Design and generation of antibody-drug conjugates using non-canonical amino acids

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy
of Freie Universität Berlin
by
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from Montpellier, France

November 2023
Berlin, Germany

Research of the present study was conducted at the Institute for Cell Therapy and Immunology, branch Bioanalytics and Bioprocesses (IZI-BB) of the Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e.V. (Am Mühlenberg 13, 14476 Potsdam-Golm, Germany) and at the Institute of Pharmacy of the Freie Universität Berlin (Königin-Luise-Straße 2+4, 14195 Berlin, Germany) from November 2018 to November 2023, under the doctoral supervision of Prof. Dr. Maria Kristina Parr (Institute of Pharmacy, Freie Universität Berlin).

Herewith I, Nathanaël Rakotoarinoro, certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me. This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

1st reviewer: Prof. Dr. Maria Kristina Parr (FU Berlin).
2nd reviewer: Prof. Dr. Hans Henning von Horsten (HTW Berlin).

Date of defense: January 25, 2024.

Table of Contents

1 Frame and aims of the work

Antibody-drug conjugates. Antibody-drugs conjugates (ADCs) are an emerging class of anticancer drugs. The concept of ADCs can be seen as conferring higher cytotoxicity to the antibody, or higher specificity to the drug. The antibody part of the ADC recognizes the tumor-associated antigen on the tumor cell surface. Once the ADC is internalized, the drug, covalently attached to the antibody via a linker, is released and kills the tumor cell. Their high toxicity combined with their high specificity, as well as their stability and tolerability, make ADCs one of the most promising options for cancer treatment. To date, thirteen antibody-drug conjugates (ADCs) have been approved by various regulatory organizations, such as the Food and Drug Administration (USA), the European Medicines Agency (EU), the Pharmaceuticals and Medical Devices Agency (Japan), and the National Medical Products Administration (China) for the treatment of solid cancers (Aidixi, Elahere, Enhertu, Kadycla, Padcev, Trodelvy, Tivdak) and hematological cancers (Adcetris, Besponsa, Blenrep, Mylotarg, Polivy, Zynlonta). Of these thirteen ADCs, nine were first approved during the course of this work, and all major pharmaceutical companies have ADCs in their clinical pipelines.

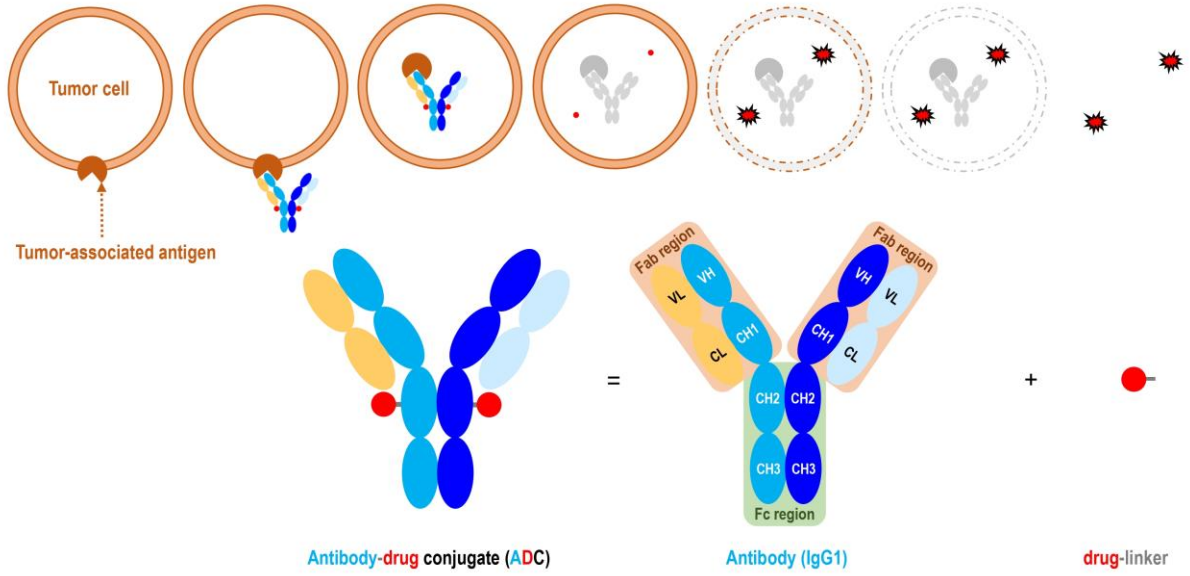

Figure 1. Antibody-drug conjugates. Schematic representation of an ADC, consisting of the antibody and the drug-linker (bottom), and an overall representation of its mechanism of action, in the case of an intracellularly cleavable linker (top). The ADC binds to the tumor-associated antigen and is subsequently internalized. Upon internalization, the linker is cleaved, releasing the drug which kills the tumor cell. Fab stands for fragment antigen-binding, Fc for fragment crystallizable, IgG for immunoglobulin G. VH is the heavy chain variable domain, CH1 the heavy chain constant domain 1, CH2 the heavy chain constant domain 2, and CH3 the heavy chain constant domain 3. VL is the light chain variable domain and CL is the light chain constant domain. The shape and size of the depicted elements are not representative, and the mechanisms depicted here are not exhaustive.

The ADC design is of crucial importance both in terms of production and its therapeutic value. Therefore, it is essential to carefully select and design the antibody (format, target), the drug (type, target), the linker (type), and the conjugation site(s) to ensure their developability, maximize the chances of clinical success, and provide the best possible outcome for the patient.

Non-canonical amino acids. The non-canonical amino acids, distinct from the 20 canonical amino acids, can be ribosomal or non-ribosomal. The ribosomal non-canonical amino acids can be natural (Pyrrolysine, Selenocysteine) or non-natural. The unique structures and chemical functions of the latter enable tailor-made designs of protein conjugates. They can be incorporated at one or more chosen sites of the protein of interest during its translation using an orthogonal system and nonsense suppression.

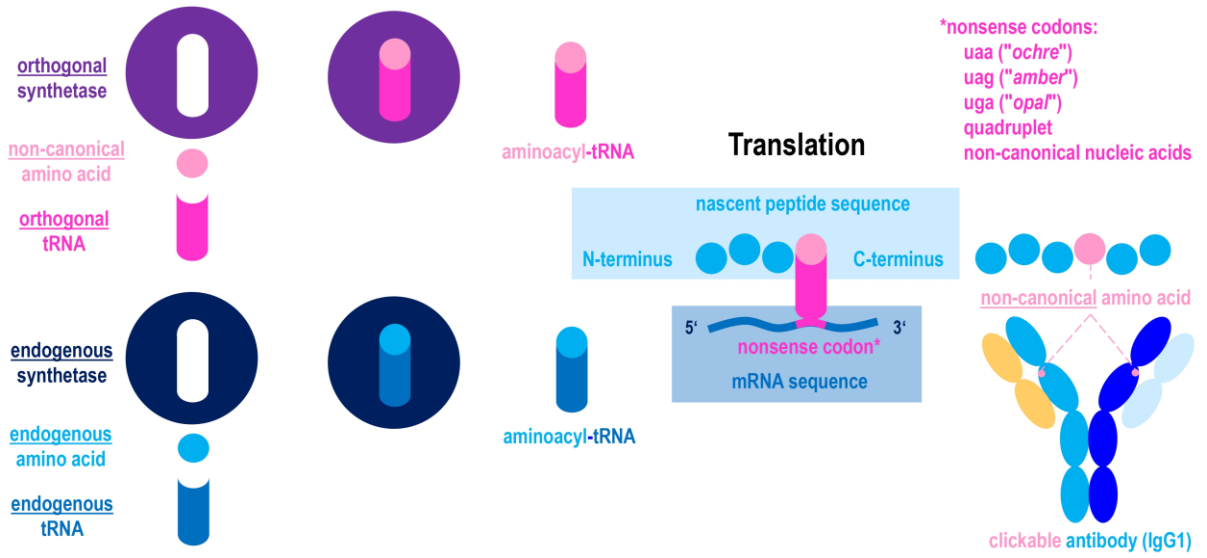

Figure 2. Non-canonical amino acids and orthogonal translation. Schematic representation of the acylation of an amino acid to tRNA by a synthetase (on the left), followed by translation (on the right). The orthogonal aminoacylated tRNA suppresses the stop codon, and the non-canonical amino acid (pink color) is introduced into the nascent peptide sequence. tRNA stands for transfer RNA (RNA, for ribonucleic acid), mRNA for messenger RNA, N-terminus for amino-terminus, C-terminus for carboxy-terminus, 5' is upstream for the mRNA and 3' downstream, and IgG1 stands for immunoglobulin 1. The shape and size of the depicted elements are not representative, and the mechanisms depicted here are not exhaustive.

In addition to enabling unique designs with minimal modifications, the chemical functions allow for bio-orthogonal conjugation reactions under physiological conditions without the need for pre-functionalization steps or enzyme introduction. Therefore, the conjugation efficiency is not dependent on the functionalization efficiency or enzyme quality. Furthermore, the absence of functionalization eliminates the need for additional steps of handling, quantification, and characterization of the candidates, and promotes high-throughput screening.

