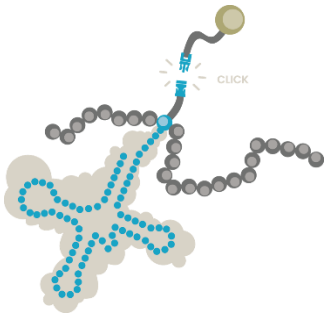
Precision Protein Engineering using Non-canonical Amino Acid Incorporation

Finding solutions for convenient and easy bioconjugation of recombinant proteins utilized in medical applications

White Paper

Executive Summary



This whitepaper introduces the **enGenes-e^{X}pand** technology, the site-specific incorporation of non-canonical amino acids (NCAAs) by amber-stop codon suppression. This technology has vast applications in fields like protein engineering, drug discovery, and biotechnology, by enabling the synthesis of modified proteins with tailored properties, as well as the cost-effective incorporation of NCAAs into proteins for bioconjugation through click-chemistry. This approach, which overlaps with biorthogonal chemistry, offers specific, sensitive, rapid, and easy-to-handle methods for protein modification under physiological conditions. Click chemistry has been applied in diverse areas such as imaging, labelling, sensing, drug design, and enzyme technology, highlighting its potential in protein engineering and medical industries.

Introduction

The use of no NCAAs in protein engineering has been a significant area of research, with applications in various fields. Galindo *et al.,* 2020 and Wiltschi *et al.,* 2016 both highlight the potential of NCAAs, which can be incorporated into proteins using amber codon suppression. This technique allows for the site-specific integration of NCAAs, enabling the addition of unique chemical functionalities to proteins. This approach uses an amber suppressor $tRNA_{CUA}$ to read the amber codon and an aminoacyl-tRNA synthetase to charge it with the NCAA. A major drawback is the low yield of mutant proteins compared to the wild type, due to competition from release factor. Efforts to improve NCAA incorporation efficiency have had moderate success. In search of increased efficiency for scalable industrial applications the **enGenes-e^{X}pand** technology was introduced, which decouples recombinant protein production from cell growth, and significantly increases NCAA incorporation and protein yields compared to conventional *E. coli* BL21(DE3). The target proteins were expressed at high levels with excellent fidelity and preserved function (Galindo *et al.,* 2020). The resulting modified proteins can then be used in a range of applications, including drug delivery (Danielewicz *et al.,* 2023) and structural biology studies (Tyagi *et al.,* 2015). These studies collectively underscore the versatility and potential of precision protein engineering using amber codon suppression and the NCAAs.

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Innovative technologies

enGenes-e^{X}pand Technology

The enGenes-**e^{X}pand** technology significantly improves the yields of protein with incorporated NCAAs. By introducing an orthogonal pair ($tRNA_{CUA}$ to read the amber codon and an aminoacyl-tRNA synthetase) and integrating as well **e^{X}press™** Technology. The combination of both **-e^{X}pand** and **-e^{X}press** technology allows for increased yields of difficult-to-express protein with NCAAs from µg to mg of product, suitable for drug delivery, variolization studies, solid-surface analytics, and vaccine applications. The enGenes-**e^{X}pand** platform provides site-specific functionalization to a wide range of proteins in physiological conditions.

Key Features:

- Site-specific modification without disrupting native structure
- Functionalization for dyes, drugs and other proteins using click chemistry
- Optimized fermentation and purification of the functionalised protein

Scientific Foundations

The development of this technology is based on specific process characteristics.

1. Customized Protein Design:

- The amber stop codon is introduced to position based on structural analysis of protein, sequence alignment analysis and homology comparison.
- Elements supporting high-efficiency transcription and translation of the protein by adjusting promotor, ribosome binding site or addition of suitable signal peptide.

2. Improved Manufacturing Processes:

Optimized fed-batch fermentation processes are employed to achieve efficient production, limited basal expression and ensure efficient nutrient supply and growth conditions.

3. Side-Product- Free Purification:

The introduction of an affinity tag at the C-terminus ensures the removal of truncated forms of protein with NCAAs.

