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DNA-FACE™ - An *Escherichia coli*-based DNA Amplification-Expression Technology for Automatic Assembly of Concatemeric ORFs and Proteins

Piotr M. Skowron and Agnieszka Zylicz-Stachula

Abstract

DNA-FACE™ (DNA Fragment Amplification & Concatemeric Expressed Nucleic Acids and Proteins) is a universal biotechnological platform, developed as *Escherichia coli* (*E. coli*) system. It is based on the ordered, head-to-tail directional ligation of the amplified DNA fragments. The technology enables the construction of targeted biomolecules - genetically programmed, concatemeric DNA, RNA, and proteins, designed to fit a particular task. The constructed, “artificial” (never seen in Nature) tandem repeat macromolecules, with specialized functions, may contain up to 500 copies of monomeric units. The technology greatly exceeds the current capabilities of chemical gene synthesis. The vector-enzymatic DNA fragment amplification assembles the DNA segments, forming continuous Open Reading Frames (ORFs). The obtained ORFs are ready for high-level expression in *E. coli* without a need for subcloning. The presented method has potential applications in pharmaceutical industry and tissue engineering, including vaccines, biological drugs, drug delivery systems, mass-production of peptide-derived biomaterials, industrial and environmental processes. The technology has been patented worldwide and used successfully in the construction of anti-HBV vaccines, pro-regenerative biological drugs and, recently, the anti-SARS-CoV-2 vaccine. The anti-SARS-CoV-2 vaccine, developed using the DNA-FACE™ technology, is non-toxic and induces strong immunological response to recombinant human spike and nucleocapsid proteins, as shown in animal studies.

Keywords: DNA-FACE™; DNA amplification, concatemer, gene expression, amplification vector, expression vector, protein overproduction, biotechnology, HBV, SARS-CoV-2, vaccine, biological drug, tissue regeneration

1. Introduction

The recombinant DNA technology, born in early 1970 ties, has nowadays become mature and highly sophisticated. It plays an indispensable role in medicine, industry, and scientific research. Its rapid proliferation into a wide variety of

techniques and molecular solutions for genetic engineering is largely owed to its historically first and still dominating recombinant DNA “vehicle” – *E. coli*. These bacteria were used for the construction of the most robust genetic expression systems, such as those based on: T7-*lac* hybrid promoter, bacteriophage lambda *pL* and *pR* promoters or arabinose operon promoter. Other advantages of the systems include fast bacterial growth, cost-effectiveness and deep understanding of the molecular basis of recombinant proteins biosynthesis, as *E. coli* is the most thoroughly studied microorganism. Bioactive molecules, including DNA, RNA, and proteins, designed *de novo*, are being utilized in increasingly diverse scientific, biomedical, industrial and environmental applications. The rapidly developing synthetic biology generates growing demands for synthetic genes of various types, from native to those with a highly modified sequence. The capability of generating DNA molecules of any sequence or size is especially important in biomedical research.

DNA sequences of the designed, synthetic genes may require long repetitive DNA fragments. However, the chemical synthesis of such fragments still poses a great challenge. Thus, there is a current need for the development of new technologies, enabling modular construction of complex synthetic genes.

Vast majority of synthetic recombinant DNA constructs are intended for recombinant protein production. Besides the need for recombinant proteins, the protein fragments, polypeptides, functional domains, protein-derived or natural peptides are being increasingly used, not only for functional studies but also in a wide variety of applied sciences, like material engineering and medicine. The intensive research on peptide-based biomaterials as biologically active tools has resulted in the development of a wide range of peptides and polypeptides with extended functionalities. Both natural and engineered versions of the bioactive peptides have found applications in the construction of biosensors, drug-delivery systems, and medicine [1–4].

To allow detailed investigation and scaled-up production of new macromolecular biomaterials (built from the repetitive DNA, RNA, or protein segments), a reliable method is needed to obtain a high yield of complex recombinant genes and their expression products. Some of the methods enabling construction of such sophisticated recombinant DNA molecules are based on the targeted plasmid vectors, capable of carrying of the multiple, joined (concatemeric) genes. Such concatemerization of the designed DNA, RNA and peptides may improve their stability and boost the biosynthesis level. What is even more interesting, concatemerization can also bring novel and sometimes unexpected features, such as an increase of bioactivity, a formation of bionanoparticles or more precise targeting by means of a gradual release of the bioactive molecule monomers or oligomers at the intended destination.

In order to solve the problems encountered in the techniques existing so far, a new genetic engineering method was developed. The method is dedicated to the formation of DNA concatemers necessary to produce “artificial”, repetitive genes, encoding concatemeric RNAs and proteins (of any nucleotide (nt)/amino acid sequence), in a format suitable for a high-level genetic expression [5–7]. The technology is world-wide protected by patents: Polish no. 228341, (2018; first filed in 2015 [8]), United States no US 10,874,735 B2 (2020 [9]), European no EP 3 134 426 B1 (2020 [10]), Japanese no P6692796 (2020 [11]); Israeli – temporary no 248011 (2021 [12]) and patent applications: Chinese no CN 106488983 A (2016 [13]) and Indian no 201647039411 (2020 [14]).

2. DNA-FACE™ - a DNA fragment amplification-expression technology

2.1 The concept of the DNA-FACE™ method for directional DNA fragment amplification and protein concatemers construction

The rapid development of synthetic biology has generated a high demand for synthetic “artificial” genes that do not exist in Nature. Whatever their application, the construction of such synthetic genes may require the use of repetitive DNA fragments. However, one of the major limitations of the chemical synthesis of DNA is the difficulty in assembling repeated segments into longer DNA sequences. The ability to construct DNA molecules of any sequence or size is crucial for numerous applications, especially in the areas of biomedical and biotechnological research.

The biosynthesis-based strategies, that can ensure control over joining repeated DNA segments (multimerization or concatemerization), which would enable head-to-tail arrangement of the monomers of DNA, RNA, and peptides, require the development of special DNA manipulation methods. Otherwise, the obtained arrangements of monomeric units would be random and result in a mixture of head-to-head, tail-to-tail orientations of DNA fragments within the assembled multimeric DNA. Such randomized monomer arrangement could render any DNA construct useless for any rational applications, as it would disable a constructed DNA molecule from performing its desired function of encoding the genetic information about specific RNA and protein. For example, even a single undesired tail-to-tail segment within a constructed DNA multimer causes a nonsense amino acid sequence translation within this segment or even further downstream, an appearance of stop codons with inverted DNA segment, or a prematurely terminated translation of the constructed gene. Thus, controlling the mode of multimerization is pivotal in downstream processes after DNA multimerization. Furthermore, the controlled head-to tail-arrangement of the multimerized DNA segments provides stability of the recombinant DNA plasmid-vector and allows for a constructed operon expression.

Several alternative strategies for the construction of concatemeric genes have been developed so far [15–29]. However, most of the established technologies suffered from several problems, such as (i) limitations on the sequence of the DNA segment serving as the monomer; (ii) technical difficulties in joining of the DNA segments; (iii) an excessively complicated reaction, leading to the necessity of tedious DNA manipulations (iv) an inadequate copy number of the monomers within the formed multimer; (v) inability to repeat another round of the DNA fragment multimerization, if a desired monomer copy number within the resulting concatemeric DNA was not obtained. There were also two critically important problems; (vi) completion of a DNA concatemer formation without the ability to express coded RNA and proteins; (vii) codon discontinuity in the newly created ORF, which would prevent its expression and the production of the final result (a polypeptide/protein, containing multiple, linked together, bioactive peptides with programmed functions, without off-frame segments) [15–26].

