 aa where X was V, G, and A or L. These constructs ranged from about 50 to 72 KDa, carrying an intein domain of about 200 amino acids (Fig. 2A). Successful expression was reported for each construct (Table 1, #1 to #5). The ELP carrier was expected to facilitate the purification of the AMP domain after intein cleavage. It was removed by the ITC (Inverse Transition Cycling) procedure, without the need for further downstream processing (Table 1, #1 to #4). Intriguingly, one of these ELP-AMP fusion proteins was described as endowed with antimicrobial activity (Table 1, #5).

Two of the other ELP-AMP reported fusions carried the cationic elastin-like polypeptide (CELP) made of 36 pentapeptidic repeats adjacent to an enterokinase proteolytic site for the AMP release (Fig. 2A). In both constructs, the composition of the ELP domain was the same, where the X guest residue was V, F, and K (7:1:1) and, the presence of lysine conferred the cationic feature to the ELP backbone. The yield of the released and ITC purified AMPs per 100 ml of culture were comparable (Table 1, #6, and #7).

Analyzing the described AMP-ELP constructs (Fig. 2B), one of them showed an ELP backbone of 90 pentapeptidic repeats where X was V, A, and G (Table 1, #8 and Fig. 2B). It was reported that the ELP length had a dramatic effect on the fusion protein products and, in this case, the longest ELP domain was selected (Hu et al., 2010). The yield of this fusion protein was 69 mg/L of culture and the AMP recovery after the hydroxylamine chemical cleavage and purification was 1.7 mg/L (Table 1, #8).

The other four AMP-ELP fusion constructs were designed and produced with a very long ELP backbone of 1000 aa made of 200 pentapeptidic repeats where X was A (Fig. 2B). Two of them carried the formic acid chemical cleavage site to release the N-terminal AMP (Table 1, #2a, and #9). Thus, ELP was used as the purification tag as in the previous examples. However, in this case, after the chemical cleavage and the ITC purification, a chromatographic step was required to improve the recovery of the AMPs (Pereira et al., 2021). This approach showed a yield about 2-times higher for the Moricin CM4, relative to that obtained with the ELP-intein-AMP fusion setup (Table 1, #2 and Fig. 2A). The other two AMP-ELP fusion proteins were intended as a kind of AMP "conjugates" to obtain materials endowed with antibacterial activity, such as micro-particles and free-standing films since it was described that the whole AMP-ELP macromolecules possessed antibacterial activity when tested by a modified agar diffusion method. The yield was 50 and 108 mg/L for each fusion protein, respectively (Table 1, #10, and #11).

The last approach for ELP fusion with AMP consisted in placing the AMP between two ELP blocks (Fig. 2C). The N-terminal ELP is described as a "sacrificial" 50 repeats pentapeptidic block that is expected to protect the host from the toxic side-effects of the AMP while increasing the expression levels as well as enabling the site-specific CNBr cleavage (Table 1, #12 and #13). In this block, the X guest residues were V and E conferring an acidic nature to this domain. Following this strategy, these authors designed and produced several constructs, bearing this sacrificial block followed by the AMP. Two different C-terminal ELP domains were selected for the fusion, one with an amphiphilic di-block structure and the other with a cationic nature (Fig. 2C). These products were expressed at higher yields compared to the other reported fusion constructs, ranging from 380 to 600 mg/L (Table 1, #12, #13 and #13a).

The last strategy consisted of the chemical conjugation between ELP and AMP by click chemistry and by EDC/NHS coupling (Fig. 2D). In both cases, a cationic ELP was selected for conjugation and it was employed as a scaffold on which the chemically synthesized AMP was

covalently bound to confer antimicrobial properties to the derived material (Table 1, #19, and #20).

The AMP-ELP fusion expressed in plants had the shortest ELP domains corresponding to 28 repeats (Table 1, #14 to #18). Unexpectedly, only the uncleaved AMP-ELP fusions showed antimicrobial activity, whereas after enzymatic cleavage no activity was detected. The authors ascribed the loss of activity of the released AMP to a loss of structural integrity maintained by the ELP fusion partner close to the smaller AMP (Ghidey et al., 2020).