CHO-based cell-free protein synthesis. Monoclonal antibodies used in ADCs are synthesized in mammalian cells for both research and industrial production. Human embryonic kidney

(HEK) cells are commonly used in research due to their transfection ease, while stably transfected Chinese hamster ovary (CHO) cells are preferred for industrial production due to their growth and productivity. These systems ensure the production of high-quality antibodies, which is crucial for regulatory compliance in industrial production and permits the correct comparison of antibody candidates in research. The CHO-based cell-free protein synthesis, based on translationally active CHO lysates and CHO microsomes, is of high relevance since antibodies are produced in CHO cells at the industrial scale. In the case of cell-free protein synthesis, the cells are cultivated to prepare a lysate. Aside from mimicking CHO events, cell-free synthesis enables features that are not possible in cell-based systems, such as the specific radioactive and orthogonal synthesis of a protein of interest, which allows for its specific quantification, detection, and evaluation in a mixture of CHO endogenous proteins. Hence, the CHO-based cell-free protein synthesis represents a promising analytical tool for investigating the CHO translation, endoplasmic reticulum (ER)-related post-translational modifications, folding, and assembly of the protein of interest, as well as their effects on protein properties.

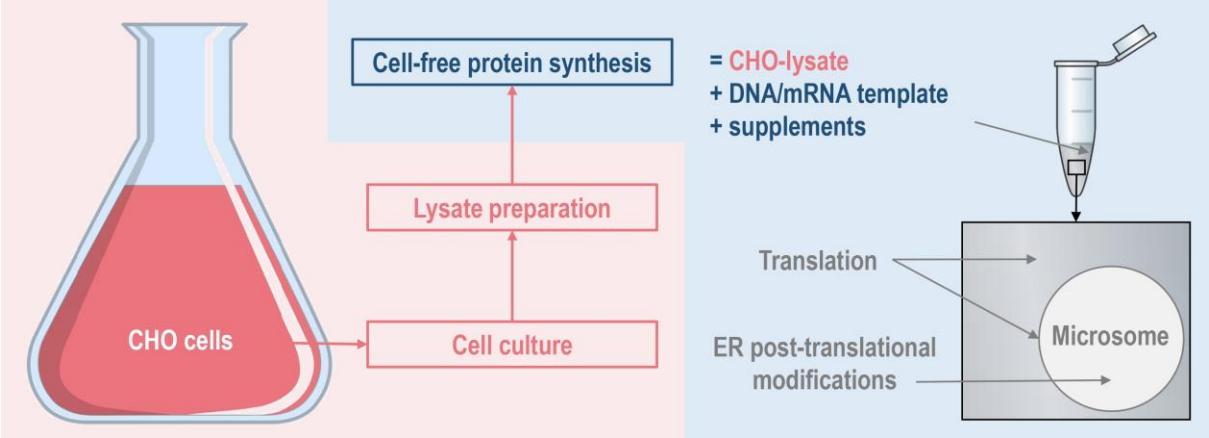

Figure 3. CHO-based cell-free protein synthesis. Schematic representation of the process (in pink) leading to cell-free protein synthesis, followed by a schematic representation of the cell-free protein synthesis (in blue). CHO stands for Chinese hamster ovary, DNA for deoxyribonucleic acid, RNA for ribonucleic acid, and ER for endoplasmic reticulum. The shape and size of the depicted elements are not representative.

Aims of the work. The aim of this doctoral work was to leverage the unique specificities and advantages of these non-canonical amino acids to address recurrent and unresolved challenges in ADCs, thereby enhancing their developability. Consequently, the idea of focussing the work on the antibody conjugation site was chosen. On the other hand, the other aim of this doctoral work was to harness the unique characteristics of this CHO-based cell-free protein synthesis to support the development of such ADCs. Thus, the idea of creating tools for the analysis of these antibodies containing non-canonical amino acids as well as their orthogonal synthesis in CHO systems was chosen.

2 Context and background of the work

2.1 ADC design: antibody conjugation site matters

2.1.1 Antibodies and effect of the antibody conjugation site on ADC properties

Antibodies in ADCs. The main role of antibodies in the context of ADCs is to bind to a defined tumor-associated antigen and to be internalized to enable intracellular release of the drug. Almost all antibodies used in ADCs are of the immunoglobulin (IgG) type 1, 2, or 4[1]. IgGs provide extended half-life and therefore tumor exposition by their binding to the neonatal fragment crystallizable (Fc) receptor (FcRn), enabling recycling and transcytosis, and by their size (~150 kDa for IgG1, IgG2, and IgG4), avoiding renal clearance[2]. In addition, IgGs provide to ADCs an excellent tolerability, due to their ability to withstand and compensate for the hydrophobicity of the conjugated drugs. Apart from their binding to tumor antigens, eliminating the antigen-associated cellular signaling, IgG1s also bind to the complement component 1 (C1) q (C1q), leading to the recruitment of the C1 complex and thus complement-dependent cytotoxicity (CDC). They also bind to Fcγ receptors (FcγRs) on monocytes, macrophages, neutrophils, and natural killer (NK) cells, resulting in antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC)[2]. In contrast, IgG2s with their low affinity for FcγRIIIa, IgG4s with their low affinity for FcγRIIIa and C1q, and engineered IgG1s are chosen to restrict the ADC mechanism of action to antigen binding and drug's killing effect, while still benefiting from FcRn binding. Among the ADCs in clinical trials and approved at the start of this work, eighty-four were based on IgG1s, four on IgG2s, five on IgG4s, and two remained undisclosed[1]. Eighty-nine of these antibodies target fifty-seven different antigens, while the targets of the six remaining antibodies remain undisclosed.

Effect of the antibody conjugation site on ADCs properties. Positions are chosen for their high surface accessibility to ensure maximum conjugation efficiency, necessary for developability, and essential for industrial production. Positions that reduce or prevent antibody binding to its receptors (tumor-associated antigen, FcRn, FcγRs, C1q, protein A) are conventionally avoided. In addition to these positions, recent data has shown that another parameter needs to be considered in the choice of the antibody conjugation site. Unlike IgG2s and IgG4s, IgG1s present the drawback of being cleaved in their hinge region in the tumor microenvironment[3]. This mechanism of resistance renders antibody effector functions no longer possible[3–5]. Although the involved metalloproteinases mainly induce a single cleavage, a full cleavage can also occur[4], releasing the (fragment antigen-binding)$_2$ ((Fab)$_2$) from the Fc part. In the case of ADCs, if the conjugated drug is located at the C-terminal side of the cleavage

site, and full cleavage occurs, the part of the antibody that binds to the antigen to be internalized is devoid of the drug and therefore ADC loses its drug-related toxicity.

2.1.2 Drugs and effect of the drug location on ADC properties

Drugs in ADCs. Once the ADC is internalized by the tumor cell and the drug is released, the role of the drug is to kill the tumor cell. The drugs used in approved or clinically advanced ADCs have different mechanisms. Pyrrolobenzodiazepines/indolinobenzodiazepine dimers (e.g. AstraZeneca's "PBD", ImmunoGen's "IGN"), calicheamicins (e.g. Pfizer/UCB's N-Acetyl-γ-calicheamicin), and duocarmycins (e.g. BMS' "BMS-936561", Byondis' "ByonZine") target DNA. Amatoxins (e.g. Heidelberg Pharma's α-amanitin) target the RNA polymerase II. Camptothecins (e.g. Gilead Sciences' "SN-38", Daiichi Sankyo's "DX-8951f") target the topoisomerase I. Anthracyclines (e.g. Genentech's "PNU-159682") target the topoisomerase II. Auristatins (e.g. Seagen's monomethyl auristatin E and F), maytansinoids (e.g. ImmunoGen's "DM1" and "DM4"), and tubulysins (e.g. AstraZeneca's "AZ13599185") target microtubules.

Effect of the drug location on ADC properties. The majority of drugs used in ADCs are hydrophobic or extremely hydrophobic, such as pyrrolobenzodiazepines, calicheamicins, and duocarmycins. The high hydrophobicity of these drugs is responsible for the high hydrophobicity of the ADC. This has both therapeutic and production-related consequences. In terms of production, this hydrophobicity leads to a higher propensity for aggregation and instability[6,7], rendering the ADC completely ineffective and unusable. From a therapeutic perspective, this hydrophobicity results in hepatic uptake, particularly by Kupffer cells and endothelial sinusoidal cells, leading to low pharmacokinetics and ADC efficacy[8,9]. Aside from the drug-to-antibody ratio (DAR), VanBrunt et al.[10] demonstrated that the antibody conjugation site also influences the hydrophobicity of the ADC. Trastuzumab was found to be less hydrophobic when conjugated with four drugs ("PBD", pyrrolobenzodiazepine at positions HC-K274AECK, IgG1-Eu[11] numbering, and LC-E81AECK, Kabat[12] numbering) (DAR4) than with two drugs ("PBD" at position HC-T359AECK, IgG1-Eu numbering) (DAR2). Later, Tumey et al.[13] demonstrated that conjugating an auristatin derivative ("Aur0101") at the position HC-K334C (IgG1-Eu numbering) resulted in a hydrophobicity shift (by hydrophobic interaction chromatography) of only 47 seconds compared to the unconjugated antibody, while this shift was 244 seconds (fivefold increase) for conjugation at the position HC-L443C, highlighting the extreme importance of the drug location. Charged variants of drugs, such as monomethyl auristatin F (charged version of monomethyl auristatin E), "DM1" (charged version of "DM4"), or amphiphilic drugs like amatoxins, can be used to overcome this hydrophobicity. However,

these drugs do not exhibit bystander effects and therefore have significantly reduced efficacy compared to their hydrophobic counterparts[1].