A simple and efficient method was developed by us to make concatemeric “artificial” proteins or to emulate the old novel – “Frankenstein” proteins, composed of multiple functional parts, dedicated to suit a particular task. The DNA-FACE™ technology enables both homoconcatemers and heteroconcatemers formation, which highly enhances the pre-programmed functionality of the resulting “artificial” proteins (Figure 1). The technology allows for insertion of the synergistically acting bioactive

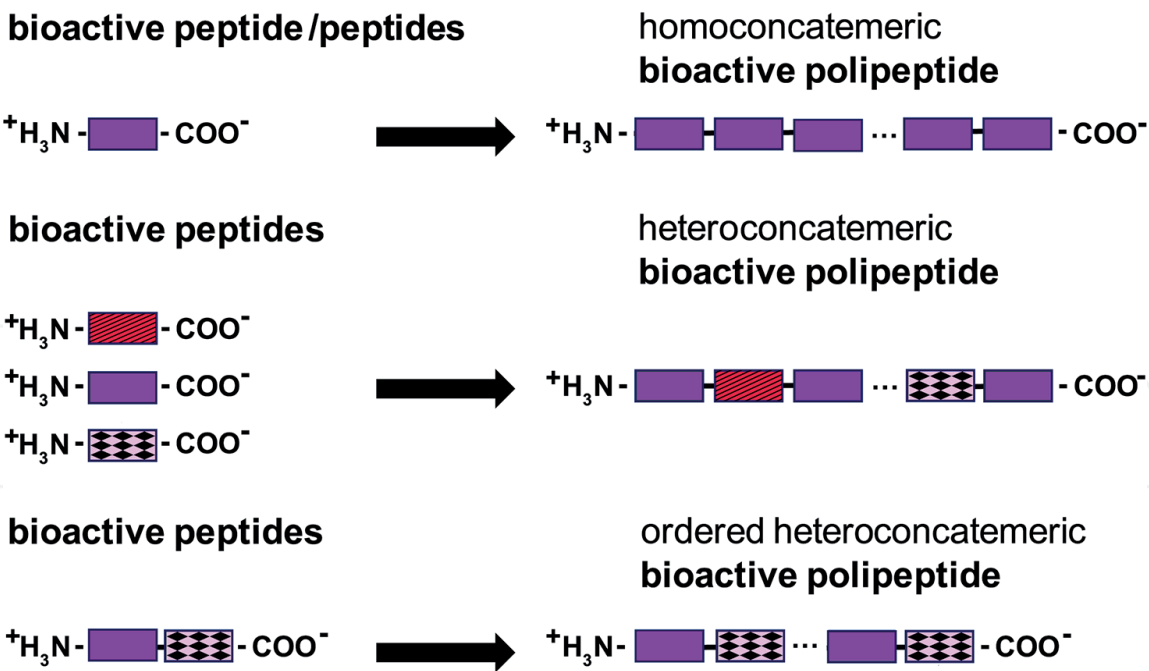


Figure 1.
Potential protein products are obtainable with the DNA-FACE™ technology.

peptides into the nascent concatemeric “Frankenstein” protein. The examples of such bioactive peptides are: different epitopes or antigen domains incorporated during a vaccine construction or combinations of various pro-regenerative peptides/protein segments. The technology is based on the custom vector-enzymatic system, which employs: (i) atypical Type IIS restriction endonucleases (REases). These Type IIS REases possess unique features: the ability to recognize 4-7 base pairs (bp) DNA sequence and to cleave at a fixed distance outside this sequence. Out of the known Type IIS REases, the DNA-FACE™ uses SapI (or its isoschizomers), which generate 3-nt protruding DNA ends; (ii) DNA ligase and (iii) dedicated amplification-expression vectors. DNA-FACE™ offers a significant improvement from earlier strategies [17]. It highly improves the construction of DNA concatemers, additionally allowing for the formation of continuous, multimeric ORFs as well as concatemeric proteins, with the desired monomer copy number and polymer/co-polymer length. **Figure 2** shows schematically the DNA fragment amplification reaction and its potential for employing multiple amplification cycles.

2.2 Molecular components and mechanism of the directional DNA fragment amplification reaction

2.2.1 DNA-interacting enzymes used and the mechanism of amplification reaction

All the DNA-FACE™ amplification-expression DNA vectors share variants of a universal DNA amplification module. The module may be custom modified and transferred into other DNA vectors, either prokaryotic or eukaryotic, containing alternative antibiotic resistance genes, origins of replication, transcriptional promoters, and translation initiation signals, among others. The amplification module contains two convergently oriented recognition sequences of the Type IIS REase - SapI, able to recognize asymmetric 7-bp 5'-GCTCTTC-3' and cleave the upper DNA strand to the 3' direction, at a distance of 1-nt and the bottom strand at a distance of 4-nt, thus leaving 3-nt 5' cohesive ends. The SapI (and its isoschizomers) are unique among the discovered, atypical Type IIS REases. The SapI DNA recognition sequence is long and the protruding ends of the SapI cleaved substrate form a codon

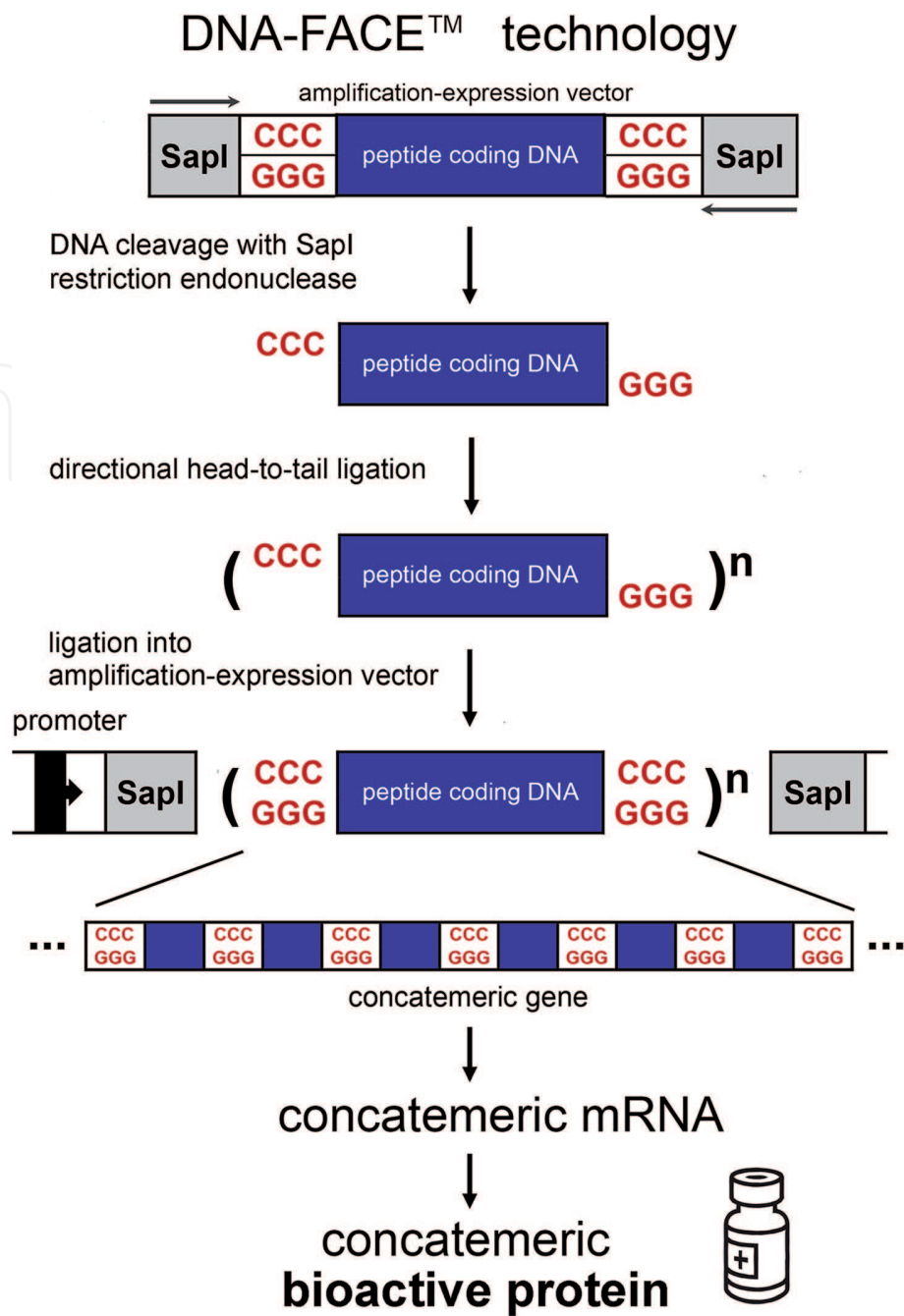


Figure 2.
Principles of the DNA-FACE™ technology.

length upon ligation. The long DNA recognition sequence highly decreases the probability of its accidental and undesired appearance with both the amplification-expression vector used and the DNA fragment to be amplified. Furthermore, the key feature of the SapI is a very rare occurrence of the 3-nt long protruding ends, that enable codon length formation in-between linked the amplified coding DNA segments, thus ensuring continuity of the ascending ORF. The SapI sites are separated by the orthodox Type II SmaI REase recognition sequence (5'-CCC|GGG-3'). SmaI cleaves its recognition sequence, leaving blunt ends. This is a convenient setup for cloning of any synthetic DNA fragments, as typically they are synthesized/delivered as double-stranded (ds) forms. The amplification-expression module provides three cloning options for a DNA fragment to be amplified (i) cohesive end cloning of the SapI generated 5'-CCC/5'-GGG sticky ends, (ii) blunt-end cloning of the SapI cohesive ends, previously filled in by T4 DNA polymerase/deoxyribonucleotides triphosphates; (iii) blunt-end cloning into SmaI site.

