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BIOMADE INNOVATION

Revolutionizing industrial peptide production with Numaswitch®

Applications for peptides are growing in a wide range of industries, from active pharmaceutical ingredients (APIs) in pharmaceuticals to their use in agriculture and aquaculture. However, most industries lack a reliable production platform for peptides due to their inherent characteristics, such as degradation sensitivity and aggregation tendency. With our Numaswitch™ approach, we have managed to circumvent these limitations and created a production platform that is not only reliable, but also more cost-efficient, scalable and sustainable, even for complex peptides and proteins.

The increasing importance of peptides

Peptides are used in a wide range of industries, from pharmaceuticals to nutrition and agriculture. Their importance as active pharmaceutical ingredients (APIs) is steadily increasing. In 2018, 5% of all APIs were peptides with a market value of 25 billion US dollars, with applications predicted to grow 7.9 % CAGR until 2027. [1,2] However, the industrial production of peptides still mainly relies on high-cost chemical synthesis, due to the lack of an efficient and cost-effective alternative. The use of toxic and hazardous solvents in chemical synthesis raises concerns regarding sustainability and safety. [3–5] Inspired by nature, the Numaswitch™ technology was developed, making use of the innate nano machineries

of *E.* coli for the biosynthesis of peptides. High product yields can be achieved, since Numaswitch™ avoids common problems such as peptide proteolysis, degradation, aggregation and cytotoxicity effects. The technology takes advantage of switchable Inclusion Bodies (IB), which are refolded quantitatively and make the end product accessible at high titres.[6,7]

Problem solving inspired by nature

Our technologies rely on the protein hemolysin A (HlyA). HlyA is the substrate that is being transported through the Gram-negative cell wall by the type 1 secretion system (T1SS) of *E. coli*. Looking in more detail into HlyA (Fig, 1), three functional domains are found: A) the hydrophobic N-terminal domain (hND), containing a hydrophobic membrane insertion domain, B) a region characterized by repetitive Ca2+-binding domains, so called GG repeats (these GG repeats are part of the consensus sequence GGXGXDXUX with X being any amino acid and U being any large and hydrophobic amino acid), and C) the C-terminal secretion signal (SS). HlyA aggregates in the cytoplasm of the E. coli cell. In the presence of T1SS, the SS domain of HlyA is recognized and the secretion of the protein is induced. Interestingly, the GG repeats bind to Ca2+ ions when the concentration in the HlyA environment exceeds the KD value for that binding event of around 10 µM. The binding of Ca2+ causes HlyA to fold properly. While the intracellular Ca2+ concentration of around 100 nM leads to the aggregation of HlyA (so-called inclusion bodies), when HlyA is secreted into the extracellular space of E. coli where the Ca2+ concentration is about 2 mM, HlyA proteins fold.8 This mechanism, in which Ca2+ ions functioning as ionic switches, is used in the Numaswitch™ technology as the expression platform for pepteins® (peptides & small proteins of ~20-500 aa).

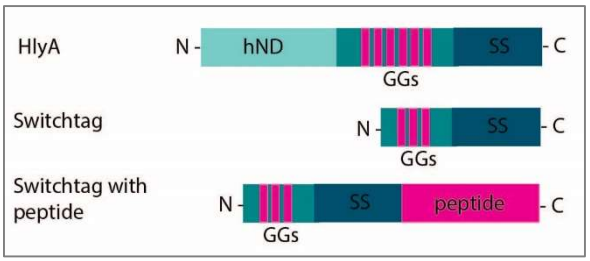

Figure 1: The HlyA protein contains a hND domain, GG repeats and a SS domain. In the context of Numaswitch™, the Switchtag™ protein contains GG repeats and is linked to the target of interest, making the IBs accessible for proper, quantitative folding (renaturation) in the presence of C2+ ions. Made with Biorender.com