2.1.3 Linkers and effect of the linker location on ADC properties

Linkers in ADCs. The linkers covalently connect the drug to the antibody. In the case of non-cleavable linkers, drug release relies solely on the proteolytic degradation of the antibody in the lysosome[1]. Thus, the released drug contains a portion of the linker. These non-cleavable linkers are only suitable if the remaining linker portion do not interfere with the drug's efficacy. Cleavable linkers, more common, contain a self-immolative moiety (such as para-aminobenzyl carbamate, known as PABC or para-aminobenzyl ether, known as PABE[14]) between the cleavage site and the drug. This allows for the release of the drug without residual linker[1]. The cleavage mechanism can be based on reduced pH, reducing conditions, tumor-associated proteases, tumor-associated molecules, or UV/Vis/IR irradiation. In the case of a pH-based mechanism, linkers containing a hydrazone[15], carbonate[16], or silyl ether[17] are cleaved by the reduced pH in lysosomes and in the tumor microenvironment. In the case of a mechanism based on reducing conditions, linkers containing a disulfide bridge[18,19] are cleaved by the reduced glutathione present in the cytoplasm. In the case of a mechanism based on tumor-associated proteases present in lysosomes, linkers containing dipeptide[20] (e.g. valine-citrulline, valine-alanine), triglycyl[21], cBu-Cit[22] are cleaved by cathepsins, linkers containing β-glucuronide[23] by β-glucuronidases, linkers containing β-galactoside[24] by β-galactosidases, linkers containing pyrophosphate[25] by phosphatases and pyrophosphatases, and linkers containing arylsulfate[26] by sulfatases. In the case of a mechanism based on tumor-associated molecules, linkers containing 1,2,4-trioxolane[27] are cleaved by elevated levels of ferrous iron present in the cytoplasm, and linkers containing dsProc[28] by Cu(I)-BTTAA present in the tumor microenvironment. In the case of an irradiation-based mechanism, linkers containing heptamethine cyanine fluorophore[29] are cleaved by irradiation with near-infrared light at λ = 650–900 nm, and linkers containing PC4AP[30] are cleaved by irradiation with ultraviolet radiation (λ = 365 nm) followed by intramolecular addition with a nearby amine.

Effect of the linker location on ADC properties. Aside from the drug hydrophobicity, issues related to conjugation sites have been observed for the linkers. The linkers containing the valine-citrulline dipeptide, normally cleaved by the tumor-associated proteases such as lysosomal cathepsins, were also shown to be cleaved unspecifically by murine extracellular carboxylesterase 1C[20,31]. Tian et al.[32] have shown that the conjugation site can influence this unspecific cleavage. When the drug-linker containing this valine-citrulline dipeptide was conjugated at the position HC-S119AcF (IgG1-Eu numbering), the unspecific drug loss in rat

and mouse plasma was significantly higher than when the drug-linker was conjugated at the adjacent position HC-A118AcF, less surface-exposed. Although this unspecific cleavage is limited to mice and rats only, and does not concern humans, it is important to take this into account in the pre-clinical analysis of ADC properties. Besides, the linkers containing maleimides, used in linkers as attachment sites for antibody cysteines, also faced issues. The retro Michael-type addition reaction leads to ADC linker deconjugation, due to maleimide exchange with cysteine residues of plasma proteins such as albumin[33]. Tumey et al.[13] have shown that with low surface-exposed antibody conjugation sites, no or low deconjugation occurs, even when succinimide hydrolysis did not take place. Although solutions have since been developed (hydrolysis of the succinimide ring by surrounding basic amino acids[13,33], by post-conjugation incubation in alkaline conditions[34], by special linkers[35,36], or using maleimide alternatives[37]), it also shows the importance that the antibody conjugation site might have on a linker and therefore on ADC efficacy.

2.1.4 Site-specific conjugation methods and enabled conjugation sites

Site-specific conjugation methods for ADCs. The initial ADCs were based on nearly random conjugation, either involving surface-exposed lysine residues or interchain cysteines[1]. The heterogeneity of the generated ADC hinders their functional and biophysical characterization. Since then, stakeholders in the field have shifted towards conjugations at defined antibody sites. Among the methods based on cysteines, residues HC-A118[33,38], HC-S239[39–41], HC-S442[41], LC-Q124[42], and LC-V205[33,38] for example are replaced by cysteines to enable conjugation. According to the position, it may require a reduction and re-oxidation[33,38–41], or no functionalization steps in rare cases[42]. Among the methods based on the use of non-canonical amino acids, residue HC-A118 is replaced by acetyl-phenylalanine (AcF)[32,43–45], azido-phenylalanine (AzF)[25,46,47], or cyclopentadienyl-ethoxy-carbonyl-lysine (CypK)[48,49]; residue HC-S119 by AcF[32]; residue HC-S136 by azido-methylphenylalanine (AMF)[50]; and residue HC-K274 by azido-ethoxy-carbonyl-lysine (AECK)[10,51] or cyclopropene-lysine (CpHK)[52] to enable conjugation. Among the methods based on glycans, fucose[53,54], sialic acid[55,56], or N-acetylglucosamine (GlcNAc)[57] are chemically[53] or enzymatically[54–57] modified to enable conjugation. Among the methods based on peptide motifs, KKQG[58–60], KCXPXR[61] (X=any amino acid), or LPETG[62] are introduced to enable conjugation via the use of a transglutaminase, formylglycine enzyme, or sortase, respectively.

Enabled antibody conjugation sites. While all these site-specific methods are suitable for industrial production and therapeutic applications, many of them only allow drug-linker conjugation at limited sites of antibodies, such as at the N-terminus, at the C-terminus, at the

glycans, or at specific motifs, thus limiting design possibilities. In contrast, chemical methods based on engineered cysteines and non-canonical amino acids allow for addressing more positions and generating tailor-made ADCs. Since non-canonical amino acids do not react with endogenous cysteines during synthesis, they allow for even more positions and therefore, designs.

2.2 ADC generation: orthogonal synthesis of antibodies

2.2.1 Mammalian cells: the gold-standard for antibody synthesis

Since antibodies used in ADCs are of the IgG type, mammalian systems are primarily used in research as well as in production. In research, where transient transfections are more common, Human embryonic kidney (HEK) cells are mainly used due to their ease of transfection with polyethylenimine (PEI). In large-scale production, where cells are stably transfected and selected, Chinese hamster ovary (CHO) cells are primarily used due to their growth rate and high synthesis yield. These mammalian cells have machinery and quality control systems[63–65] that allow the synthesis of IgGs of extremely high quality. The machinery enables, among other things, N-glycosylation that is identical to or nearly close to human IgGs, proline and cysteine isomerization, formation of disulfide bonds, folding/unfolding assisted by foldases, all of which lead to the desired product (biophysical and functional characteristics). After translation and translocation into the endoplasmic reticulum, the antibody is transported to the Golgi apparatus before being secreted. The quality of the secreted antibodies is ensured through quality controls throughout the whole modification process. In the endoplasmic reticulum for example, the continuous quality control routes proteins that have successfully passed this control to the Golgi apparatus. Proteins identified as incorrect are for example returned to the cytoplasm (endoplasmic reticulum-associated degradation, ERAD) or sent to the lysosomes, (endoplasmic reticulum-to-lysosomes-associated degradation, (ERLAD) where they are degraded. This whole quality control prevents the generation of incorrect antibodies that could create immunogenic reactions in patients. Further upstream in research and development, this quality control allows the reliable characterization and comparison of candidates. In synthesis systems that do not allow for the separation of correct forms from incorrect forms (prokaryotic cells, prokaryotic and eukaryotic cell-free systems), it is not possible to determine for example whether differences in functionality are due to a suboptimal folding, assembly or modification of the product from one side, or is due to the product itself from another side. This, in turn, does not allow for a reliable comparison of candidates and leads to the mistaken exclusion of otherwise promising candidates. Just as the drug-linker conjugation efficiency to the antibody for the characterization of ADC candidates, the quality of the generated antibodies is crucial

for the biophysical and functional characterization of antibody and ADC candidates. More than that, only mammalian cells allow the generation of human or human-like N-glycosylation, with a few rare exceptions[66].