Whatever cloning option is used, the general protocol needs to be followed for all the amplification-expression vectors: (1) selection of bioactive peptides from a natural source or design of the monomeric DNA fragment to be amplified; (2) generation of the monomer by chemical synthesis of DNA, PCR amplification or REases excision from natural DNA; (3) cloning of the DNA fragment to the selected amplification-expression vector.

The cloning process is preferentially conducted using the cohesive end approach (mentioned above). The asymmetric 5' -CCC/5' -GGG cohesive ends can be generated (a) through their addition at 5' - and 3' - termini of the dsDNA monomer during chemical synthesis or (b) *in vitro*, from SapI recognition sequences, added during chemical synthesis of the monomer and further clipped-off with SapI REase or (c) by PCR amplification with primers, containing SapI sites at their 5' - overhangs and clipping-off with SapI or (d) by excision of a SmaI-cloned monomer from an amplification-expression vector.

Subsequent stages of the DNA-FACE™ procedure include: (i) purification of the dsDNA fragment equipped with SapI-compatible 5' -CCC/5' -GGG cohesive ends, ordered self-ligation of DNA monomers in directional, head-to-tail orientation, driven by asymmetric cohesive ends, (ii) ligation of generated concatemers mixture or of a selected gel-purified concatemer into the SapI-cleaved amplification vector, (iii) transformation into a suitable *E. coli* host strain, tolerant to atypical DNA sequences, such as DH5alpha, Top10, JM109, Endura™; (iv) selection of *E. coli* clones containing a concatemeric ORF segment with the desired number of monomers; (v) expression of the concatemeric ORF directly from an amplification-expression vector, containing strong transcription promoter, resulting in concatemeric protein biosynthesis or (vi) excision of the concatemer with SapI from the vector, which results in a DNA concatemeric segment equipped with SapI-cohesive ends and repeating steps (i–iv), until a desired number of monomeric DNA segments within a concatemer is obtained (**Figure 2**).

2.2.2 Amplification-expression vectors

Four categories of DNA amplification-expression vectors were designed for the purpose of the DNA-FACE™ technology: (I) pAMP series of six vectors for concatemeric protein biosynthesis in *E. coli* cytoplasm, (II) DNA amplification-expression pET21AMP-HisA vector for IPTG-controlled concatemeric protein biosynthesis in *E. coli* cytoplasm, (III) pET28AMP_SapI-Ubq vector for cytoplasmic biosynthesis of concatemeric proteins fused with ubiquitin at N-terminus and (IV) pET28AMP_PhxA or pET28AMP_MalE vectors for secretion of concatemeric proteins, produced under IPTG control, to the *E. coli* periplasm.

The pAMP series ((I); **Figure 3**) was constructed on the basis of the vector pACYC184 and its derivative pRZ4737 (W. S. Reznikoff) [5, 6, 30]. For the pAMP DNA vectors, six versions of the amplification modules were used, which differed by the presence/absence of His6_tag for metal affinity chromatography in three different reading frames (**Figure 3**). The pAMP DNA vectors are compatible with the *colE1* origin vectors, such as pET-series, and can be maintained in the same *E. coli* cell, if needed [5–14].

The pET21AMP-HisA vector ((II); **Figure 4**) is based on the pET-21d(+) expression vector (Novagen, EMD Millipore Corporation).

The pET28AMP_SapI-Ubq vector ((III); **Figure 5**) was designed as a modification of the pET-28d(+) expression vector (Novagen, EMD Millipore Corporation) and enables the fusion of a concatemeric protein with ubiquitin [5–7]. As concatemeric proteins contain repeated peptide modules, depending on their sequence, they may not form typical, natural protein structures, with hydrophobic amino acids residues forming a “core” surrounded by exposed polar and charged amino acids residues. This may affect their solubility, thus for some applications a fusion

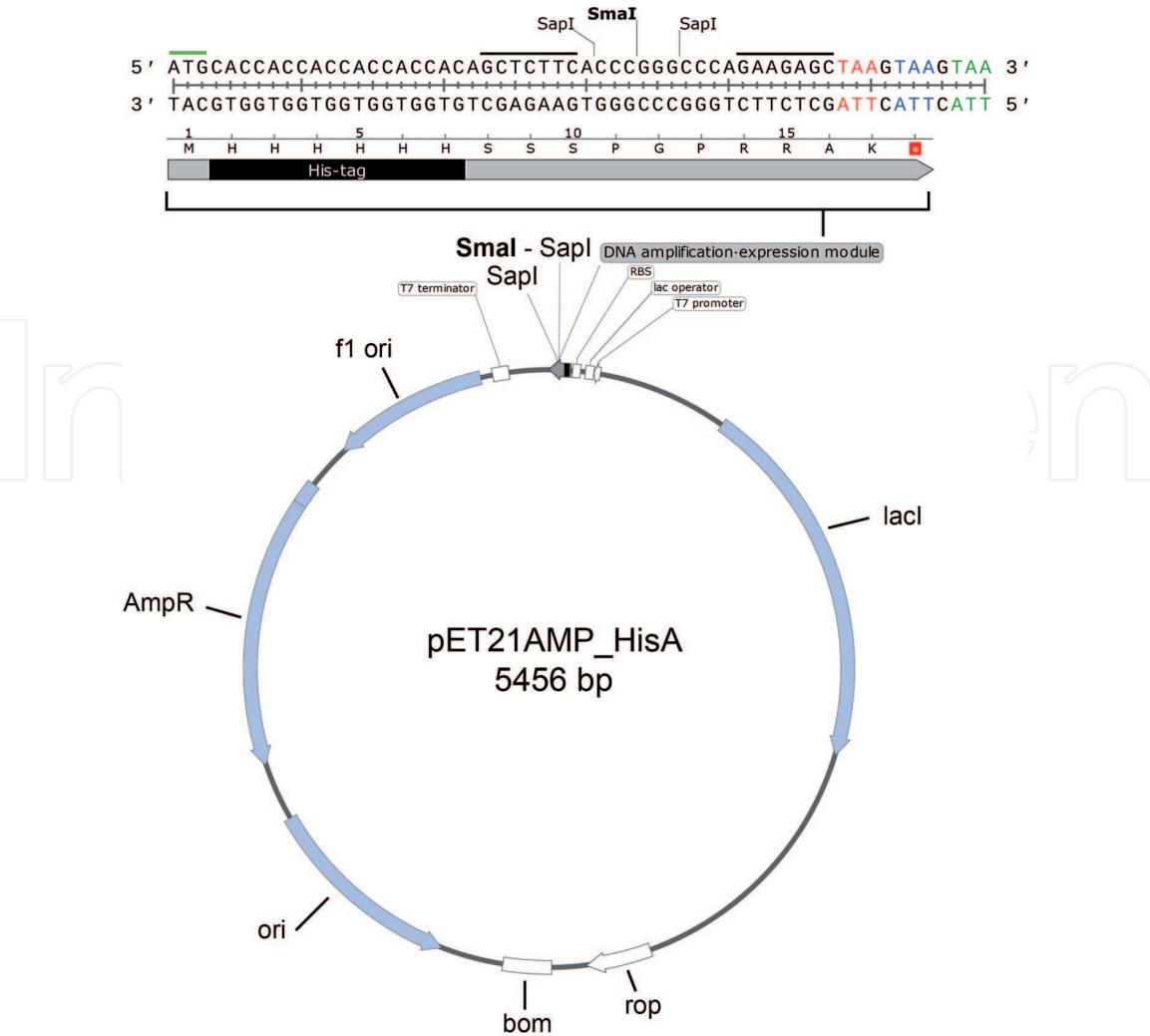


Figure 4. The pET-derived, amplification-expression DNA vector, designed and constructed for the DNA-FACE™ technology. The pET21AMP-HisA DNA vector (GenBank MK606521) contains (a) colE1 origin, (b) f1/M13 origin, (c) very strong T7-lac transcription promoter, inducible by lactose or IPTG, (d) amplification module HisA and (e) ampicillin resistance gene.

expression optimized synthetic 21-bp DNA fragment, encoding the epitope TKPTDGN was cloned into the amplification-expression pAMP1-HisA vector. The detailed procedure of cloning of the synthetic 21-bp DNA fragment and its further amplification (based on the DNA-FACE™ technology) was described by Skowron et al. [5]. The results are shown in **Figure 7** (see the first round of amplification).

