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Numaswitch – The First Viable Alternative for Chemical Synthesis of Peptides and Pepteins

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Introduction

Progress in peptide and protein therapeutics increased the need for rapid and cost-effective production platforms. Yet, the high cost of production remains the major obstacle to the broader use of polypeptide therapeutics. Especially, pepteins of 30-300 amino acids length, covering such important families as long-chained peptides, disulfide-rich peptides, growth factors, peptide fusions and antibody fragments are of high interest and neither chemical synthesis nor traditional recombinant approaches are a reliable and cost-efficient production platform. To this end, we developed such a production platform called Numaswitch [1]. Here, bifunctional Switchtag proteins were developed combining two essential functions (Figure 1). In the first step, Switchtags allow the high titer expression with the accuracy of ribosomal biosynthesis (> 99.99%) as inclusion bodies (IB) protecting the target and cells by aggregation and facilitating easy upscaling of the processes. In the second step, Switchtags act as solubility tags that convert targets quantitatively in water-soluble, functional molecules.

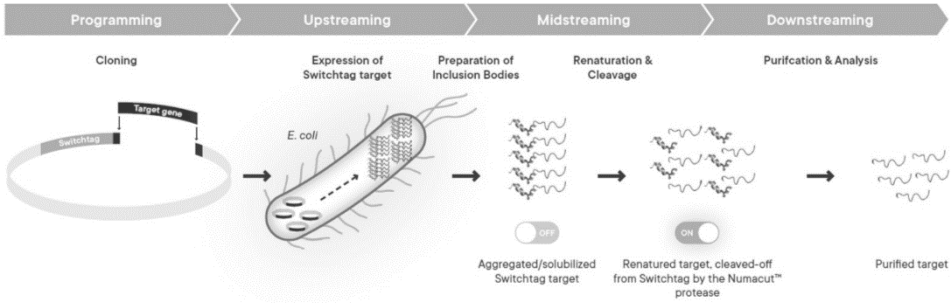

Fig. 1. Numaswitch approach enabling high-titer peptein expression. Adapted from [1].

The development of Switchtags was possible by the application of Ca^{2+} binding domains, so called GG repeats. Being unfolded proteins at the stage of solubilized IBs, the binding of Ca^{2+} to Switchtags (and fusion proteins including target molecules of 2-500 amino acids) enables the quantitative refolding into the native conformation. Inside the cytoplasm of *Escherichia coli* (*E. coli)* and in the absence of Ca^{2+}, Switchtags are unstructured and aggregation-prone proteins. Obviously, Ca^{2+} is acting chaperon-like preventing intermolecular interactions (and thereby aggregation and/or precipitation) of unfolded proteins increasing the overall refolding efficiencies significantly.

Our data show that Switchtags are also applicable for the efficient production of complex, antimicrobial and aggregation-prone targets being fused to Switchtags, like disulfide-rich pepteins, antimicrobials or growth factors. Additional limitations with recombinant approaches are the production of pepteins containing unnatural amino acids [2]. With Numaswitch many of such obstacles can be overcome and many non-natural amino acids can be inserted either during the biosynthesis (via amber stop codon strategy) or by developed protocols (*C*-terminal amidation, *N*-terminal acetylation, side-chain elongations, etc.) [3,4].

As case study (Figure 2), we introduced an unnatural amino acid in Liraglutide (Lira-Bpa), produced the disulfide bridge containing LaM2 light chain antibody fragment and the antimicrobial bifunctional dipeptide conjugate Dermaseptin-Thanatin (DS-THA).

Results and Discussion

To address the current challenges, we generated Switchtag screening libraries of all three above mentioned pepteins and performed expression studies in *E. coli* cells. In agreement with our aim to provide a generally applicable expression platform, we found high-titer expression of the Switchtag target fusion proteins as inclusion bodies. This although hold true for pro-Liraglutide, where an alanine was replaced with the non-canonical amino acid *p*-Benzoyl-L-Phenylalanine (Bpa) by genetic code expansion. Interestingly, the co-expressing of Bpa-tRNA synthetase and the corresponding tRNA had only minor influence on the Switchtag expression level (~ 90%). Refolding from inclusion bodies normally comes along with complex denaturing/refolding procedures and high dilutions (µg/ml). The high renaturation rates observed of up 94% indicate that the Switchtag for the first-time grants access to the advantage of inclusion bodies while acting as a solubility-tag in aqueous solution after execution its molecular switch function by the addition of Ca^{2+}. After renaturation, the pepteins were cleaved-off from the Switchtag, natively and without any traces, using our highly active and specific Numacut TEV protease platfrom (https://numaferm.com/product/). Purification was carried out by state-of-the-art chromatography methods.