2.2.2 Orthogonal synthesis in mammalian cells

In the chemical method using non-canonical amino acids, the non-canonical amino acid is introduced into the antibody during translation, and the synthesized antibody is then ready-to-click. To carry out the incorporation of the non-canonical amino acid into the IgG, the non-canonical amino acid is first supplemented in the cell culture medium. Once in the cell, the non-canonical amino acid is aminoacylated on the orthogonal transfer ribonucleic acid (tRNA) by the orthogonal aminoacyl-tRNA synthetase (abbreviated as synthetase), both produced by the transiently or stably transfected cell. In the case of synthesis in mammalian cells for ADC applications, an orthogonal pair (synthetase and tRNA) from archaeal systems such as *Methanosarcina mazei*[10,48,49,51,52] or bacterial systems such as *Escherichia coli*[25,32,43–47] is used, native or engineered. The non-canonical amino acid and the orthogonal compounds must not cross-react with endogenous amino acids, synthetases, or tRNAs. Once the orthogonal tRNA is aminoacylated with the non-canonical amino acid, it recognizes a nonsense codon (stop codon) on the mRNA encoding the antibody. The nonsense codon, amber (uag), ochre (uaa), or opal (uga) is placed at the position where the non-canonical amino acid has to be introduced in the antibody. Consequently, the non-canonical amino acid is introduced in the nascent sequence during translation. This event is called suppression since the nonsense codon is suppressed by the aminoacylated orthogonal tRNA. The generated product, containing the non-canonical amino acid, is called the suppression product. However, when the orthogonal system is not optimal, the translation terminates, due to the nonsense codon. This event is called termination and the generated product is called termination product. Although the orthogonal system also targets endogenous nonsense codons, Roy et al.[51] have shown that amber (uag) suppression does not impact the growth, viability, yields, or quality of the produced antibody. Reduced cell viability was only observed with some non-canonical amino acids, due to the non-canonical amino acid itself, independently of the orthogonal system or amber suppression. They also described its suitability for industrial application, outperforming with 3 g/L the yields previously reported[10,32,43]. Aside from enabling unique ADC designs, these non-canonical amino acids enable direct and highly efficient conjugation under physiological conditions, without the need for pre-functionalization steps or enzyme introduction, which are associated with extensive handling, quantification, and characterization steps, and are susceptible to affecting the conjugation efficiency. This promotes reliable assessment and parallelization of

candidates for research applications and represents an attractive way to avoid the introduction of additional batch-to-batch variations for industrial production. More than that, the conjugation efficiencies[10,50] far outperform those of other conjugation methods in the field.

2.2.3 CHO-based cell-free protein synthesis: an ermerging analytical tool

The CHO-based cell-free protein synthesis is of high relevance since CHO cells are the most frequently used system for the industrial production of antibodies. In contrast to cell-based protein synthesis, where the cell directly synthesizes the protein of interest, cell-free protein synthesis involves the extraction of cell contents to synthesize the protein of interest. This CHO-based cell-free protein synthesis is based on the use of translationally active lysate and endogenous microsomes[67–77]. These microsomes are vesicles derived from the endogenous endoplasmic reticulum, enabling post-translational modifications such as N-glycosylation (high mannose), signal peptide cleavage, and disulfide-bridge formation[73], among others. In addition to mimicking cellular events, cell-free synthesis enables features that are not possible in cell-based systems. This includes, for example, the specific radioactive[69–78] and orthogonal[77] synthesis of a protein of interest, which allows for its specific quantification, detection, and evaluation in a mixture of endogenous proteins[69–78]. This is enabled, notably, by the digestion of endogenous mRNA during the lysate preparation and the possibility to change and to control the source of amino acids available for translation. Furthermore, the discontinuous nature of the microsomes and the absence of a continuous energy regeneration system allow for the generation of the different structural states of a protein of interest accumulated within the lumen of the microsome[73]. This might be used to determine for example if an antibody variant has a more challenging or, conversely, easier folding capacity, or if a variant is more likely to undergo post-translational modifications, or less likely. It might also be used to assess the influence of a structural state on the function or conjugability. Finally, the open nature of this approach allows for syntheses under the same conditions, as the cell content and the supplements are the same among the different reactions. This allows for example syntheses with known and defined concentrations of orthogonal components (synthetases, tRNA, non-canonical amino acids)[77], independently of cell events (internalization, transfection, endogenous transcription, translation). Although the first CHO cell-free syntheses were described in 1972[79] and 1974[80], they have only been described twelve other times[67–78] until the start of this work, almost exclusively by the same group[67–77]. CHO-based cell-free synthesis of antibodies within microsomes was described for the first time by Stech et al.[73], and CHO-based cell-free orthogonal synthesis by Zemella et al.[77]. However, CHO-based cell-free orthogonal synthesis of antibodies as well as related technologies has not been described.

3 Peer-reviewed publications of the work

3.1 Publication I (research article)

3.1.1 General information

• *Title of the research article (digital object identifier)*

A disruptive clickable antibody design for the generation of antibody-drug conjugates (doi.org/10.1093/abt/tbad023).

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• *Abstract*

Background: Antibody-drug conjugates are cancer therapeutics that combine specificity and toxicity. A highly cytotoxic drug is covalently attached to an antibody that directs it to cancer cells. The conjugation of the drug-linker to the antibody is a key point in research and development as well as in industrial production. The consensus is to conjugate the drug to a surface-exposed part of the antibody to ensure maximum conjugation efficiency. However, the hydrophobic nature of the majority of drugs used in antibody-drug conjugates leads to an increased hydrophobicity of the generated antibody-drug conjugates, resulting in higher liver clearance and decreased stability. Methods: In contrast, we describe a non-conventional approach in which the drug is conjugated in a buried part of the antibody. To achieve this, a ready-to-click antibody design was created in which an azido-based non-canonical amino acid is introduced within the Fab cavity during antibody synthesis using nonsense suppression technology. The Fab cavity was preferred over the Fc cavity to circumvent issues related to cleavage of the IgG1 lower hinge region in the tumor microenvironment. Results: This antibody design significantly increased the hydrophilicity of the generated antibody-drug conjugates compared to the current best-in-class designs based on non-

canonical amino acids, while conjugation efficiency and functionality were maintained. The robustness of this native shielding effect and the versatility of this approach were also investigated. Conclusions: This pioneer design may become a starting point for the improvement of antibody-drug conjugates and an option to consider for protecting drugs and linkers from unspecific interactions.

• *Keywords*

antibody-drug conjugates. native shielding. non-canonical amino acids. Fab cavity. hydrophilicity. hydrophobicity.

• *Journal / Volume, Issue / Date*

Antibody Therapeutics (Oxford Academic) / Volume 6, Issue 4 / 2023.

https://doi.org/10.1093/abt/tbad023

3.1.2 Accepted manuscript (2023)

A disruptive clickable antibody design for the generation of antibody-drug conjugates

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Abstract

Background: Antibody-drug conjugates are cancer therapeutics that combine specificity and toxicity. A highly cytotoxic drug is covalently attached to an antibody that directs it to cancer cells. The conjugation of the drug-linker to the antibody is a key point in research and development as well as in industrial production. The consensus is to conjugate the drug to a surface-exposed part of the antibody to ensure maximum conjugation efficiency. However, the hydrophobic nature of the majority of drugs used in antibody-drug conjugates leads to an increased hydrophobicity of the generated antibody-drug conjugates, resulting in higher liver clearance and decreased stability. Methods: In contrast, we describe a non-conventional approach in which the drug is conjugated in a buried part of the antibody. To achieve this, a ready-to-click antibody design was created in which an azido-based non-canonical amino acid is introduced within the Fab cavity during antibody synthesis using nonsense suppression technology. The Fab cavity was preferred over the Fc cavity to circumvent issues related to cleavage of the IgG1 lower hinge region in the tumor microenvironment. Results: This antibody design significantly increased the hydrophilicity of the generated antibody-drug conjugates compared to the current best-in-class designs based on non-canonical amino acids, while conjugation efficiency and functionality were maintained. The robustness of this native shielding effect and the versatility of this approach were also investigated. Conclusions: This pioneer design may become a starting point for the improvement of antibody-drug conjugates and an option to consider for protecting drugs and linkers from unspecific interactions.