Further, the selected 5-mer was subjected to the second round of amplification (**Figure 7**; see the second round of amplification). The amplification-expression vectors were designed in such a way, that no SapI recognition sites were left within the amplified DNA segment. Such a vector design makes it possible to use a multi-meric DNA fragment, obtained in the first round, as a “monomer”.

Subsequently, an alternative or hybrid route of a DNA fragment amplification was tested. A possibility of a combination of chemical synthesis of the pre-formed HBV epitope-coding DNA, pushed to its technical limits with DNA-FACE™ method, was investigated. The limits of such chemical synthesis strongly depend on the DNA sequence and the size of the DNA fragment to be concatemerized. The model HBV epitope coding DNA turned out to be a rather “friendly” case, as compared to the other designed DNA sequences. Testing several commercial services, chemically synthesizing the designed DNA molecules, a maximum of 25-mers within a single synthetic gene was obtained. The 25-mer was then used as a “monomer” in DNA-FACE™ biotechnology amplification. As a result, bacterial clones

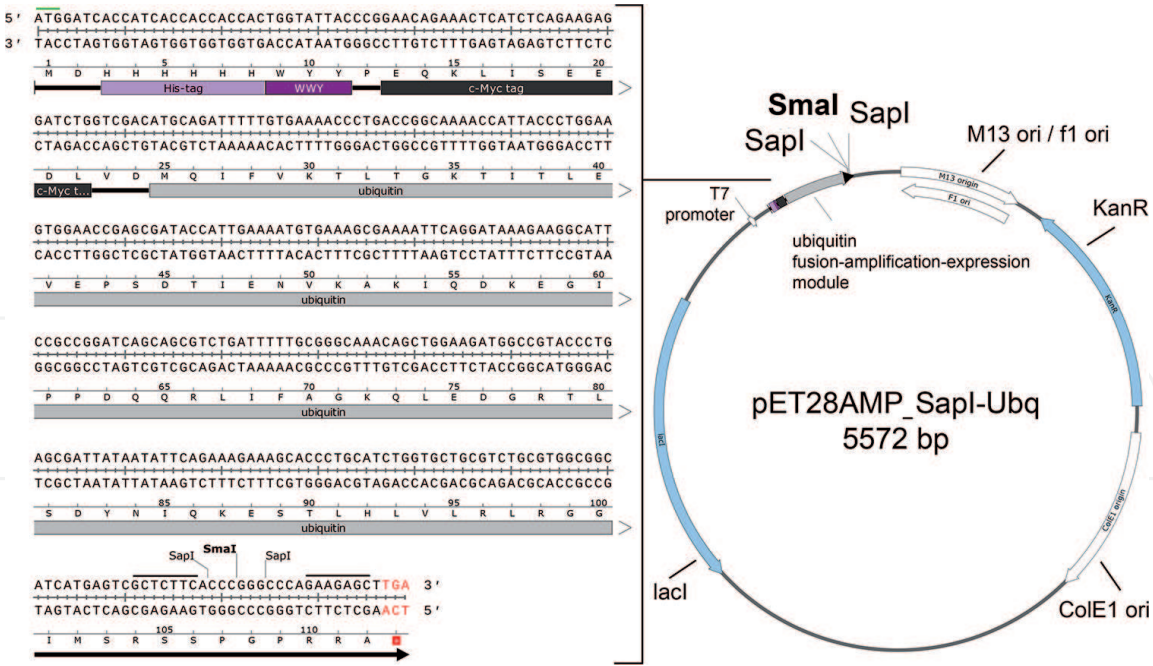


Figure 5.
The pET-derived pET28AMP_SapI-Ubq DNA vector, designed and constructed for the DNA-FACE™ technology. The pET28AMP_SapI-Ubq DNA vector (GenBank MK606527) is composed of: (a) colE1 origin, (b) f1/M13 origin, (c) T7-lac transcription promoter, inducible by lactose or IPTG, (d) the DNA fragment amplification module His6_c-Myc_WYY_ubiquitin_SapI-Sma-SapI, enabling ubiquitin gene fusion and (e) kanamycin resistance gene.

containing up to 500-copies of the 21-bp HBV epitope were obtained (Figure 7; see the alternative round of amplification [5, 7–14]).

During the next stage of the technology testing, the ability of the amplification-expression vector to yield an efficient translation of a highly atypical concatemeric gene, was investigated. The selected pAMP-HisA constructs, exemplified by 10-mer, 13-mer, 15-mer, 20-mer and 30-mer, were expressed (Figure 8). The expression of the recombinant constructs with up to 450 repeats, (composed of the 7-aa monomers), was tested.

It is known that the upper limit of molecular weight of a single polypeptide, biosynthesized by *E. coli*, is app. 150–200 kDa. However, due to a potential “slippage” of the translation machinery on mRNA repeat and a possible premature translation termination, a “smear” on SDS-PAGE gels was typically observed. The “smear” was located near the expected position of the recombinant protein, with the size corresponding to its molecular weight (Figure 8) [5]. Nevertheless, even a mixture of translation products is expected to be fully functional in planned applications, as each monomeric unit is semi-independent in genetically programmed functions, such as comprising an immunologically condensed “artificial” antigen, built from immunoactive epitopes only.

Afterwards, the DNA-FACE™ biotechnology was validated in the construction of prospective, pro-regenerative drugs and in the concatemeric proteins designed for remediation of the environment and new generation biosensors. Taken together, over 50 concatemeric ORFs and the resulting concatemeric proteins were constructed. Among those, a series of prospective pro-regenerative drugs was developed [5, 6]. For this purpose, the amplification (concatemerization) of four types of the designed DNA fragments was performed. The selected DNA fragments encoded either the laboratory-developed/predicted peptides or the peptides originally derived from wound healing stimulatory proteins.

The first selected peptide -TSRGDHELLGGGAAPVGG, which originated from the angiopoietin-related growth factor (AGF), was used for the construction of a poly-signal protein [5]. The peptide was linked to the elastase recognition sequence