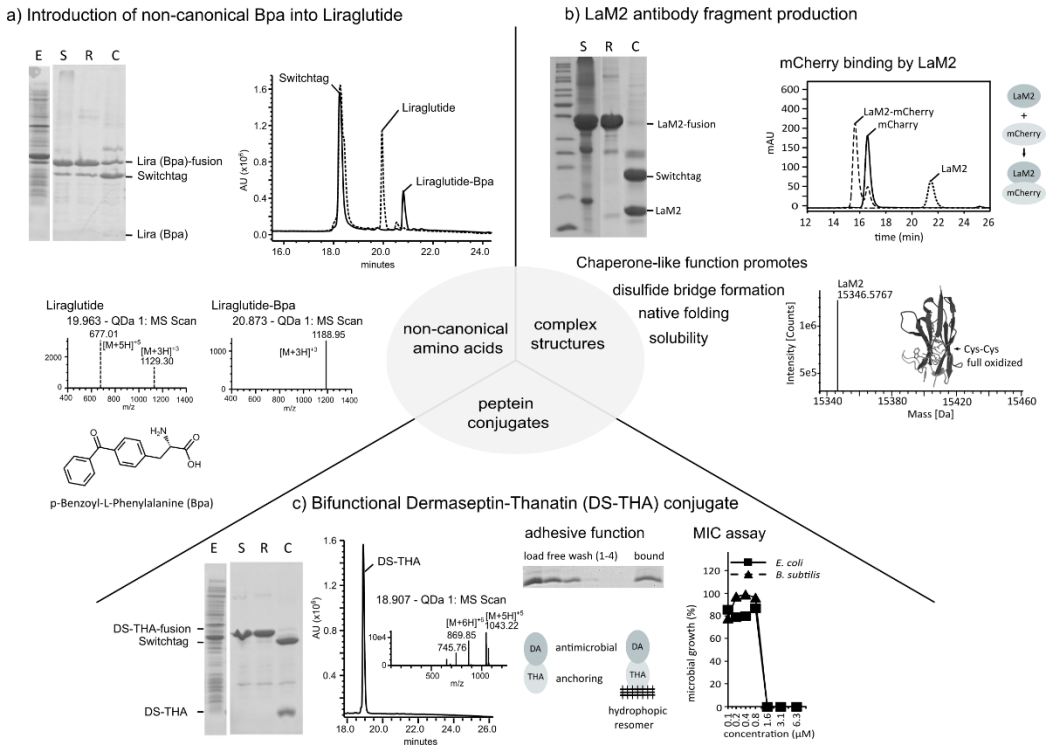

Fig. 2. Application of Numaswitch approach. a) Introduction of the non-canonical amino acid Bpa into pro-Liraglutide. b) Production of complex, disulfide-bridge containing LaM2 light chain antibody fragment. c) Production of the antimicrobial bifunctional peptein conjugate DS-THA. SDS-PAGE illustrated show the expressed (E), the solubilized (S), renatured (R) and by Numacut TEV protease cleaved (C) Switchtag fusion proteins. Introduction of Bpa was confirmed by HPLC/MS, functionality of LaM2 was verified by SEC analysis via binding assay with mCherry. The adhesive function of DS-THA was confirmed by pull down assays and its antimicrobial activity was determined for E. coli and B. subtilis (MIC 1.6 µM).

Table 1. Characteristics of Switchtag-based Liraglutide-Bpa, LaM2 and Dermaseptin-Thanatin production.

Peptein	*Liraglutide-Bpa*	*LaM2*	*Dermaseptin-Thanatin*
Type	non-canonical amino acid	Cys-Cys bridge(s)	antimicrobial peptein conjugate
Size (kDa)	2.7	15.4	5.2
Cys residues	-	2	2
Renaturation (%)	92	83	94
Purity (%)	> 99[a]	> 98[b]	> 99[a]
Titer (g/L)	≥ 2.0[c]	4.5[d]	2.5[d]

[a]determined by RP-HPLC; [b]determined by UV/VIS spectrometry; [c]expected or [d]obtained titer of purified peptein per liter fermentation broth

Mass spectrometry analysis of pro-Liraglutide-Bpa revealed that Bpa was successfully introduced by genetic code expansion, suggesting that the Switchtag approach can act as a promising platform for generation of pepteins harboring non-canonical amino acids. Furthermore, mass spectrometry analysis confirmed that the LaM2 antibody fragment was obtained in the full oxidized state; meaning, that the cysteine-bridge was formed. In addition, functionality of the LaM2 antibody fragment was confirmed by mCherry binding via size exclusion chromatography [5]. Although pepteins are expected to fold correct to the native conformation under thermodynamic control, the folding is very inefficient and dominated by intermolecular interactions yielding to aggregated and/or precipitated targets, especially for cysteine-containing pepteins [6]. The Switchtags act as solubility tags keeping the target in solution and preventing premature aggregation/precipitation. Hereby, the Switchtags gives the peptein more time to form the correct fold and even the right isomers for disulfide-rich candidates (sometimes in the presence of redox shuffle systems).

We also produced, purified, and analyzed the bifunctional antimicrobial peptide DS-THA. DS-THA consist of a hydrophobic, antimicrobial, anchoring domain Thanatin (THA) and an antimicrobial peptide Dermaseptin (DS) [7]. Expression of such a combination is difficult to realize in recombinant systems without the loss of productivity due to the tendency of aggregation and the target toxicity towards the expression host. Although the produced DS-THA is toxic towards *E. coli* with a minimal inhibitory concentration (MIC) of 1.6 µM, growth was not affected and high amount of Switchtag fusion proteins were formed.

Switchtag fusion proteins are expressed in high titers and refolded up to quantitatively, even for complex pepteins. In addition, due to the highly pure refolded Switchtag proteins, process-related impurities (host cell DNA, proteins, and endotoxins) are easily removed before, after or during the refolding step allowing high production titers and purities (Table 1).

Overall, our data show that Numaswitch serves as high-titer expression platform for peptides and pepteins independently of the length, functionality and physicochemical properties.

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