Introduction

To date, a total of thirteen antibody-drug conjugates (ADCs) have been approved for cancer therapy by various regulatory organizations, such as the Food and Drug Administration (USA), the European Medicines Agency (EU), the Pharmaceuticals and Medical Devices Agency (Japan), and the National Medical Products Administration (China). The concept of ADCs can be seen as conferring greater cytotoxicity to the antibody, or higher specificity to the drug. The antibody part of the ADC recognizes the tumor-associated antigen on the tumor cell surface. Once the ADC is internalized, the drug, covalently attached to the antibody via a linker, is released and kills the tumor cell. Among the thirteen approved ADCs, eleven are based on IgG1, while two of them are based on IgG4. The use of these antibody formats allows a long half-life and stability of the ADCs. Since the mammalian cell machinery allows excellent quality control of proline isomerization, disulfide-bridge formation, N-glycosylation, and other post-translational modifications required for the antibody quality, antibodies are industrially produced in chinese hamster ovary (CHO) cells for eleven of the currently approved ADCs, or in NS0 and Sp2/0 cells for the two remaining. The majority of drugs used for ADCs currently approved or having reached clinical trials target DNA (anthracyclines, benzodiazepines, calicheamicins, duocarmycins), RNA polymerase II (amatoxins), topoisomerase I (camptothecins), or microtubules (auristatins, maytansinoids, tubulysins). A linker, which may be cleavable or non-cleavable, covalently links the drug to the antibody. These drug-linkers are generally produced chemically. Once the drug-linker and the antibody are produced, they are conjugated by chemical, enzymatic, or a combination of enzymatic and chemical methods. Conjugation efficiency is a key issue in research and development since homogeneous products are required to draw meaningful conclusions about functional and biochemical properties. For production, reduced conjugation efficiency has a significant time and cost impact. To ensure maximum conjugation efficiency, surface-exposed conjugation sites are conventionally chosen. However, the drugs used for ADCs are often highly hydrophobic. This leads to an increased hydrophobicity of the generated ADCs, resulting in higher liver clearances by the Kupffer cells and sinusoidal endothelial cells (1) (2), which then reduces the therapeutic efficacy. Aside from the therapeutic aspect, this hydrophobicity also decreases ADC stability due to a higher propensity to aggregate. In contrast, we opted for a non-conventional approach, which can be seen as analogous to a Trojan Horse. We decided to conjugate the drug in a buried part of the antibody and specifically chose the cavity in the Fab region over the cavity in the Fc region (Figure 1a). Indeed, one issue of the IgG1-based antibodies is the cleavage in their lower hinge region in the tumor microenvironment by metalloproteinases (3). While the IgG1 remains full-

length upon single chain cleavage, due to strong non-covalent interactions between both CH3 domains, the full cleavage separates the Fc part from the (Fab)2 part. Hence, if the drug is located on the Fc region and full cleavage occurs, the Fab region, capable of targeting the tumor cell, no longer has a cytotoxic effect.

Enzymatic and combination of enzymatic and chemical methods enable addressing the N-terminus, C-terminus, or limited positions within the antibody, implying very limited design possibilities. On the other hand, chemical methods based on engineered cysteines and non-canonical amino acids allow for addressing more positions and generating tailor-made ADCs. The non-canonical amino acids, due to their unique structures and reactive groups, enable new designs and conjugation strategies. Therefore, this technology was used to create a new design. Although the orthogonal system also targets endogenous nonsense codons, Roy et al. (4) have shown that amber (uag) suppression does not impact the growth, viability, yields, or quality of the produced antibody. Reduced cell viability was only observed with some non-canonical amino acids, due to the non-canonical amino acid itself, independently of the orthogonal system or amber suppression. They also described its suitability for industrial application, outperforming with 3 g/L the yields previously reported (5) (6) (7). Hence, the amber suppression technology was chosen to create a monoclonal antibody (mAb) design in which the non-canonical amino acid is located in a buried position of the Fab. This mAb design was assessed in respect of synthesis, conjugation, as well as of biochemical and functional properties of the generated ADCs.

Results and discussion

Antibody design. Based on 3D structures of human IgG1 (8), murine IgG1 (9), humanized IgG1-based Fab (10) (11) (12) (13) (14), and humanized IgG2-based Fab (15), we aimed to identify positions whose amino acid residue 1) has its α-carbon/β-carbon bond or β-carbon/γ-carbon bond oriented towards the inside of the Fab cavity, 2) while being sufficiently far from the center of the cavity, to provide enough space for the drug-linker and to ensure the maximum shielding effect. For the selected positions, the possible rotamers of the lysine and tyrosine residues were then evaluated. Among the canonical amino acids, lysine is structurally the closest to azido-ethoxy-carbonyl-lysine (AECK), a non-canonical amino acid used for the generation of ADCs (4) (7). Similarly, tyrosine is structurally the closest canonical amino acid to p-azido-phenylalanine (AzF), another non-canonical amino acid used for the generation of ADCs (16) (17) (18). Positions were numbered according to the IgG1-Eu described by Edelman et al. (19). We determined the position HC-152 in combination with AECK as the best-suited design (Figure 1b).

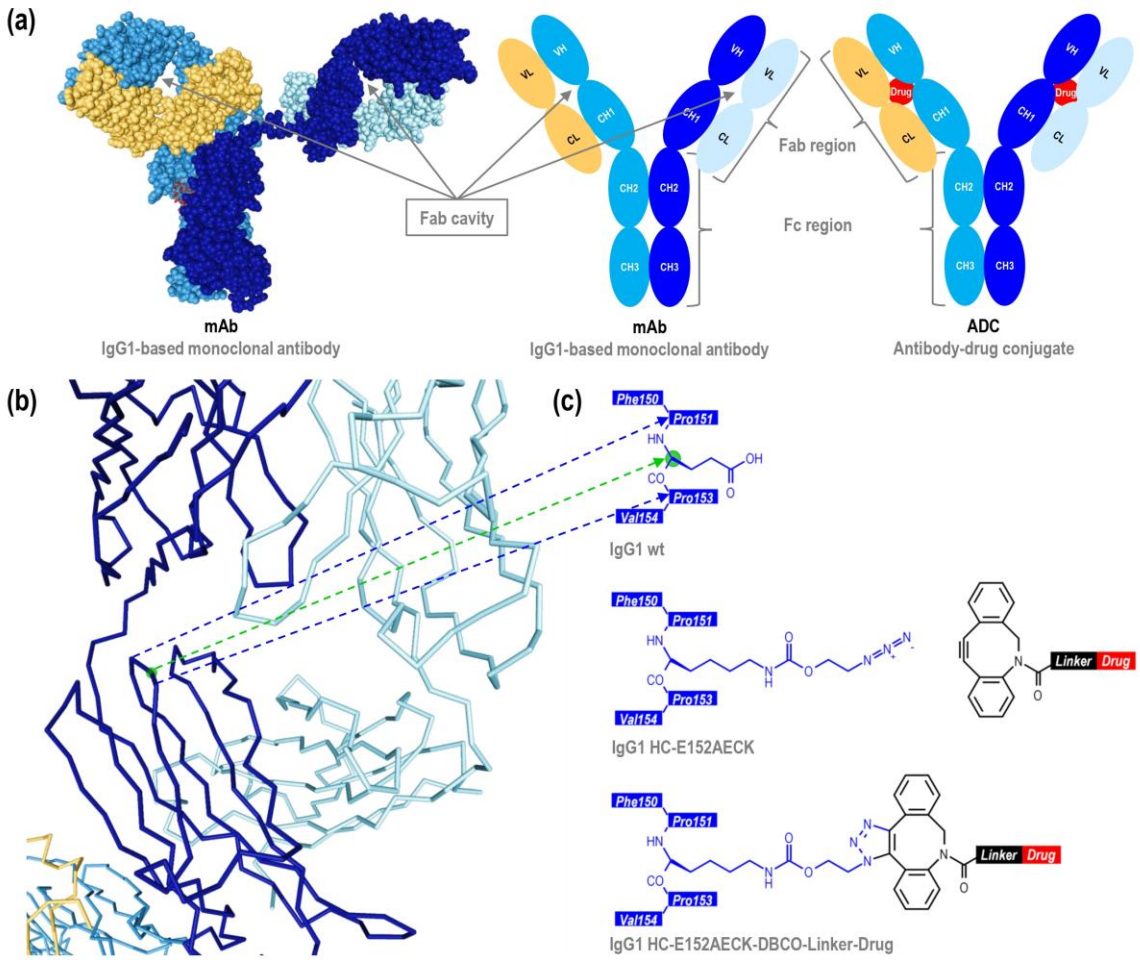


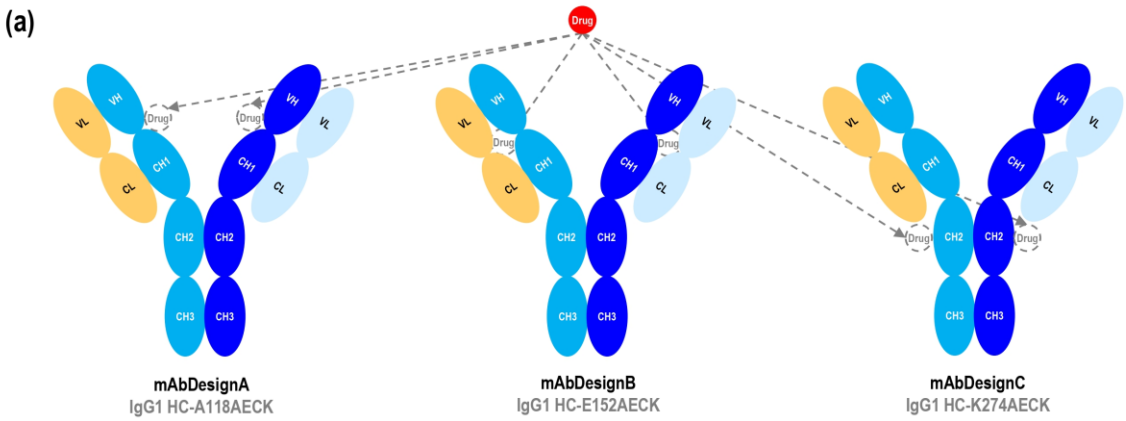
Figure 1. Antibody design (a) 3D (PDB: 1HZH) and schematic representations of a monoclonal antibody, and schematic representation of an antibody-drug conjugate with the drug within the Fab cavity. **(b)** 3D representation (PDB: 1HZH) of one of the two Fab regions. Each point represents the α-carbon of the amino acid. The amino groups, the carboxy groups, and the side chains are not represented to simplify the visualization. The sphere represents the α-carbon of the glutamic acid HC-152. **(c)** Schematic representations of the native antibody sequence with the sphere representing the α-carbon of the glutamic acid HC-152, the antibody sequence with AECK introduced at the position HC-152, and the antibody sequence with AECK at the position HC-152 conjugated to a DBCO-based drug-linker. Only one of the two triazole regioisomers (1,4) is represented. Amino acids are numbered according to the IgG1-Eu described by Edelman et al. (19).