5. CONCLUSION & FUTURE PERSPECTIVE

Most of the studies described the use of the ELP as a tag either to purify the whole fusion protein or to selectively isolate the AMP from the ELP itself exploiting the ITC. Different strategies to release the AMP from the fusion protein were described, e.g., the intein-based excision as well as the chemical and the enzymatic cleavage (see Table 1). The employment of the ELP carrier as an alternative and effective route for the active AMPs production was proposed in most cases. The other expected applications are related to the employment of the ELP fusion proteins as the basic components for new biomaterials endowed with antimicrobial activity. Table 2 briefly summarizes the possible applications.

Several AMPs with diverse features were selected from different authors for the fusion with the ELP carrier. Their lengths span from one to six dozens of amino acids. Most of them are cationic and they show different secondary and tertiary structures. Almost all the described recombinant fusions of AMPs with ELPs were successfully expressed in T7-based expression systems.

In the described constructs, the bioactive AMP domain was placed at either the N-terminus or C-terminus end of the ELP, as well as in the middle of two ELP blocks. All these fusion proteins were successfully expressed with a variable yield, depending on the recombinant construct and culture conditions. Most of them resulted in the active AMP domain recovery and, intriguingly, some fusion proteins showed antimicrobial activity too, irrespective of the N-terminal or C-terminal placement of the AMP. All the AMP-ELP uncleaved fusion constructs that were expressed in plants demonstrated strong antibacterial activity.

In summary, the structure of the ELPs employed for the fusion with AMPs consisted of repetitions of the pentapeptidic motif VPGXG from bovine elastin ranging from 36 to 200 repeats and resulting in fusion constructs of the total mass varying from about 20 to 90 KDa. From the point of view of the amino acid composition, only a few types of ELP carriers were

employed. The guest X amino acid was mainly V, G, and A, sometimes L and F were introduced, and K and E were used to confer basic or acidic nature to the ELP block, as well as to allow for chemical conjugation. Other described variations in the pentapeptidic motif were VPAVG, VPGSG, and IPGVG. A hybrid ELP containing blocks of silk-derived repeats (SELP) was also described as the carrier for AMPs.

ELPs were reported to be effective to avoid the adverse effect of the fused AMPs on the expression host as well as to improve the expression yield. However, the length of the ELP domain was recognized as a key parameter affecting the yield of the fusion proteins. The presence of the ELP domain was described to facilitate protein solubility, avoiding inclusion body formation.

Overall, ELPs have been shown to be a versatile platform to express different kinds of AMPs and recover their functionality. However, the reported examples showed little variability regarding both the ELP sequences that were employed and the expression systems that were used for their production. The interesting finding that some AMP domains conferred antimicrobial activity to the whole fusion construct points to ELP as a versatile scaffold to support the AMP itself, opening the way to the realization of new materials endowed with antimicrobial properties.

From this point of view, the potential of ELPs as a modular carriers for AMPs appears still underdeveloped so there is room for the design of unexplored combinations to improve the production and the performance of new constructs and their derived materials. New approaches offer intriguing opportunities, such as machine-learning algorithms, to optimize the antimicrobial sequences by improving the activity and avoiding microbial resistance. New technologies such as 3D printing are also attractive to make a qualitative leap in manufacturing. The adoption of such modern techniques spanning form *in silico* to experimental production holds the key to the wide application of this kind of versatile recombinant fusions in the field of active materials and coatings for medical devices and beyond.

ACKNOWLEDGEMENT

The authors are grateful to Prof. Giorgio Manzini that critically reviewed the manuscript. This study was supported by the Horizon 2020 Innovative Training Network AIMed under the Marie Skłodowska-Curie, grant agreement No 861138.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Fig. 1