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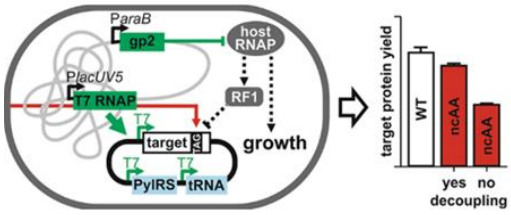
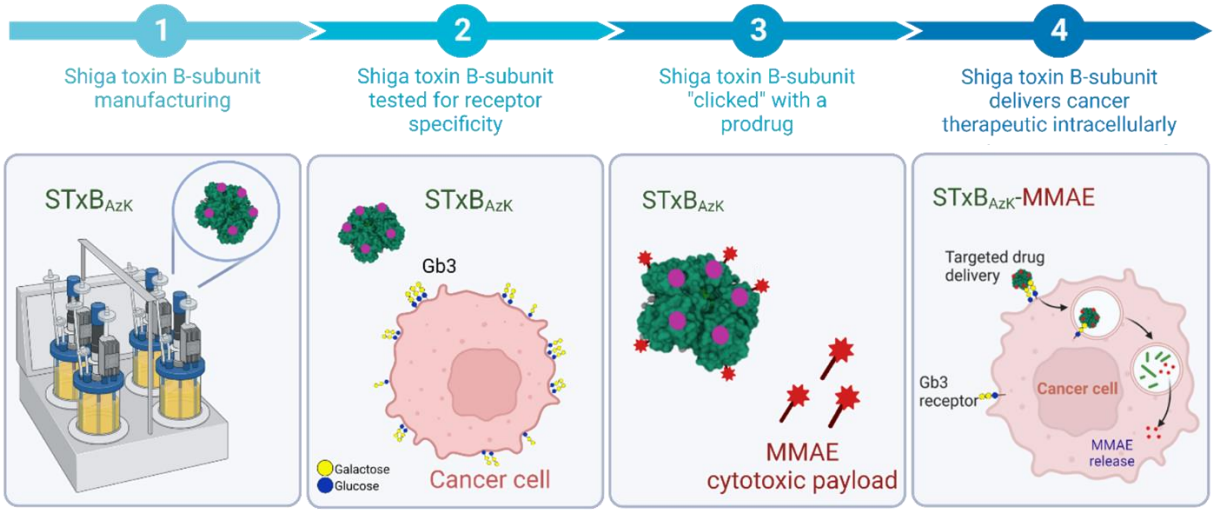
Case Studies and Results

Scale Production:

Studies using the enGenes-**e^{X}pand** platform have demonstrated the successful production of a biologically active functionalized protein, with site-specific modification. The platform combined with **-e^{X}press** technology drives difficult-to-express protein yield from µg to mg of product yield.



Proof-of-concept:



- Studies included manufacturing Shiga toxin B-subunit (STxB) with NCAAs called AzK (azido lysine derivative) incorporated in the structure (Danielewicz *et al.,* 2023).
- Ability to recognise cancer receptors after the incorporation, was tested in vitro,
- which then was followed by the drug attachment. This small molecule is often used in antibody drug engineering and is composed of DBCO-PEG$_4$ that enables

(award-winning) click-chemistry to $STxB_{AzK}$, Valine-citrulline-PAB-linker is enzymatically cleaved by Cathepsin B and Monomethyl Auristatin E (MMAE) is the toxic payload released to the cytosol after cleavage.

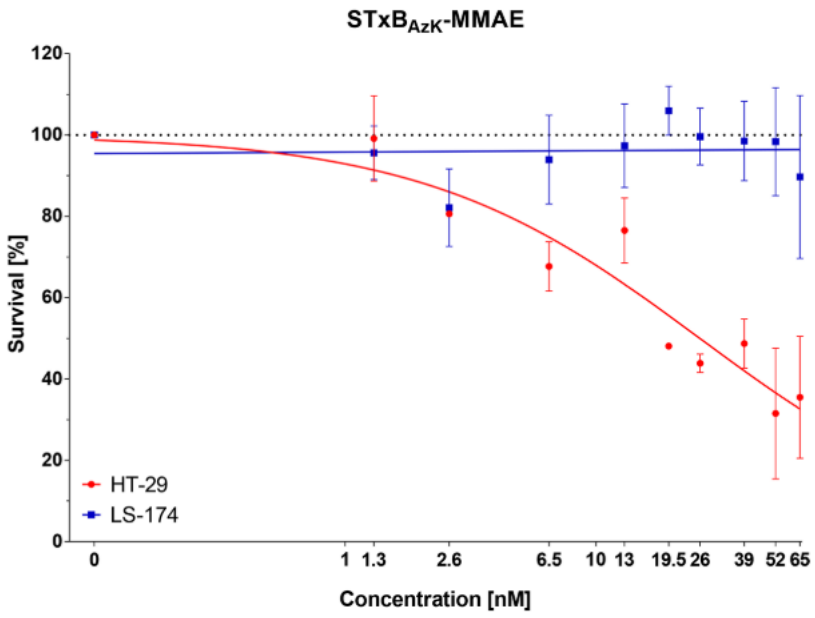
- Performance of drug delivery by functionalized $STxB_{AzK}$ was assessed with standard cell proliferation assay on colon-type adenocarcinoma (HT-29 and LS-174).