For AzF however, rotamers at this position that are suitable for conjugation may not guarantee an inward orientation of the drug. Therefore, AzF was not further investigated. Position HC-152 has the advantage of being located C-terminal of the proline residue HC-151, a checkpoint for IgG folding. Isomerization of this proline to cis-conformation is essential for the folding of the CH1 domain and its assembly with the CL domain (20). Furthermore, it is located N-terminal of the proline residue HC-153, also one of the three cis prolines of the CH1 domain. As a result, the side chain orientation of the amino acid HC-152 is more likely to be energetically maintained. In combination with AECK containing an azido group,

dibenzocyclooctyne (DBCO) containing the alkyne counterpart was chosen to conjugate the drug-linker to the antibody by strain-promoted azide-alkyne cycloaddition (SPAAC) (Figure 1c). Unlike bicyclononyne (BCN) which forms a single triazole regioisomer upon SPAAC, or simple alkyne which forms a single triazole regioisomer upon copper-catalyzed azide-alkyne cycloaddition (CuAAC), DBCO enables the generation of two possible regioisomers, thus maximizing the chances of addressing this difficult-to-access position.

Antibody synthesis. To determine whether the production of this antibody design would be suitable for industrial application, two additional antibody designs based on different positions for the incorporation of AECK were tested as references. The first reference design is based on position HC-118 (IgG1-Eu numbering). This position was described for the incorporation of the non-canonical amino acids acetyl-phenylalanine (AcF) (5) (6) (21) (22) (23), AzF (16) (17) (18), and cyclopropene-lysine (CypK) (24) (25) to generate ADCs, allowing an enhanced ADC stability over the position HC-119 (6). Indeed, this is the position used in ARX517 and ARX788 (A118AcF), respectively anti-PSMA and anti-HER2 ADCs currently investigated in clinical phases I and II. More widely, this is also the position used in the THIOMAB™ technology based on engineered cysteines (A118C) (26) (27). The second reference design is based on position HC-274. This position was described for the incorporation of AECK (4) (7) and cyclopentadienyl-ethoxy-carbonyl-lysine (CpHK) (28) to generate ADCs, showing the highest ADC hydrophilicity over several positions (7). Both positions HC-118 and HC-274 were reported as suitable positions for the industrial production of antibodies containing non-canonical amino acids using amber suppression (4) (6) (7). In this article, mAb design A refers to the antibody design in which the non-canonical amino acid AECK is introduced at position HC-118, mAb design B at position HC-152, and mAb design C at position HC-274 (Figure 2a). Since human embryonic kidney (HEK) cells are more commonly used in research for antibody synthesis, due to their ease of transfection with polyethylenimine (PEI), and CHO cells are mainly used for industrial production, the feasibility assessment of the designs was performed in both systems. A *Methanosarcina mazei* orthogonal system in combination with amber suppression was chosen to introduce the non-canonical amino acid AECK, and two IgG1-based monoclonal antibodies (mAb1 and mAb2) were used as models. As a proof-of-concept, antibody synthesis was performed by transient transfections. Four plasmids, encoding the antibody heavy chain, the antibody light chain, the orthogonal *Methanosarcina mazei* AECK-transfer ribonucleic acid (AECK-tRNA), and the orthogonal *Methanosarcina mazei* AECK-tRNA synthetase (AECK-RS), were co-transfected in equimolar ratios. Yields were quantified by affinity chromatography using the MabSelect™ SuRe™ column. The protein A of this

column is modified to only recognize the heavy chain at its CH2-CH3 interface, in contrast to the native protein A which additionally recognizes the variable domain of the heavy chain for VH3-type antibodies. As a result, the suppression product can be selectively purified and quantified in a single step.



(b)

mAb	Design	Amino acid and codon context Position HC-118		Amino acid and codon context Position HC-152		Amino acid and codon context Position HC-274		iPASS score	Yields HEK cells	Yields CHO cells
mAb1	DesignA	SS**X**ST	aguucu**uag**ucuaca	FPEPV	uuuccu**gag**ccugug	EVKFN	gaagug**aag**uucaau	1.01	20.0 mg/L	8.6 mg/L
mAb1	DesignB	SSAST	aguucugcaucuaca	FP**X**PV	uuuccu**uag**ccugug	EVKFN	gaagug**aag**uucaau	0.85	17.7 mg/L	7.4 mg/L
mAb1	DesignC	SSAST	aguucugcaucuaca	FPEPV	uuuccu**gag**ccugug	EV**X**FN	gaagug**uag**uucaau	2.00	12.8 mg/L	5.5 mg/L
mAb2	DesignA	SS**X**ST	agcagu**uag**ucuaca	FPEPV	uuuccu**gag**ccugug	EVKFN	gaagug**aag**uucaau	0.65	5.3 mg/L	2.4 mg/L
mAb2	DesignB	SSAST	agcagugcaucuaca	FP**X**PV	uuuccu**uag**ccugug	EVKFN	gaagug**aag**uucaau	0.85	5.1 mg/L	1.5 mg/L
mAb2	DesignC	SSAST	agcagugcaucuaca	FPEPV	uuuccu**gag**ccugug	EV**X**FN	gaagug**uag**uucaau	2.00	2.1 mg/L	0.7 mg/L

Figure 2. Antibody synthesis (a) Schematic representations of the mAb designs A, B, and C, with their intended drug location. **(b)** Amino acid context, codon context, theoretical iPASS score, and antibody yields in CHO and HEK systems for designs A, B, and C with mAb1 and mAb2. The reference amino acid or reference codon is indicated as underlined. **X** represents the non-canonical amino acid. iPASS scores were calculated based on the amber codon context by using the website shiny.bio.lmu.de:3838/iPASSv2. An iPASS score of ≥ 1 should indicate above-average relative non-canonical amino acid incorporation efficiency in HEK cells with the orthogonal *Methanosarcina mazei* pyrrolysine-tRNA/pyrrolysine-RS pair. Antibody yields were quantified from the culture supernatants by affinity chromatography using the MabSelect™ SuRe™ column in an ÄKTA pure 25 L system. Transient transfections were independently performed three times. Amino acids are numbered according to IgG1-Eu described by Edelman et al. (19).

Analysis of the synthesis showed that yields obtained from design B were between those of designs A and C, for both mAb1 and mAb2, in both HEK and CHO systems (Figure 2b). Interestingly, iPass (identification of permissive amber sites for suppression) scores, calculated based on amber codon context and supposed to predict suppression efficiency in HEK cells with the orthogonal *Methanosarcina mazei* pyrrolysine-tRNA/pyrrolysine-RS pair (29), did not correspond at all with the obtained yields (Figure 2b), emphasizing the fact that although codon context is important for suppression efficiency, other factors are much more determining.

ADC Hydrophobicity. To evaluate ADC hydrophobicity, hydrophobic interaction chromatography (HIC) was utilized. This mild condition-based method enables the analysis of ADCs in a non-denatured state. DBCO-C4-PEG3-VC-PABC-AE (named SLAE, for standard linker auristatin E) was conjugated to purified antibodies. Auristatin E (AE) used in SLAE is widely known as monomethyl auristatin E, the main reference for ADC technologies. After conjugation, the generated ADCs were then purified, and HIC-ultraviolet (HIC-UV) analysis showed that ADCs based on design B were the most hydrophilic among the three investigated designs (Figure 3). The drug-to-antibody ratio = 2 (DAR2) species of design B were even more hydrophilic than the DAR1 species of design A. Interestingly, design C, which was reported to lead to the less hydrophobic ADCs among several designs (7), here led to the less hydrophilic ADCs.