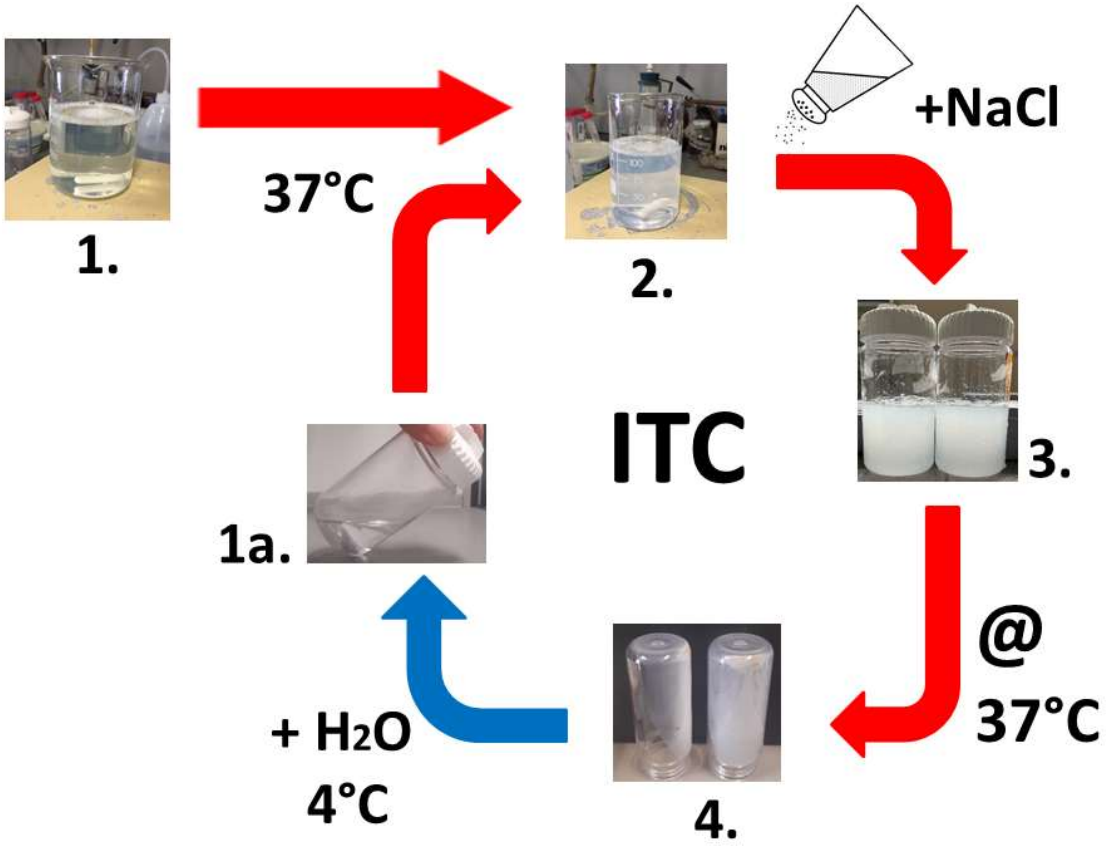

Fig. 2

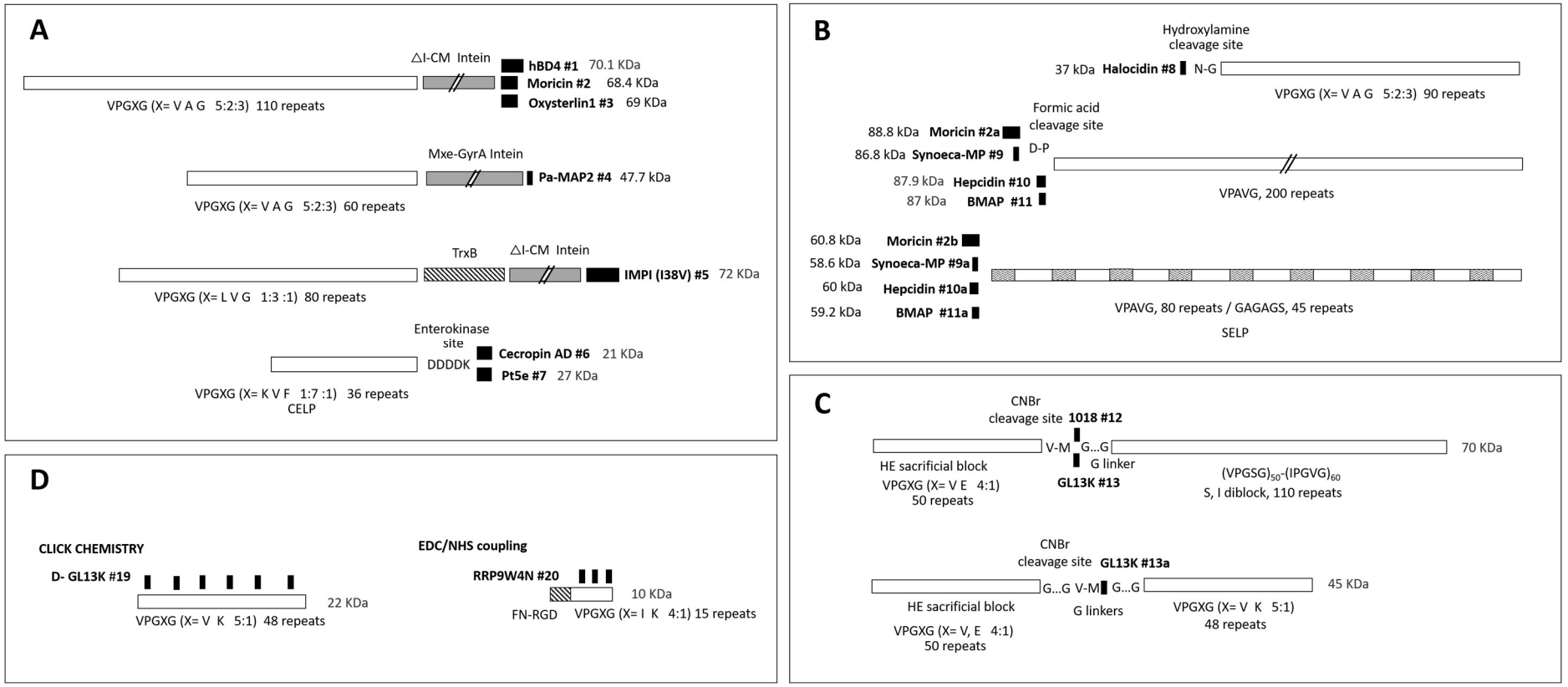
A
△I-CM Intein
hBD4 #1 70.1 KDa
Moricin #2 68.4 KDa
Oxysterlin1 #3 69 KDa
VPGXG (X= V A G 5:2:3) 110 repeats
Mxe-GyrA Intein
Pa-MAP2 #4 47.7 kDa
VPGXG (X= V A G 5:2:3) 60 repeats
TrxB
△I-CM Intein
IMPI (I38V) #5 72 KDa
VPGXG (X= L V G 1:3 :1) 80 repeats
Enterokinase site
DDDDK
Cecropin AD #6 21 KDa
Pt5e #7 27 KDa
VPGXG (X= K V F 1:7 :1) 36 repeats
CELP
B
Hydroxylamine cleavage site
37 kDa Halocidin #8 N-G
VPGXG (X= V A G 5:2:3) 90 repeats
Formic acid cleavage site
88.8 kDa Moricin #2a
86.8 kDa Synoeca-MP #9
87.9 kDa Hepcidin #10
87 kDa BMAP #11
D-P
VPAVG, 200 repeats
60.8 kDa Moricin #2b
58.6 kDa Synoeca-MP #9a
60 kDa Hepcidin #10a
59.2 kDa BMAP #11a
VPAVG, 80 repeats / GAGAGS, 45 repeats
SELP
C
CNBr cleavage site 1018 #12
V-M
G...G
G linker
70 KDa
HE sacrificial block
VPGXG (X= V E 4:1) 50 repeats
GL13K #13
(VPGSG)50-(IPGVG)60
S, I diblock, 110 repeats
CNBr cleavage site GL13K #13a
G...G V-M G...G
45 KDa
HE sacrificial block
VPGXG (X= V, E 4:1) 50 repeats
G linkers
VPGXG (X= V K 5:1) 48 repeats
D
CLICK CHEMISTRY
D- GL13K #19
22 KDa
VPGXG (X= V K 5:1) 48 repeats
EDC/NHS coupling
RRP9W4N #20
10 KDa
FN-RGD
VPGXG (X= I K 4:1) 15 repeats

Table 1.