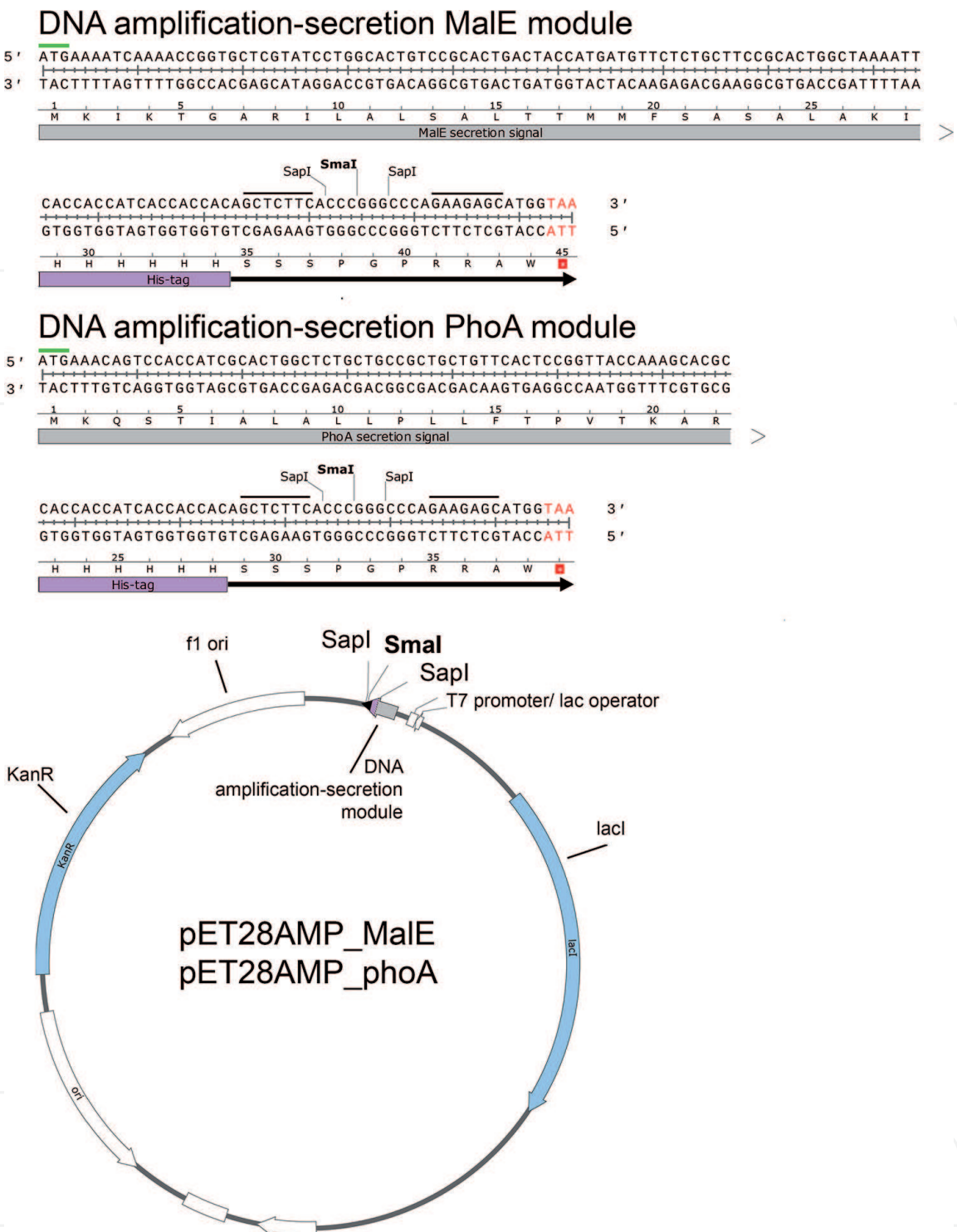


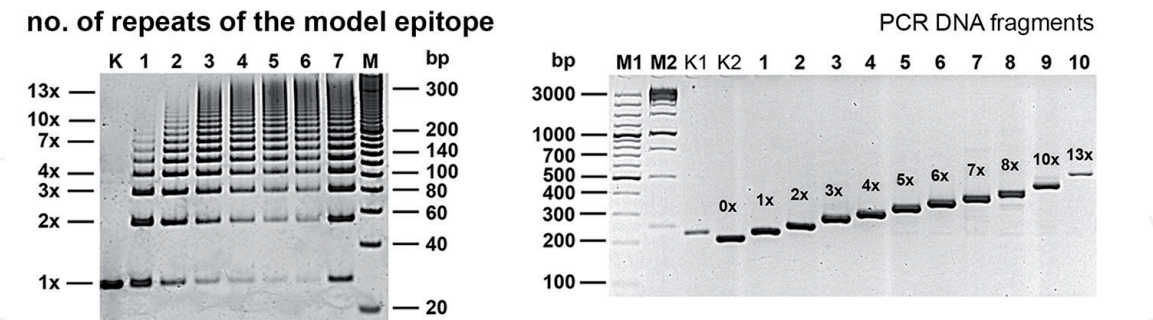
Figure 6. The pET-derived, amplification-expression-secretion DNA vectors: pET28AMP_PhoA and pET28AMP_MalE, designed and constructed for the DNA-FACE™ technology. The pET28AMP_PhoA DNA vector (GenBank MK606526) and the pET28AMP_MalE vector (GenBank MK606522) contain: (a) colE1 origin, (b) f1/M13 origin, (c) T7-lac transcription promoter, inducible by lactose or IPTG, (d) the amplification module His6_PhoA_SapI-Sma-SapI or His6_MalE_SapI-Sma-SapI, (e) kanamycin resistance gene.

to facilitate a gradual enzymatic release of the monomers/oligomers, cleaved by the elastase present in human serum [5, 6]. The AGF is known to promote epidermal proliferation, new blood vessel formation, and wound healing in the skin.

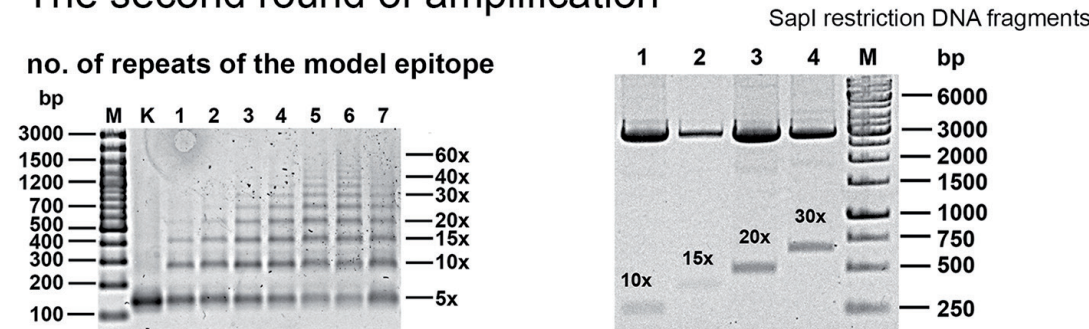
The second type of the selected peptides – the RGD and RGDGG peptides – originated from fibronectin. These motifs function as crucial cell-binding factors.

DNA-FACE™ technology: Proof of Concept

The first round of amplification



The second round of amplification



Alternative round of amplification

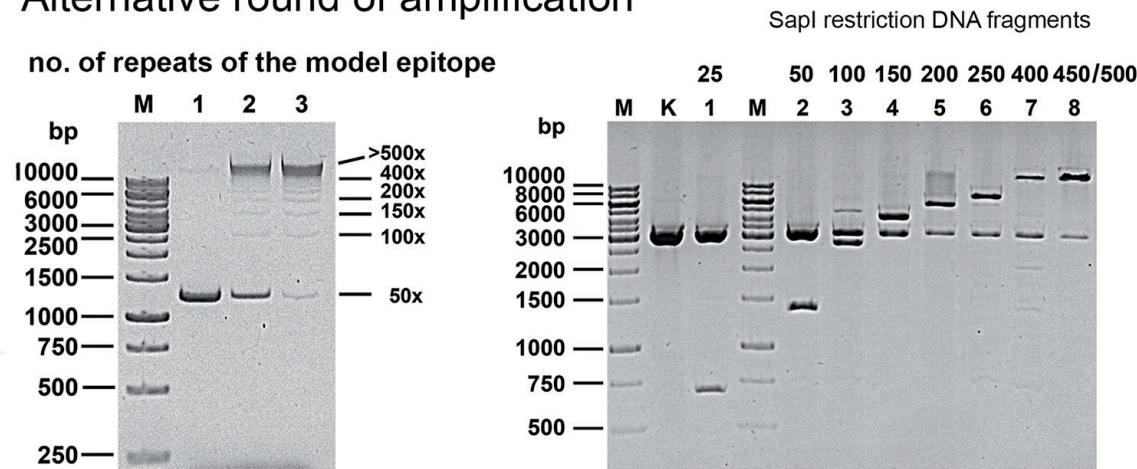


Figure 7. The DNA-FACE™ technology proof of concept – amplification of the model epitope encoding DNA fragment. The first round of amplification: the synthetic DNA fragment, encoding the model epitope, was cloned into the amplification-expression pAMP1-HisA vector as described by Skowron et al. [5]. Then, the PCR amplification of the appropriate DNA segment was performed. The PCR product was cleaved with Sapl and subjected to autoligation at 16°C using T4 DNA ligase and aliquots were taken at intervals of 5, 10, 20, 40, 80, and 160 min. A series of DNA segments of increasing length was obtained. The resulting concatemers were pooled and cloned in pAMP1-HisA, cleaved with Sapl. The obtained bacterial clones were analyzed by colony PCR [5]. The second round of amplification: the selected 5-mer was subjected to the second round of amplification [5]. The appropriate DNA fragment was excised from the E. coli clone plasmid and subjected to autoligation. The reaction products were pooled, ligated back to the pAMP1-HisA. The plasmid DNAs from the positive bacterial clones were cleaved by Sapl and the obtained restriction fragments were analyzed electrophoretically. Alternative round of amplification: the synthetic 25-mer was subjected to the amplification as described by Skowron et al. [5]. The autoligation products were ligated to the pAMP1-HisA. The plasmid DNAs from the positive bacterial clones were cleaved by Sapl and the obtained restriction fragments were analyzed electrophoretically.