Welcome to Numaswitch®

For Numaswitch™, we make use of a fine-tuned version of the HlyA protein containing mainly GG repeats, named Switchtag®, which is linked to the peptein of interest (Fig 1). The production of the target can be divided into simple steps (Fig. 2). At first, we insert the genes for both the Switchtag™ and the desired target peptide as a fusion gene into a plasmid. Competent E. coli cells are then transformed with this circular DNA and the peptein of interest is biosynthesized, linked to the Switchtag®. Due to their nature as discussed above, the fusion proteins will form IBs. After cell lysis, the pepteins, still linked to the Switchtag™, are in their aggregated off state. A sufficient Ca2+ concentration in the buffer will trigger the ionic switch which leads to renaturation of the functional pepteins. Following this, the Switchtag™ can be cleaved from the peptide of interest and purified using different purification approaches. Applying this technology, we ensure that high yields and purity levels are reached, even for pepteins that are normally hard to express (for example β-amyloid and targets containing multiple cysteine bridges). Most importantly, and due to its ease of operation, this technology allows us to produce pepteins in a

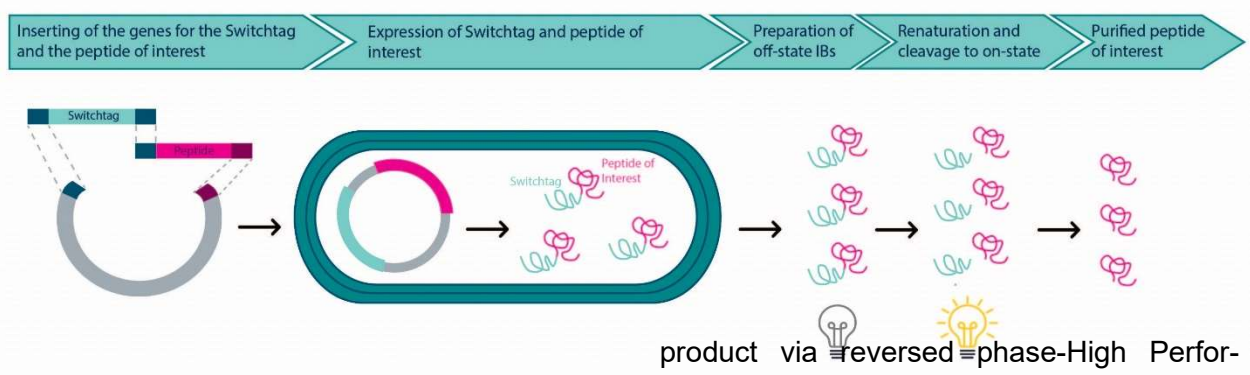

Figure 2: Schematic overview of the Numaswitch technology. From left to right: The genes for the Switchtag™ and the target are inserted into an expression DNA circle called plasmid. Competent *E. coli* cells are transformed with that plasmid. The cells will use the DNA to produce the peptein of interest linked to the Switchtag™, they are present as IBs. After the IBs are released from the cells and are present in their off-state, they are renaturated by Ca2+ ions (on-state) and the Switchtag™ is cleaved from the peptein of interest. The peptein is finally purified, and the level of purity is analyzed by EMA and FDA conform methods. Made with Biorender.com

cost and time efficient way, while reaching target yields as high as > 2g per fermentation liter. Furthermore, high initial purities and product quality are achieved with non-complex processes (HPLC-free), reducing long term cost of goods by more than 90%. Due to the strong Numaswitch™ platform, initial target samples can be produced within weeks, outperforming alternative tailor-made recombinant approaches.

Teriparatide production – a well-known example

We have successfully applied the Numaswitch™ technology for a range of pepteins used in different industrial applications. One example is Teriparatide (PTH1-34), a peptide used for osteoporosis treatment and being commercially sold under the trade name Forteo® [9]. With our established high cell density fermentation protocol, more than 20g Switchtag® Teriparatide protein per liter fermentation broth (as IBs) was produced. Remarkably, more than 95% of the IBs were recovered by refolding them in the presence of Ca^{2+} ions. Purification by a single chromatographic step (Cation Exchange Chromatography) resulted in over 99% product purity. Analysis of the product via reversed-phase-High Performance Liquid Chromatography (RP-HPLC) revealed a purity of >99.6% (Fig. 3). The identity was proven by mass spectrometry and peptide mapping. All specifications for active pharmaceutical ingredients for human applications were met (Table 1). It is worth mentioning that the released products have non-detectable endotoxin levels, despite using *E. coli* as the expression host. This is possible since the aggregated proteins can be washed before the refolding step, eliminating all endotoxins present.