N°	AMP domain	AMP Sequence and information	AMP source and main features	Construct reference and main features
#1	human b-defensin 4 (hBD4) 50aa	EFELDRICGYGTARCRKKCRSQEYRIGR CPNTYACCLRKWDESLLNRTKP https://dbaasp.org/peptide-card?id=18490	From *Homo sapiens* cationic (+6), disulfide bonds Gram+, Gram-, Cancer, Mammalian Cell	**#1:** DOI: 10.1016/j.micres.2010.01.002 Intein-based release Released AMP yield: 1.8 mg/L Tt below 30° C in high-salt solution
#2 **#2a** **#2b**	Moricin CM4 ABP-CM4, 35aa	RWKIFKKIEKVGQNIRDGIVKAGPAVAV VGQAATI https://dbaasp.org/peptide-card?id=3460	From *Bombix mori* cationic (+6) Active against Cancer, Fungus, Mammalian Cell	**#2:** DOI: 10.1016/j.micres.2010.01.002 Intein-based release Released AMP yield: 0.6 mg/L Tt below 30° C in high-salt solution **#2a:** DOI: 10.1021/bm5016706 Chemical cleavage (formic acid) - Released AMP yield: 1.5 mg/ 100 mg fusion protein Tt 31.6°C **#2b:** DOI: 10.3390/app11125352 Active fusion yield: 120 mg/L Tt below 37° C
#3	Oxysterlin1 39aa	GSKRWRKFEKRVKKIFEETKEALPVVQ GVVAVATAVGRR https://dbaasp.org/peptide-card?id=10889	From *Oxysternon conspicillatum* Cationic (+7), amphiphilic Gram+, Gram-, Fungus, Mammalian Cell	**#3:** DOI: 10.13345/j.cjb.200625 Intein-based release Released AMP yield: 1.2 mg/L Tt below 37° C in high-salt solution
#4	Pa-MAP2 28aa	LKAAAAAAKLAAKAAKAALKAAAAA AKL https://dbaasp.org/peptide-card?id=9012	Synthetic (inspired from *Pleuronectes americanus* Pa-MAP) Cationic (+6) Gram+, Gram-, Virus, Cancer, Mammalian Cell	**#4:** DOI: 10.1016/j.jbiotec.2016.07.021 Intein-based release Released AMP yield: 96 mg/L Tt below 30° C in high-salt solution
#5	IMPI Insect Metalloprot ease Inhibitor (mutant I38V) 69aa	IVLICNGGHEYYECGGACDNVCADLHI QNKTNCPIINVRCNDKCYCEDGYARDV NGKCIPIKDCPKIRS NCBI Reference Sequence ID: XP_031769425.1	Mutant from *Galleria mellonella* Anionic (-1) 5 Disulfide bonds	**#5:** DOI: 10.3389/fbioe.2019.00150 Intein-based release Active fusion yield: 5 to 20 mg/L of bioactive fusion construct Tt not reported

#6	Cecropin AD 37aa	KWKLFKKIEKVGQRVRDAVISAGPAVA TVAQATALAK https://dbaasp.org/peptide-card?id=6471/	Synthetic cationic (+7) amphipathic Gram+, Gram-, Mammalian Cell	**#6:** DOI: 10.1016/j.pep.2012.04.007 Enzymatic release Released AMP yield:1.2 mg / 100 ml Tt below 30° C in high-salt solution
#7	Pt5e (phosvitin C-term - derived mutant) 55aa	SRMSKTATIIEPFRKFHKDRYLAHHSAT KDTSSGSAAASFEQMQKQNRFLGNDIP https://pubmed.ncbi.nlm.nih.gov/24028820/	Mutant from *Danio rerio* phosvitin Cationic (+4) Gram+, Gram-	**#7:** DOI: 10.1016/j.fsi.2016.09.044 Enzymatic release Released AMP yield: 1.47 mg / 100 ml Tt below 30° C in high-salt solution
#8	Halocidin (subunit A) 18aa	WLNALLHHGLNCAKGVLA https://dbaasp.org/peptide-card?id=3	From tunicate *Halocynthia aurantium* cationic (+1), α-helical structure Gram+	**#8:** DOI: 10.1007/s12010-009-8850-2 Chemical cleavage (hydroxylamine) Released AMP yield: 1.7 mg / 69 mg total protein Tt below 40° C in high-salt solution
#9 **#9a**	Synoeca MP 14aa	INWIKIGKKIIASL https://dbaasp.org/peptide-card?id=18234	From *Synoeca surinama* Cationic, and amphiphilic α-helical Gram+, Gram-, Fungus, Mammalian Cell	**#9:** DOI: 10.3390/ph14100956 Chemical cleavage (formic acid) Released AMP yield: 0.5 mg / 100 mg of fusion Tt ~ 33° C
				#9a: DOI: 10.3390/app11125352 Active fusion yield: 73 mg/L Tt not reported, T increase accelerates gelation
#10 **#10a**	Hep25C (Hepcidin) 25aa	DTHFPICIFCCGCCHRSKCGMCCKT https://dbaasp.org/peptide-card?id=2042	Human cationic (+2), disulfide bonds Gram+, Gram-, Cancer, Fungus	**#10:** DOI: 10.1016/j.nbt.2018.07.001 Active fusion yield: 50 mg/L. Tt 32.3 °C in H2O, 29.7 °C in PBS
				#10a: DOI: 10.3390/app11125352 Active fusion yield: 90 mg/L Tt not reported, T increase accelerates gelation
#11 **#11a**	BMAP-28(1-18),18aa	GGLRSLGRKILRAWKKYG https://dbaasp.org/peptide-card?id=11938	Synthetic, truncated derivative of myeloid antimicrobial peptide 28 from *Bos taurus* Cationic (+7) Gram+, Gram-, Fungus	**#11:** DOI: 10.1021/acsbiomaterials.0c01262 Active fusion yield:108 mg/L Tt 32.7 in H2O, 29.0 °C in PBS
				#11a: DOI: 10.3390/app11125352 Active fusion yield: 70 mg/L Tt not reported, T increase accelerates gelation
#12	1018 12aa	VRLIVAVRIWRR https://dbaasp.org/peptide-card?id=7111	Synthetic Cationic (+5)	**#12:** DOI: 10.1021/acs.biomac.0c00865 chemical cleavage (CNBr)