Applications:

Functionalized product (e.g. Shiga toxin B subunit, OKT3)[3,5] applicable for drug delivery.

Click chemistry used for fluorophore labelling and cell compartment visualization[3,5,6,7]

(a)



(b)

Cell line	Compound	IC_{50} [nM]
HT-29 ($Gb3^+$)	$StxB_{AzK}$-MMAE	25.89
LS-174 ($Gb3^-$)	$StxB_{AzK}$-MMAE	/

Cytotoxic activity of STxB$_{AzK}$-MMAE on human colorectal adenocarcinoma cell lines HT-29 (Gb3+ high abundance) and LS-174 (Gb3- low abundance) tumour cells. (a) Dose-dependent reduction of HT-29 (red) and LS-174 (blue) cell survival following the addition of STxB$_{AzK}$-MMAE in a standard cell proliferation assay (MTT) for 72 h compared to treatment with PBS. Data represent three independent experiments, n = 3. (b) IC50 values for STxB$_{AzK}$-MMAE efficacy following dose−response cytotoxicity curves presented in (a).

Future Outlook

The enGenes-**e^{X}pand** technology offers a robust solution for the site-specific incorporation of noncanonical amino acids into proteins, enhancing yields and facilitating bioconjugation for medical applications. This advancement paves the way for innovative developments in protein engineering, drug discovery, and biotechnology, making precision protein modification more efficient and scalable.

Conclusion

The enGenes-**e^{X}pand** technology demonstrates significant advancements in the site-specific incorporation of NCAAs, offering enhanced yields and efficient bioconjugation methods. The successful integration of noncanonical amino acids into proteins, while maintaining high fidelity and functionality, underscores the technology's value in advancing medical and scientific research.

Authors

Dr.nat.techn. Natalia Danielewicz, Senior Scientist at enGenes Biotech GmbH.

References

1. Casas, M. G.; Stargardt, P.; Mairhofer, J.; Wiltschi, B. Decoupling Protein Production from Cell Growth Enhances the Site- Speci Fi c Incorporation of Noncanonical Amino Acids in E. Coli. ACS Synth. Biol. 2020, 9 (11), 3052–3066. https://doi.org/10.1021/acssynbio.0c00298.
2. Wiltschi, B. Incorporation of Non-Canonical Amino Acids into Proteins in Yeast. Fungal Genet. Biol. 2016, 89, 137–156. https://doi.org/10.1016/j.fgb.2016.02.002.

3. Danielewicz, N.; Rosato, F.; Tomisch, J.; Gr, J.; Wiltschi, B.; Striedner, G.; Winfried, R.; Mairhofer, J. Clickable Shiga Toxin B Subunit for Drug Delivery in Cancer Therapy. 2023. https://doi.org/10.1021/acsomega.3c00667.
4. Tyagi, S.; Lemke, E. A. Single-Molecule FRET and Crosslinking Studies in Structural Biology Enabled by Noncanonical Amino Acids. Curr. Opin. Struct. Biol. 2015, 32, 66–73. https://doi.org/10.1016/j.sbi.2015.02.009.
5. Rosato, F.; Pasupuleti, R.; Tomisch, J.; Meléndez, A. V.; Kolanovic, D.; Makshakova, O. N.; Wiltschi, B.; Römer, W. A Bispecic, Crosslinking Lectibody Activates Cytotoxic T Cells and Induces Cancer Cell Death. 2022, 1–47.
6. Haigh, J. L.; Williamson, D. J.; Poole, E.; Guo, Y.; Zhou, D.; Webb, M. E.; Deuchars, S. A.; Deuchars, J.; Turnbull, W. B. A Versatile Cholera Toxin Conjugate for Neuronal Targeting and Tracing. Chem. Commun. 2020, 56 (45), 6098–6101. https://doi.org/10.1039/d0cc01085e.
7. Pasupuleti, R.; Rosato, F.; Kolanovic, D.; Makshakova, O. N.; Römer, W.; Wiltschi, B. Genetic Code Expansion in E. Coli Enables Production of a Functional 'Ready-to-Click' T Cell Receptor-Specific ScFv. N. Biotechnol. 2023, 76 (May), 127–137. https://doi.org/10.1016/j.nbt.2023.05.007.