Table 1: Release analytics of Teriparatide produced with Numaswitch®.

Property	Specification	Measured
Identity (4117.7 Da)	1030.4 [M + 4H]$^{+4}$	1030.4 [M + 4H]$^{+4}$
Peptide mapping (aa)	23-30, 23-34, 5-22, 5-19	Confirmed
Purity	≥ 95%	99.6%
Net/gross weight	> 80%	> 88.7%
Acetate	> 95% Acetate	96 (mol%)
Endotoxins	< 5 EU/mg	< 0.4 EU/mg
rHCP	< 500 ng/mg	< 100 ng/mg
rHCD	< 200 pg/mg	< 10 pg/mg
Functionality	As WHO standard	confirmed

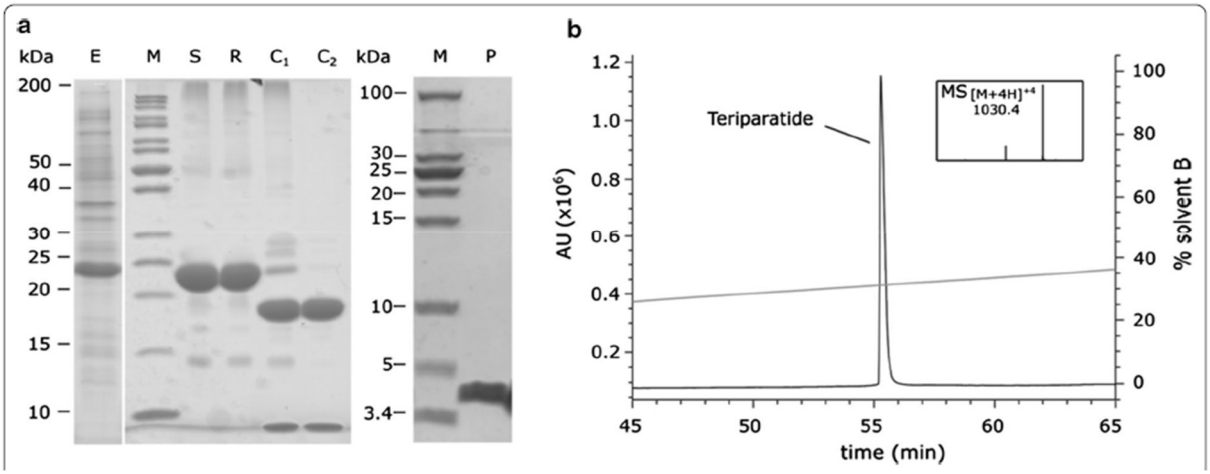

Figure 3: Teriparatide produced with Numaswitch. a) SDS-PAGE analysis of products from different process steps. From left to right: *E. coli* cells after high cell density fermentation (E), protein marker with marked molecular weights (M), solubilized Teriparatide linked to Switchtag (S), Teriparatide linked to Switchtag after renaturation (R), Teriparatide after cleavage from Switchtag (C1: crude, C2: cleared), Teriparatide after purification via CEX purification and TFA/acetate exchange (P). b) Analysis of purified Teriparatide by RP-HPLC/MS.

The future of industrial peptide production

Our Numaswitch™ technology is successful in producing pepteins in a time and cost-efficient manner, supporting the growing market for peptides as APIs and other applications. Compared to alternative recombinant approaches and chemical synthesis, Numaswitch™ can reduce cost of goods by more than 90% with a fully scalable process. Commercial GMP-grade product can be made available within 6 to 9 months. In addition, the technology supports the trend towards green chemistry by significantly reducing hazardous raw materials, while the safety profile of the overall production process and the final product are enhanced. This groundbreaking, highly efficient technology will play an important role in the future for the industrial scale production of peptides, pepteins and proteins.

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NUMAFERM

BIOMADE INNOVATION

Numaferm GmbH
Merowingerplatz 1a
40225 Düsseldorf
Germany

+49 211 97631946
info@numaferm.com
www.numaferm.com