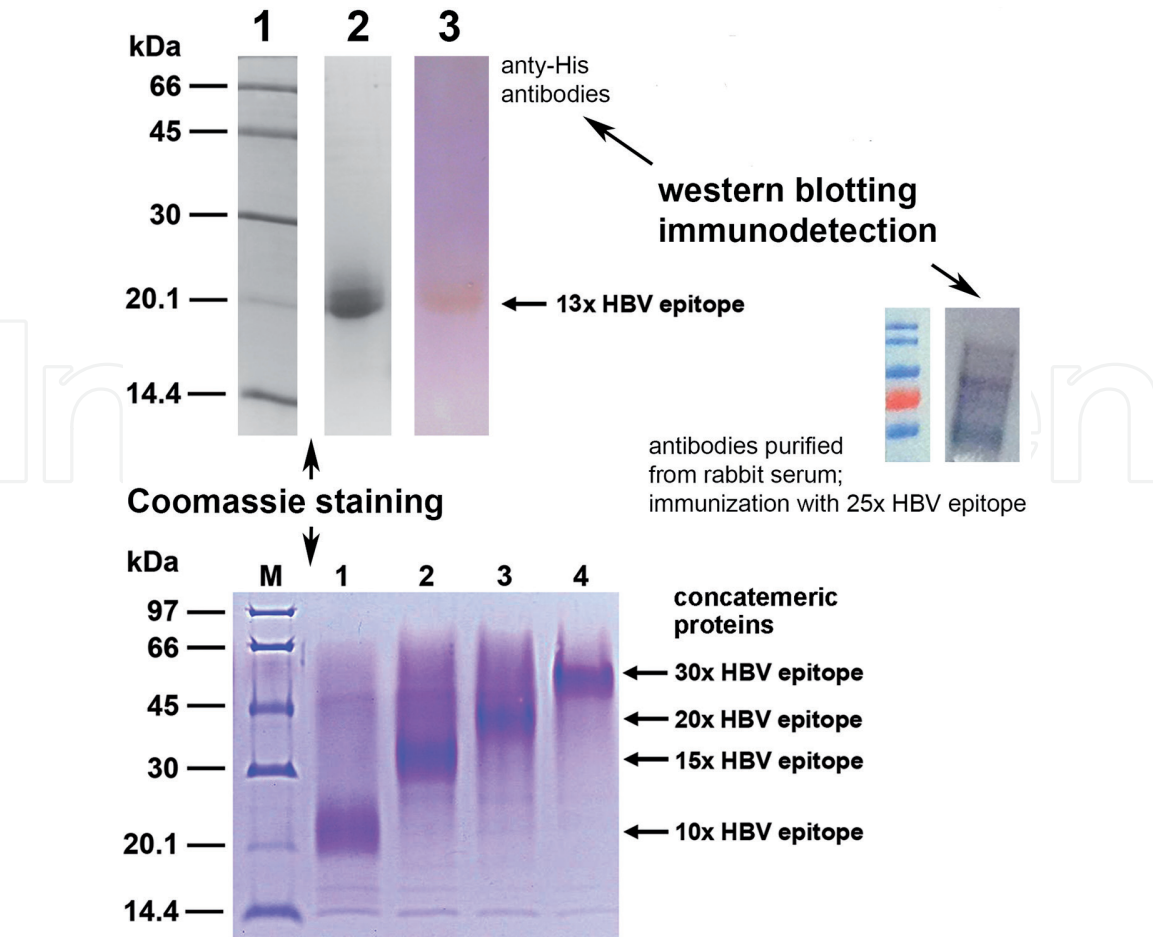


Figure 8. Concatemeric, recombinant proteins consisting of multiple repeats of a model HBV epitope, obtained with the DNA-FACE™ technology.

The RGD sequence is present in several extracellular matrix (ECM) molecules and is responsible for the mediation of cell attachment. It is known to promote cell/tissue interaction with artificial biomaterials and shows a pro-regenerative effect [32, 33].

The third designed peptide - RLIDRTNANFLGGGAAPVGGG originated from the platelet-derived growth factor (PDGF B). PDGF B functions as a mitogen for fibroblasts and smooth muscles cells and regulates embryonic development. The peptide was extended by GG helical breakers and an AAPV peptide, known to be effectively cleaved by human elastase [34–36].

The fourth peptide series: GHK, GHKGG, GHKGGGAAPVGG, KGHKGGGAAPVGG was designed on the basis of the GHK peptide, which naturally occurs in human plasma and can be released by the injured tissues. The peptide is responsible for diverse protective and healing actions. For example, it is known to improve tissue repair, stimulate blood vessel and nerve outgrowth, boost collagen, elastin, and glycosaminoglycan synthesis [37, 38].

Another explored application of the DNA-FACE™ biotechnology includes construction and evaluation of concatemeric proteins for the purpose of environment remediation and biosensors development. These proteins target toxic heavy metal ions: Pb^{2+} , Hg^{2+} , Ag^{+} , As^{3+} , Ni^{2+} , Cd^{2+} , and the uranyl ion (UO_2^{2+}). The details will be released to public domain following submission of the patent application.

2.3.2 Final note concerning concatemeric proteins constructions

It should be noted that the maximum possible monomer copy number within a constructed DNA concatemer could be lower or higher than in the case of the model

HBV epitope [5], as it strongly depends on the DNA sequence and the length of the DNA fragment to be concatemerized. Further precautions concern the downstream applications of the DNA concatemers. Namely, some of the constructed concatemeric genes/ORFs may not be efficiently or error-free transcribed or expressed in *E. coli*. This is again highly dependent on the nt sequence of the resulting mRNA as well as on its resistance to form stable secondary and tertiary structures, which may hide the translation initiation/termination signals or stall the translating ribosomes. Moreover, certain translated amino acids sequences, especially these appearing in ascending concatemeric proteins as repeated segments, may yield low expression levels due to the depletion of highly used aminoacyl-tRNAs, as well as cause the insolubility or toxicity to the recombinant host. Although these potential problems generally concern recombinant genes expression, they may be more pronounced due to the “artificial” nature of the concatemeric proteins. If needed, an implementation of additional strategies can be helpful, such as testing various cultivation/ expression conditions, fusions with non-concatemeric proteins, *E. coli* strains, alternative prokaryotic or eukaryotic expression systems, among others. It is worth noting that the DNA amplification-expression modules can be easily transferred to other DNA vectors, including eucaryotic, if necessary.

2.4 Application of the DNA-FACE™: development of a novel type of anti-SARS-CoV-2 vaccine

The DNA-FACE™ concept of construction of “artificial”, concatemeric protein with a vaccine functionality has been used to construct an anti-SARS-CoV-2 vaccine.

The co-polymerization type construct contains multiple copies of various epitopes clusters, derived from Spike and Nucleoprotein of the SARS-CoV-2 virus.

The clusters were selected using a proprietary method developed in BioVentures Institute Ltd. (Poland), which has also developed the DNA-FACE™ biotechnology.

The vaccine is the most “Frankenstein”, as it is composed of: (i) N-terminal polyepitopic clusters of various amino acid sequences and (ii) protein adjuvant to enhance the immune response. The entire protein construct has a molecular weight of approximately 70 kDa only, as compared to Spike protein (140.3 kDa) and Nucleoprotein (45.6 kDa). Nevertheless, immunogenicity and toxicity studies in animals (rabbits) have shown that the protein is not toxic and induces a strong, specific immune response (**Figure 9**). Additional advantages of such composite vaccine are: (i) no need for refrigeration, as the antigen does not contain native conformation protein, which has to be protected from denaturation, and (ii) potential for rapid module exchange if a new virus variant contains changes epitopes. Panel (a) shows a high level of biosynthesis of the recombinant anti-SARS-CoV-2 vaccine protein in *E. coli*. The recombinant gene was cloned into pETAMP1-A amplification-expression vector. Upon induction with IPTG (**Figure 9A, lane 2**), a dominant band of approximately 70 kDa becomes evident, as compared to control, uninduced recombinant *E. coli* culture (**Figure 9A, lane 1**). The induced sample was subjected to western blotting, using primary anti-His6-tag antibodies (**Figure 9B, lane 1**) or rabbit anti-anti-SARS-CoV-2 vaccine (**Figure 9C, lanes 1 and 2**). In both lines, multiple bands appear, which is expected, as rabbits carry also their own anti-*E. coli* proteins antibodies. The band pattern in the lane 2 is different - the dominant protein band of approximately 70 kDa comprises the anti-SARS-CoV-2 vaccine, the remaining bands are most probably a mixture of the vaccine degradation products, reacting *E. coli* proteins, including those, which are co-induced with IPTG. Further assay included western blotting using native Spike and Nucleoprotein proteins (purchased from an independent, foreign company), expressed in human cells,