			Gram+, Gram-, Mammalian Cell	Active fusion yield: 380 to 600 mg/L Tt (heating) 17.5 ± 0.6 (cooling) 13.0 ± 0.6
#13 **#13a**	GL13K 14aa	GKIIKLKASLKLL https://dbaasp.org/peptide-card?id=13151	Synthetic Cationic (+5) Gram+, Gram-, Insect, Mammalian Cell	**#13:** DOI: 10.1021/acs.biomac.0c00865 Chemical cleavage (CNBr) Fusion yield: 380 to 600 mg/L Tt (heating) 19.6 ± 0.5 (cooling) 13.1 ± 0.4
				# **13a:** DOI: 10.1021/acsbiomaterials.9b00247 Fusion yield: 270 mg /L Tt not reported
#14	ADP-2 (Amblyomma defensin peptide 2), 41aa	YENPYGCPTDEGKCFDRCNDSEFEGGY CGGSYRATCVCYRT https://dbaasp.org/peptide-card?id=5176	From *Amblyomma hebraeum* Anionic (-3), non-cationic defensin-like Gram+, Gram-, Fungus	**#14 to #18:** DOI: 10.1016/j.nbt.2019.12.001 Enzymatic release Active fusion yield: 4−113 mg / 200 g of plant tissue Tt below 37° C in high-salt solution
#15	DefensinTK 41aa	SPAIWGCDSFLGYCRLACFAHEASVGQ KECAEGMLCCIPNVF https://dbaasp.org/peptide-card?id=8441	From *Theloderma kwangsiensis* Anionic (-2) disulfide bonds, defensins Gram+, Gram-, Fungus, Mammalian Cell	
#16	PopuDef, 44aa	GASPALWGCDSFLGYCRIACFAHEASV GQKDCAEGMICCLPNVF https://dbaasp.org/peptide-card?id=8198	From *Polypedates puerensis* Anionic (-2) non-cationic defensin Gram+, Gram-, Mammalian Cell	
#17	Laterosporulin 49aa	ACQCPDAISGWTHTDYQCHGLENKMY RHVYAICMNGTQVYCRTEWGSSC https://dbaasp.org/peptide-card?id=5743	From *Brevibacillus sp* Anionic (-1) non-cationic defensin Gram+, Gram-	
#18	SpliDef 50aa	VSCDFEEANEDAVCQEHCLPKGYTYGI CVSHTCSCIYIVELIKWYTNTYT https://pubmed.ncbi.nlm.nih.gov/22067477/	Anionic (-5) non-cationic defensin HQ603825, APD3	
#19	D-enantiomer of GL13K 13aa	gkiiklkaslkll https://dbaasp.org/peptide-card?id=13152	Synthetic Cationic (+5) Gram+, Gram-, Insect, Mammalian Cell	**#19:** DOI: 10.1039/d0bm00155d ELP functionalization by click chemistry high yield ELP fusion Tt not reported
#20	RRP9W4N 13aa	RRPRPRPRPWWWW-NH2 https://dbaasp.org/peptide-card?id=9325	Synthetic Cationic (+6), Proline-rich Gram+, Gram-, Mammalian Cell	**#20:** DOI: 10.1016/j.actbio.2018.10.039 ELP functionalization by EDC/NHS coupling Tt below 37° C