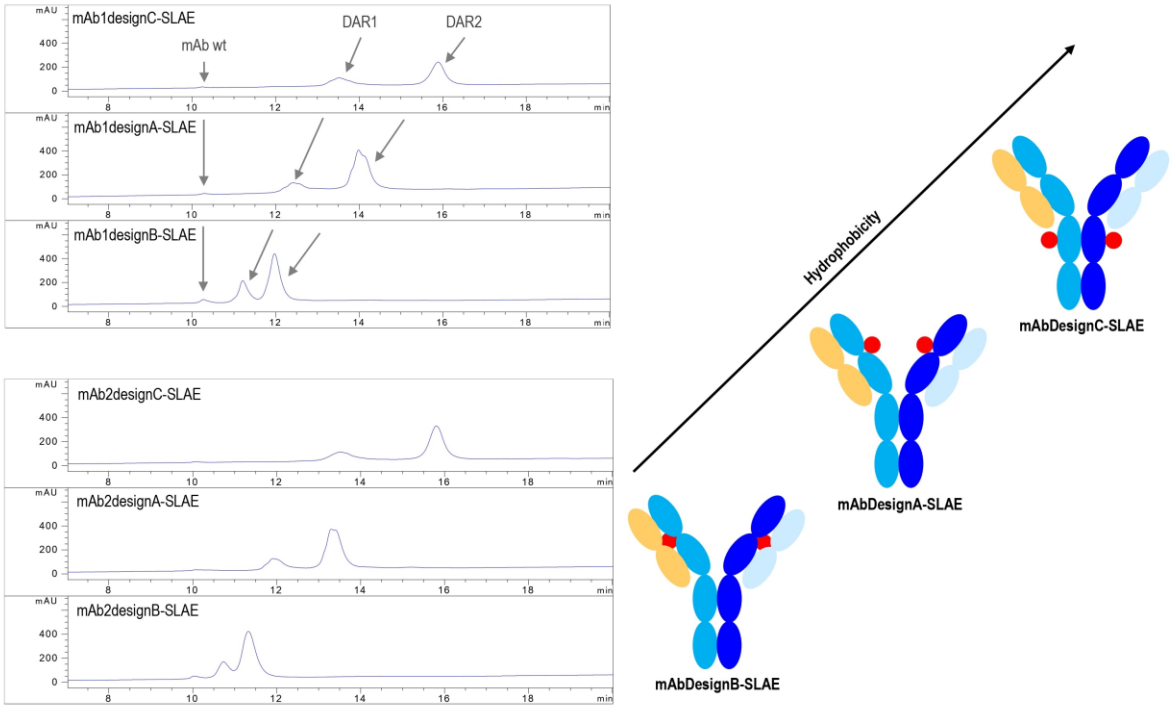


Figure 3. ADC hydrophobicity. Left: HIC chromatograms (214 nm) of ADCs based on mAb1 or mAb2, with designs C, A, and B (from top to bottom), and containing SLAE (DBCO-C4-PEG3-VC-PABC-AE). The arrows indicate the corresponding DAR species in each sample. For the chromatograms presented here, 125 ng of mAb1 wild-type (wt) or mAb2 wt were added to the sample (5 µg) as internal standard before injection. Right: schematic representations of ADCs based on designs C, A, and B (from top to bottom). The arrow indicates the increasing hydrophobicity. SLAE stands for standard linker auristatin E and is schematically represented in Figure 4a.

Shielding effect. To determine the limit of this shielding effect, antibodies were conjugated with a drug via a longer linker (Figure 4a), to place the drug further outside the cavity. For this purpose, DBCO-C6-PEG8-VC-PABC-AE, named LLAE (for long linker auristatin E) was used

to generate new ADCs. Compared to ADCs based on SLAE, ADCs based on LLAE showed an increase in retention time of 4 seconds with mAb1 and 5 seconds with mAb2 for the ADCs based on design C. For ADCs based on design A, an increase in retention time of 8 seconds with mAb1 and 9 seconds with mAb2 was observed. For ADCs based on design B, an increase in retention time of 40 seconds with mAb1 and 66 seconds with mAb2 was observed (Figure 4b). This strong hydrophobic shift for design B indicates a drastically reduced shielding effect. However, ADCs based on design B still remained much more hydrophilic than the ADCs based on designs A and C (Figure 4c), showing that even with this unusually long linker, the shielding effect is still present.

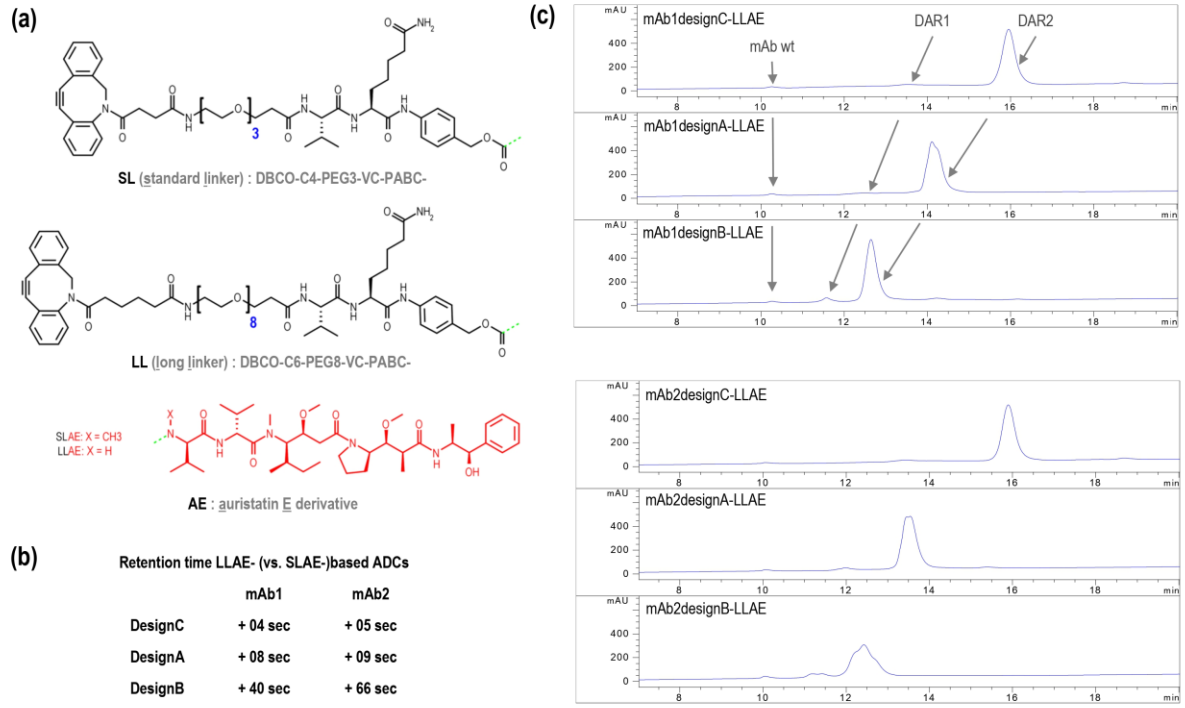


Figure 4. Shielding effect (a) Chemical structures of the standard linker (SL), long linker (LL), and auristatin E derivatives (AE). SLAE is the combination of SL and AE, in which X is a methyl substituent. LLAE is the combination of LL and AE, in which X is a hydrogen substituent. The dashes at the C-terminus of the linker and at the N-terminus of the drug represent the bond between the linker and the drug. **(b)** Differences in retention time (RT) between ADCs based on LLAE and ADCs based on SLAE were calculated as follows: RT difference = $RT_{LLAE\text{-based ADC}} - RT_{SLAE\text{-based ADC}}$. **(c)** HIC chromatograms (214 nm) of ADCs based on mAb1 or mAb2, with designs C, A and B (from top to bottom), containing LLAE (DBCO-C6-PEG8-VC-PABC-AE). The arrows indicate the corresponding DAR species in each sample. For the chromatograms presented here, 125 ng of mAb1 wt or mAb2 wt were added to the sample (5 µg) as internal standard before injection.

Conjugation efficiency. To determine conjugation efficiency, areas under the curve obtained from the HIC-UV chromatograms were analyzed. Even though the non-canonical amino acid AECK is more difficult to access in design B, the conjugation efficiencies were in fact similar between the three different designs (Table 1). Conjugation with LLAE was more efficient than

with SLAE (Table 1). This may be explained by the fact that the conjugation conditions, in particular the dimethyl sulfoxide (DMSO) concentration, were not optimal for SLAE (which is

ADC	DAR	Conjugation efficiency	DAR0 species	DAR1 species	DAR2 species
mAb1DesignA-SLAE	1.83	92%	0%	17%	83%
mAb1DesignB-SLAE	1.69	85%	2%	26%	72%
mAb1DesignC-SLAE	1.67	84%	0%	33%	67%
mAb2DesignA-SLAE	1.79	90%	0%	21%	79%
mAb2DesignB-SLAE	1.77	88%	1%	21%	78%
mAb2DesignC-SLAE	1.69	85%	0%	31%	69%
mAb1DesignA-LLAE	2.00	100%	0%	0%	100%
mAb1DesignB-LLAE	1.96	98%	0%	4%	96%
mAb1DesignC-LLAE	1.95	97%	0%	5%	95%
mAb2DesignA-LLAE	1.98	99%	0%	2%	98%
mAb2DesignB-LLAE	1.90	95%	1%	8%	91%
mAb2DesignC-LLAE	1.96	98%	0%	4%	96%

Table 1. DAR, conjugation efficiency, DAR0, DAR1, and DAR2 species of the generated ADCs. Samples (5 µg) were analyzed by HIC (214 nm) without addition of the reference mAb wt.