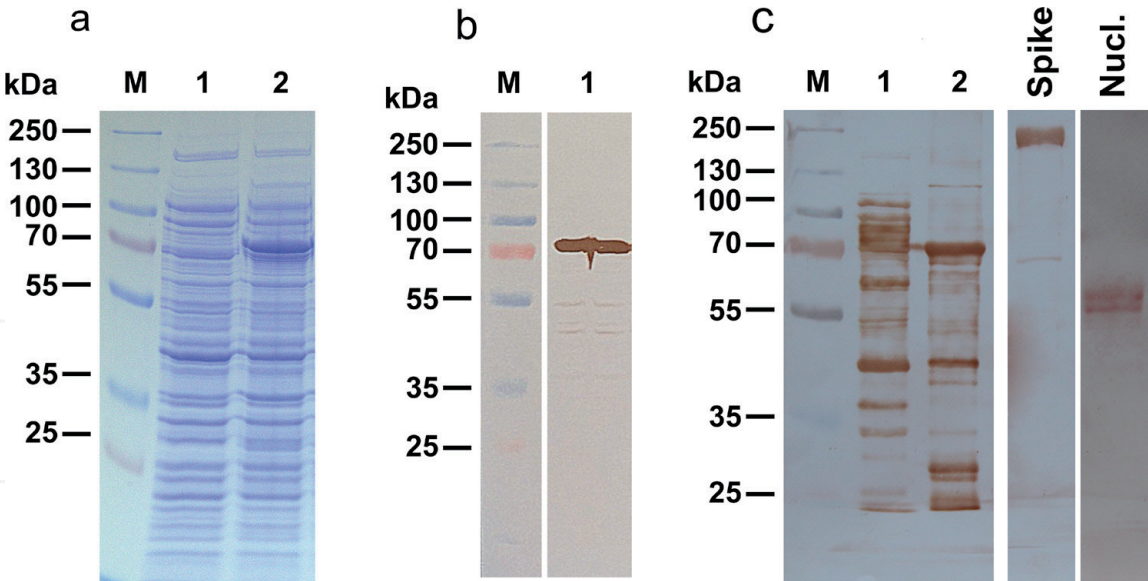


Figure 9. Biosynthesis and immunogenicity of the recombinant anti-SARS-CoV-2 vaccine. Lane M, Page Ruler™ Plus Prestained Protein Ladder (Thermo Scientific) (a) SDS-PAGE analysis of the recombinant anti-SARS-CoV-2 vaccine protein biosynthesis in *E. coli*.; lane 1, control *E. coli* lysate, without induction of the recombinant gene expression; lane 2, *E. coli* lysate, 3 hours after induction of the recombinant gene expression. (b) Western blotting and immunodetection of the recombinant anti-SARS-CoV-2 vaccine protein using anti-His-tag antibodies. (c) Western blotting and immunodetection of the recombinant anti-SARS-CoV-2 vaccine protein, using antibodies purified from the immunized rabbit serum. Lane 1, *E. coli* lysate, 3 hours after induction; lane 2, purified anti-SARS-CoV-2 vaccine protein; lane Spike, immunodetection of human, recombinant SARS-CoV-2 Spike protein; lane Nucl., immunodetection of the human, recombinant SARS-CoV-2 Nucleocapsid protein.

thus identical to those present in the virus, including posttranslational modification, absent in *E. coli*. Nevertheless, strong, specific immunological reaction was obtained. Currently, this novel type of anti-SARS-CoV-2 vaccine undergoes further full-scale evaluation, the regulated pre-clinical animal and *in vitro* tests and its clinical tests will follow shortly. Important aspect of this vaccine design is that it does not contain intact Spike and Nucleoprotein, which are known to be toxic to human immunological system, among other negative effects. Thus, it is expected, that the vaccine will have much reduced, if any, side effects upon vaccination. It needs to be emphasized that the DNA-FACE™ concept used to develop anti-SARS-CoV-2 vaccine also applies to essentially all types of microbial pathogens as well as to cancer cells.

3. Conclusions

The DNA-FACE™ biotechnology was developed for the construction of “artificial”, repetitive genes, encoding concatemeric RNAs and proteins of any nt and aa sequence. The DNA-FACE™ is capable of formatting of ordered polymers in a controlled process, containing 500 or more copies of DNA, RNA, or peptide repeats within a concatemer.

The constructed concatemeric genes yield efficient genetic expression of concatemeric proteins, which were tested in the development of:

- i. New generation of vaccines with an enhanced stimulation of the immune system, including anti-SARS-CoV-2 vaccine.

- ii. Concatemeric proteins contain modules for toxic and/or rare metal ions chelation for their industrial obtainment, environmental remediation, or organism detoxification.
- iii. Reservoirs for bioactive peptides, either developed or derived from the signaling proteins, which can stimulate tissue regeneration.
- iv. Protective, therapeutic concatemeric proteins, containing peptide activators or inhibitors of biological functions, for a new type of biological drugs development, aiming at the treatment of molecular, viral, and bacterial diseases.

The DNA-FACE™ technology is uniquely suited for wide applications in the scientific research, biotechnology, pharmaceutical, and medical industries. The method goes far beyond current chemical genes synthesis capabilities. It allows for gene and protein design solutions, which were impossible before the development of this technology. This opens new research avenues not only for studying biological systems but also for practical solutions, such as novel types of cancer inhibitors, currently under development, using DNA-FACE™. We believe that the scientific and industrial community will recognize the potential of the DNA-FACE™ technology, and several new applications of the technology will soon appear.

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Conflict of interest

The authors declare no conflict of interest.

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References

- [1] Fosgerau K, Hoffmann T. Peptide therapeutics: Current status and future directions. *Drug Discovery Today*. 2015;**20**(1):122-128. DOI: 10.1016/j.drudis.2014.10.003
- [2] Lau J, Dunn M. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Bioorganic and Medicinal Chemistry*. 2018;**26**(10):2700-2707. DOI: 10.1016/j.bmc.2017.06.052
- [3] Chow D, Nunalee ML, Lim DW, Simnick AJ, Chilkoti A. Peptide-based biopolymers in biomedicine and biotechnology. *Materials Science and Engineering R: Reports*. 2008;**62**(4): 125-155. DOI: 10.1016/j.mser.2008.04.004
- [4] Lee H, Piao D, Lee J, Choi J, Bok J, Cho C, et al. Artificially designed recombinant protein composed of multiple epitopes of foot-and-mouth disease virus as a vaccine candidate. *Microbial Cell Factories*. 2017;**16**(1):33. DOI: 10.1186/s12934-017-0648-2
- [5] Skowron PM, Krawczun N, Żebrowska J, Krefft D, Żolnierkiewicz O, Bielawa M, et al. A vector-enzymatic DNA fragment amplification-expression technology for construction of artificial, concatemeric DNA, RNA and proteins for novel biomaterials, biomedical and industrial applications. *Materials Science and Engineering C: Materials for Biological Applications*. 2020;**108**:110426. DOI: 10.1016/j.msec.2019.110426
- [6] Skowron PM, Krawczun N, Żebrowska J, Krefft D, Żolnierkiewicz O, Bielawa M, et al. Data regarding a new, vector-enzymatic DNA fragment amplification-expression technology for the construction of artificial, concatemeric DNA, RNA and proteins, as well as biological effects of selected polypeptides obtained using this method. *Data in Brief*. 2019;**28**:105069. DOI: 10.1016/j.dib.2019.105069
- [7] Skowron PM, Krawczun N, Żebrowska J, Krefft D, Żolnierkiewicz O, Bielawa M, et al. An efficient method for the construction of artificial, concatemeric DNA, RNA and proteins with genetically programmed functions, using a novel, vector-enzymatic DNA fragment amplification-expression technology. *MethodsX*. 2020;**7**:101070. DOI: 10.1016/j.mex.2020.101070
- [8] Skowron P, Zylicz-Stachula A, Żolnierkiewicz O, Skowron M, Janus Ł, Jezewska-Frackowiak J, Szymańska A. Sposób uzyskiwania białka poliepitopowego oraz wektor DNA do realizacji tego sposobu (A method of obtaining a polyepitopic protein as well as a DNA vector for embodying this method), Republic of Poland patent office no 228341, 2018
- [9] Skowron P, Zylicz-Stachula A, Żolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Nidzworski D., Szemiako K, Maciejewska N, Nowak M, Szymanska A. Method of obtaining a polyepitopic protein and polyepitopic DNA vector. United States Patent US 10,874,735 B2, 2020
- [10] Skowron P, Zylicz-Stachula A, Żolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Nidzworski D, Szemiako K, Maciejewska N, Nowak M, Szymanska A. Method of obtaining a polyepitopic protein and polyepitopic DNA vector. European Patent Office EP 3 134 426 B1, 2020
- [11] Skowron P, Zylicz-Stachula A, Żolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Krefft D, Nidzworski D, Szemiako K, Maciejewska N, Nowak M, Szymanska A. A method of obtaining a polyepitopic protein as well as a DNA vector for embodying this method. Japanese Patent Office no P6692796, 2020