Table 2

N°	Applications
#1	Production of hBD4
#2	Production of moricin
#2a	Production of cast film for skin application
#2b	Production of free-standing films
#3	Model for large-scale production of antimicrobial peptides
#4	Production of Pa-MAP 2
#5	Scale-up production active IMPI containing multiple disulfide bonds using minimal medium
#6	Production of Cecropin AD
#7	Production of antibiotic for MRSA resistant bacteria
#8	Production/purification of Halocidin
#9	Up-scalable biotechnological platform for AMP production
#9a	Production of free-standing films
#10	Production of AMP, use in microbial infections, advanced drug-delivery systems
#10a	Production of free-standing films
#11	Production of cast film for skin application candidates for new drug-free polymers endowed with antimicrobial properties.
#11a	Production of free-standing films
#12	Molecular tools in the development of self-assembling nanosystems with potential use for biotechnological and biomedical applications
#13	
#13a	Self-assembled monolayers for realization of advanced, medical devices to prevent infection
#14	AMP fusions expression in plants in high yield, easy purification of fusion peptides with high antimicrobial activity without the need for a peptide cleavage step
#15	
#16	
#17	
#18	
#19	Multifunctional coatings for implants to be employed in regenerative medical applications
#20	Antimicrobial coatings for implants and medical devices

FIGURES and TABLE CAPTIONS

Figure 1. Schematic representation of the purification process based on the ITC. The use of ELPs as purification tag exploits the thermo-responsive properties of the elastin-like domain. 1. Supernatant obtained after centrifugation of bacterial cell lysate; 2. Phase transition after heating to 37°C and 3. after NaCl addition; 4. Pellet obtained after centrifugation at 37°C; 1a. Pellet resuspension in cold water.

Figure 2. Schematic representation of the recombinant ELP fusion constructs and ELP conjugation with AMPs that are reported in the literature. (A) C-terminal ELP-AMP fusion proteins, (B) N-terminal AMP-ELP fusion proteins, (C), ELP-AMP-ELP fusion proteins and (D) ELP conjugated by chemical methods. Black box, antimicrobial domain; white box, elastin-like domain; grey box, intein domain; hatched box other domains. The name of AMP and the respective construct number correspond to those reported in Table 1.

Table 1. Antimicrobial peptides that were selected as fusion partner for an ELP carrier. Numbers refer to the ELP fusion constructs bearing the AMP. References and the main features of the fusion constructs are also reported. Information about peptides, when available, was obtained from the DBAASP (https://dbaasp.org) which is an open-access AMP data resource supported by I.Beritashvili Center of Experimental Biomedicine (IBCEB) Tbilisi, Georgia and the National Institute of Allergy and Infectious Diseases (NIAID) Office of Cyber Infrastructure and Computational Biology (OCICB) in Bethesda, MD. These data were collected and submitted by members of the DBAASP team

Table 2. Potential applications of the ELP-based fusion constructs carrying the AMPs. The number of the constructs corresponds to those indicated in Table 1 and Fig. 2.

REFERENCES

Bellotto, O., Semeraro, S., Bandiera, A., Tramer, F., Pavan, N., & Marchesan, S. (2022). Polymer Conjugates of Antimicrobial Peptides (AMPs) with d-Amino Acids (d-aa): State of the Art and Future Opportunities. *Pharmaceutics*, *14*(2). https://doi.org/10.3390/pharmaceutics14020446

Boparai, J. K., & Sharma, P. K. (2020). Mini Review on Antimicrobial Peptides, Sources, Mechanism and Recent Applications. *Protein Pept Lett*, *27*(1), 4-16. https://doi.org/10.2174/0929866526666190822165812

Cui, Z., Luo, Q., Bannon, M. S., Gray, V. P., Bloom, T. G., Clore, M. F., . . . Letteri, R. A. (2021). Molecular engineering of antimicrobial peptide (AMP)-polymer conjugates. *Biomater Sci*, *9*(15), 5069-5091. https://doi.org/10.1039/d1bm00423a

Ghidey, M., Islam, S. M. A., Pruett, G., & Kearney, C. M. (2020). Making plants into cost-effective bioreactors for highly active antimicrobial peptides. *N Biotechnol*, *56*, 63-70. https://doi.org/10.1016/j.nbt.2019.12.001