- [12] Skowron P, Zylicz-Stachula A, Zolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Krefft D, Nidzworski D, Szemiako K, Maciejewska N, Nowak M, Szymanska A: A method of obtaining a polyepitopic protein as well as a DNA vector for embodying this method. Israel Patent Office no 248011, 2021
- [13] Skowron P, Zylicz-Stachula A, Zolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Krefft D, Nidzworski D, Szemiako K, Maciejewska N, Nowak M, Szymanska A. A method of obtaining a polyepitopic protein as well as a DNA vector for embodying this method. China Patent Office no CN 106488983 A, 2016
- [14] Skowron P, Zylicz-Stachula A, Zolnierkiewicz O, Skowron M, Janus L, Jezewska-Frackowiak J, Krefft D, Nidzworski D, Szemiako K, Maciejewska N, Nowak M, Szymanska A. A method of obtaining a polyepitopic protein as well as a DNA vector for embodying this method. Indian Patent Office no 201647039411, 2016
- [15] Kempe T, Kent SB, Chow F, Peterson SM, Sundquist WI, L'Italien JJ, et al. Multiple-copy genes: Production and modification of monomeric peptides from large multimeric fusion proteins. *Gene*. 1985;**39**(2-3):239-245. DOI: 10.1016/0378-1119(85)90318-x
- [16] Kim SC, Szybalski W. Amplification of cloned DNA as tandem multimers using BspMI-generated asymmetric cohesive ends. *Gene*. 1988;**71**(1):1-8. DOI: 10.1016/0378-1119(88)90071-6
- [17] Lee JH, Skowron PM, Rutkowska SM, Hong SS, Kim SC. Sequential amplification of cloned DNA as tandem multimers using class-II restriction enzymes. *Genetic Analysis*. 1996;**13**(6):139-145. DOI: 10.1016/s1050-3862(96)00164-7
- [18] Lee JH, Minn I, Park CB, Kim SC. Acidic peptide-mediated expression of the antimicrobial peptide buforin II as tandem repeats in *Escherichia coli*. *Protein Expression Purification*. 1998;**12**(1):53-60. DOI: 10.1006/prep.1997.0814
- [19] Wang YQ, Cai JY. High-level expression of acidic partner-mediated antimicrobial peptide from tandem genes in *Escherichia coli*. *Applied Biochemistry and Biotechnology*. 2007;**141**(2-3):203-213. DOI: 10.1007/BF02729062
- [20] Lennick M, Haynes JR, Shen SH. High-level expression of alpha-human atrial natriuretic peptide from multiple joined genes in *Escherichia coli*. *Gene*. 1987;**61**(1):103-112. DOI: 10.1016/0378-1119(87)90369-6
- [21] McDaniel JR, Mackay JA, Quiroz FG, Chilkoti A. Recursive directional ligation by plasmid reconstruction allows rapid and seamless cloning of oligomeric genes. *Biomacromolecules*. 2010;**11**(4):944-952. DOI: 10.1021/bm901387t
- [22] Won J, Barron A. A new cloning method for the preparation of long repetitive polypeptides without a sequence requirement. *Macromolecules*. 2002;**35**(22):8281-8287. DOI: 10.1021/ma020892i
- [23] Shen SH. Multiple joined genes prevent product degradation in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*. 1984;**81**(15):4627-4631. DOI: 10.1073/pnas.81.15.4627
- [24] Li MV, Shukla D, Rhodes BH, Lall A, Shu J, Moriarity BS, et al. HomeRun vector assembly system: A flexible and standardized cloning system for assembly of multi-modular DNA constructs. *PLoS One*. 2014;**9**(6):e100948. DOI: 10.1371/journal.pone.0100948

- [25] Meyer DE, Chilkoti A. Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: Examples from the elastin-like polypeptide system. *Biomacromolecules*. 2002;**3**(2):357-367. DOI: 10.1021/bm015630n
- [26] Chu HS, Ryum J, Park SY, Kim BG, Kim DM, Won JI. A new cloning strategy for generating multiple repeats of a repetitive polypeptide based on non-template PCR. *Biotechnology Letters*. 2011;**33**(5):977-983. DOI: 10.1007/s10529-010-0510-7
- [27] Mi L. Molecular cloning of protein-based polymers. *Biomacromolecules*. 2006;**7**(7):2099-2107. DOI: 10.1021/bm050158h
- [28] Ott W, Nicolaus T, Gaub HE, Nash MA. Sequence-independent cloning and post-translational modification of repetitive protein polymers through Sortase and Sfp-mediated enzymatic ligation. *Biomacromolecules*. 2016;**17**(4):1330-1338. DOI: 10.1021/acs.biomac.5b01726
- [29] Rodríguez-Cabello JC, Girotti A, Ribeiro A, Arias FJ. Synthesis of genetically engineered protein polymers (recombinamers) as an example of advanced self-assembled smart materials. *Methods in Molecular Biology*. 2012;**811**:17-38. DOI: 10.1007/978-1-61779-388-2_2
- [30] Chang AC, Cohen SN. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of Bacteriology*. 1978;**134**(3):1141-1156. DOI: 10.1128/jb.134.3.1141-1156.1978
- [31] Wojtowicz-Krawiec A, Sokolowska I, Smorawska M, Chojnacka-Puchta L, Mikiewicz D, Lukasiewicz N, et al. Use of Ubp1 protease analog to produce recombinant human growth hormone in *Escherichia coli*. *Microbial Cell Factories*. 2014;**13**(1):113. DOI: 10.1186/s12934-014-0113-4
- [32] Schaffner P, Dard MM. Structure and function of RGD peptides involved in bone biology. *Cellular and Molecular Life Sciences*. 2003;**60**(1):119-132. DOI: 10.1007/s000180300008
- [33] Czupryn A., Mucha P., Skowron PM, Mazuryk J, Wiśniewska M, Koziński K, Beresewicz M, Krupska O, Załuska I, Palczewska-Groves M, Żylicz-Stachula A, Żebrowska J, Piotrowski P, Piśkuła M, Sachadyn P, Janus Ł, Rodziewicz-Motowidło S. PolyRGD peptides obtained by chemical and biotechnological method with antidiarrhoeal and/or neuroprotective effect and a set for determination of their activity. European Patent Office no EP 18000319.6 (2018) and Republic of Poland Patent Office no P.425131 (2018). Patent cloud applications pending
- [34] Skowron PM, Żylicz-Stachula A, Żebrowska J, Palczewska-Groves M, Maciejewska N, Czupryn A, Janus Ł, Mucha M, Piśkuła M, Piotrowski A, Sachadyn P, Rodziewicz-Motowidło S, Sawicka J, Dzierżyńska M, Karpowicz P. Modified amplification and expression vectors, fusion proteins, composition and their application. Republic of Poland Patent Office no P.427146 (2018). Patent cloud applications pending.
- [35] Deptuła M, Karpowicz P, Wardowska A, Sass P, Sosnowski P, Mieczkowska A, et al. Development of a peptide derived from platelet-derived growth factor (PDGF-BB) into a potential drug candidate for the treatment of wounds. *Advances in Wound Care (New Rochelle)*. 2020;**9**(12):657-675. DOI: 10.1089/wound.2019.1051
- [36] Shim AH, Liu H, Focia PJ, Chen X, Lin PC, He X. Structures of a platelet-derived growth factor/propeptide complex and a platelet-derived growth

factor/receptor complex. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**(25): 11307-11312. DOI: 10.1073/pnas.1000806107

[37] Pickart L. The human tri-peptide GHK and tissue remodeling. Journal of Biomaterials Science, Polymer Edition. 2008;**19**(8):969-988. DOI: 10.1163/156856208784909435

[38] Pickart L, Margolina A. Regenerative and protective actions of the GHK-Cu peptide in the light of the new gene data. International Journal of Molecular Sciences. 2018;**19**(7):1987. DOI: 10.3390/ijms19071987