Hu, F., Ke, T., Li, X., Mao, P. H., Jin, X., Hui, F. L., . . . Ma, L. X. (2010). Expression and purification of an antimicrobial peptide by fusion with elastin-like polypeptides in Escherichia coli. *Appl Biochem Biotechnol*, *160*(8), 2377-2387. https://doi.org/10.1007/s12010-009-8850-2

Li, Y. (2009). Carrier proteins for fusion expression of antimicrobial peptides in Escherichia coli. *Biotechnol Appl Biochem*, *54*(1), 1-9. https://doi.org/10.1042/BA20090087

Li, Y. (2011). Recombinant production of antimicrobial peptides in Escherichia coli: a review. *Protein Expr Purif*, *80*(2), 260-267. https://doi.org/10.1016/j.pep.2011.08.001

Luan, C. H., Parker, T. M., Gowda, D. C., & Urry, D. W. (1992). Hydrophobicity of amino acid residues: differential scanning calorimetry and synthesis of the aromatic analogues of the polypentapeptide of elastin. *Biopolymers*, *32*(9), 1251-1261. https://doi.org/10.1002/bip.360320914

Magana, M., Pushpanathan, M., Santos, A. L., Leanse, L., Fernandez, M., Ioannidis, A., . . . Tegos, G. P. (2020). The value of antimicrobial peptides in the age of resistance. *Lancet Infect Dis*, *20*(9), e216-e230. https://doi.org/10.1016/s1473-3099(20)30327-3

McDaniel, J. R., Radford, D. C., & Chilkoti, A. (2013). A unified model for de novo design of elastin-like polypeptides with tunable inverse transition temperatures. *Biomacromolecules*, *14*(8), 2866-2872. https://doi.org/10.1021/bm4007166

Pereira, A. M., Costa, A. D., Dias, S. C., Casal, M., & Machado, R. (2021). Production and Purification of Two Bioactive Antimicrobial Peptides Using a Two-Step Approach Involving an Elastin-Like Fusion Tag. *Pharmaceuticals (Basel)*, *14*(10). https://doi.org/10.3390/ph14100956

Rai, A., Ferrao, R., Palma, P., Patricio, T., Parreira, P., Anes, E., . . . Ferreira, L. (2022). Antimicrobial peptide-based materials: opportunities and challenges. *J Mater Chem B*, *10*(14), 2384-2429. https://doi.org/10.1039/d1tb02617h

Ramazi, S., Mohammadi, N., Allahverdi, A., Khalili, E., & Abdolmaleki, P. (2022). A review on antimicrobial peptides databases and the computational tools. *Database (Oxford)*, *2022*. https://doi.org/10.1093/database/baac011

Shah, N. H., & Muir, T. W. (2014). Inteins: Nature's Gift to Protein Chemists. *Chem Sci*, *5*(1), 446-461. https://doi.org/10.1039/c3sc52951g

Silva, A. R. P., Guimaraes, M. S., Rabelo, J., Belen, L. H., Perecin, C. J., Farias, J. G., . . . Rangel-Yagui, C. O. (2022). Recent advances in the design of antimicrobial peptide conjugates. *J Mater Chem B*, *10*(19), 3587-3600. https://doi.org/10.1039/d1tb02757c

Urry, D. W. (1988). Entropic elastic processes in protein mechanisms. I. Elastic structure due to an inverse temperature transition and elasticity due to internal chain dynamics. *J Protein Chem*, *7*(1), 1-34.

Varanko, A. K., Su, J. C., & Chilkoti, A. (2020). Elastin-Like Polypeptides for Biomedical Applications. *Annu Rev Biomed Eng*, *22*, 343-369. https://doi.org/10.1146/annurev-bioeng-092419-061127

Wibowo, D., & Zhao, C. X. (2019). Recent achievements and perspectives for large-scale recombinant production of antimicrobial peptides. *Appl Microbiol Biotechnol*, *103*(2), 659-671. https://doi.org/10.1007/s00253-018-9524-1

Yeboah, A., Cohen, R. I., Rabolli, C., Yarmush, M. L., & Berthiaume, F. (2016). Elastin-like polypeptides: A strategic fusion partner for biologics. *Biotechnol Bioeng*, *113*(8), 1617-1627. https://doi.org/10.1002/bit.25998