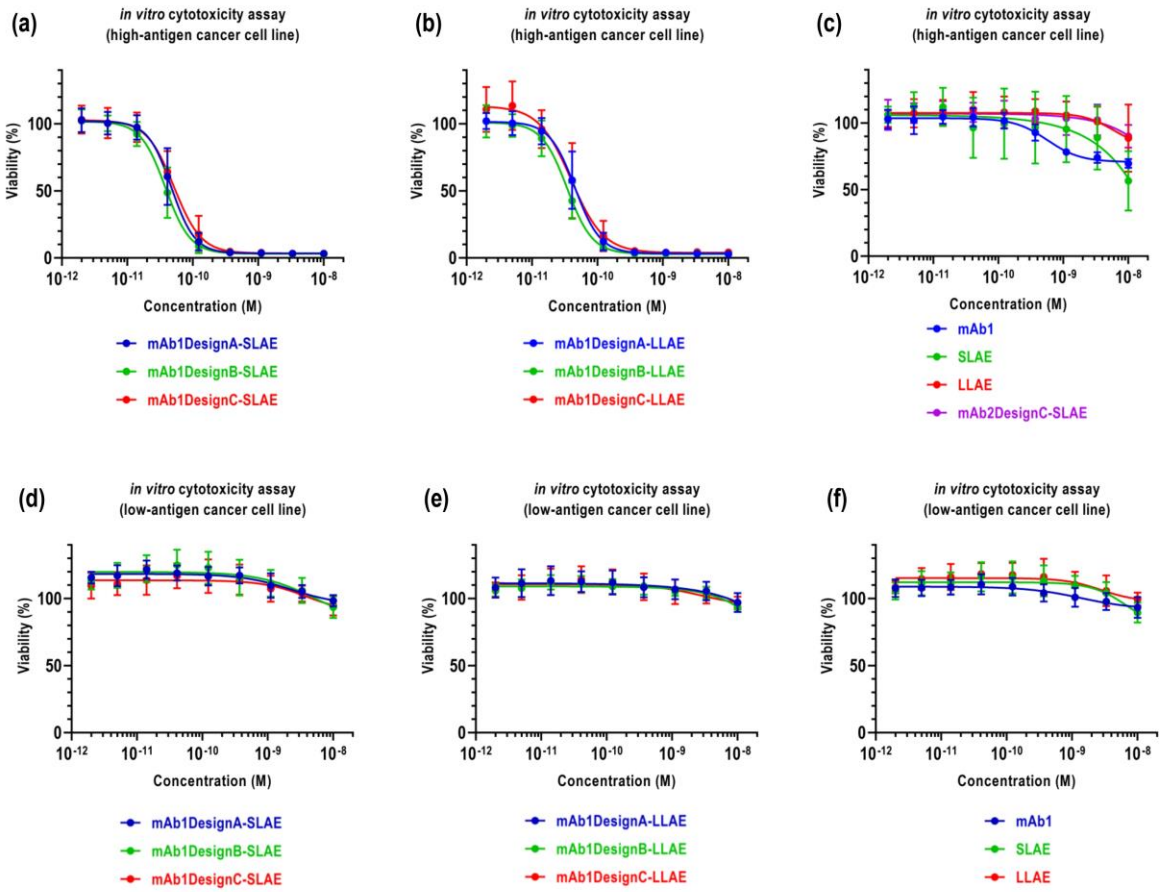


Figure 5. *In vitro* efficacy. Cytotoxic activity of **(a)** SLAE-based ADCs, **(b)** LLAE-based ADCs, **(c)** mAb wt, drug-linkers, and isotype ADC on high antigen-expressing cancer cell lines. Cytotoxic activity of **(d)** SLAE-based ADCs, **(e)** LLAE-based ADCs, **(f)** mAb wt and drug-linkers on low antigen-expressing cancer cell lines. The means and the standard deviations are the results of 3 independent experiments performed in triplicates.

less polar than LLAE). Even though LLAE resulted in more hydrophobic ADCs, due to the drug being more exposed on the surface of the antibody, it showed that 100% conjugation efficiency could be achieved (Table 1), which corroborates the fidelity of this *Methanosarcina mazei* orthogonal system (4) and the excellent conjugability of this non-canonical amino acid (7).

***In vitro* efficacy.** To determine whether shielding the drug in the Fab cavity reduces the ADC efficacy at the cellular level, *in vitro* cytotoxic assays were performed. As the cleavable linker is less accessible for design B and therefore more strongly dependent on lysosomal degradation compared to designs A and C, it was expected that ADCs based on the latter two designs show better efficacy. However, analysis of cytotoxic activity on high-antigen expressing cancer cell lines showed that ADCs based on design B perform as well as ADCs based on designs A and C (Figure 5a,b). Additionally, ADCs based on design B did not show cytotoxicity on low-antigen expressing cancer cell lines, similar to ADCs based on designs A and C (Figure 5a,b). The antibody wild-type (wt) alone, the drug-linkers alone, and the isotype ADC showed low cytotoxicity (Figure 5c).

Versatility. The proof-of-concept of this approach has been completed with auristatin E derivatives as ADC payload. To determine if this is not only applicable to auristatin E derivatives, this approach was repeated with a chemical molecule having a very different structure. DBCO-Cyanine5 (Figure 6b), a highly hydrophobic fluorophore, rarely used compared to its more hydrophilic sulfonated equivalent (DBCO-sulfo-Cyanine5), was conjugated to mAb1 designs A and B (Figure 3a) (Figure 4c). Due to its higher hydrophobicity, design C was not investigated anymore. As similarity in respect of hydrophobicity and conjugation efficiency was observed between both antibodies (Figure 3, 4, table 1), further investigations were done with either mAb1 or mAb2. To quantify and compare the hydrophobicity of ADC designs, the relative hydrophobicity (RH) was calculated based on the retention times (RT) from HIC-UV chromatograms, using the equivalent ADCs based on design A as reference (Figure 6a). For this, 125 ng of the corresponding antibody wt were added to the sample (2 µg) as internal standard before injection into the HIC-UV system. It was observed that the shielding effect is also present when conjugating DBCO-Cyanine5 (Cy5) (RH = 0.14) (Figure 6c). It seems that the shorter the linker, the stronger the shielding effect (RH = 0.62, 0.46, and 0.14 with mAb1DesignB conjugated with LLAE, SLAE, and Cy5 respectively) (Figure 6c). This suggests that the potential of this approach with our auristatin E-based drug-linkers can further be exploited.

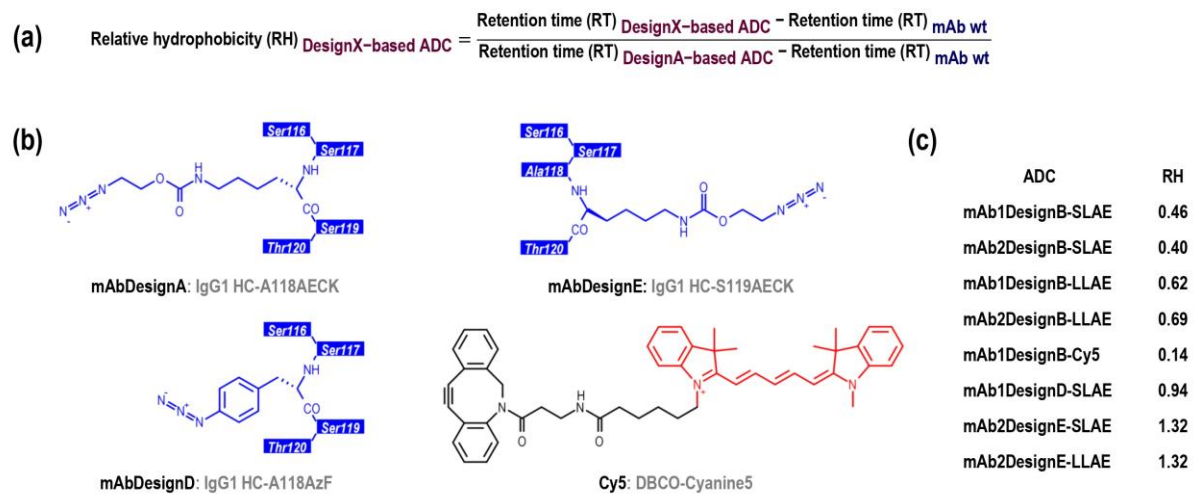


Figure 6. Relative hydrophobicity. (a) Formula of the relative hydrophobicity (RH). For the determination of the relative hydrophobicity, 125 ng of mAb wt were added to the sample (2 µg) as internal standard before injection into the HIC-UV system. Relative hydrophobicity was calculated with design A-based ADC sharing the same antibody and the same drug or fluorophore as reference. A low RH value indicates a low hydrophobicity. **(b)** Schematic representations of the antibody sequence with AECK introduced at position HC-118 (mAbDesignA), the antibody sequence with AzF introduced at position HC-118 (mAbDesignD), the antibody sequence with AECK introduced at position HC-119 (mAbDesignE), and the chemical structure of Cy5 (DBCO-Cyanine5). Amino acids are numbered according to IgG1-Eu described by Edelman et al. (19). **(c)** Relative hydrophobicity (RH) of the generated ADCs. RH of design A-based ADCs, which are equal to 1 by definition, are shown in Table S1.

Additional reference design. Since design A (AECK introduced at position HC-118) was used as a reference but unlike design C has not yet been described in the literature, an additional antibody design, here referred to as design D, was generated. In this design, the non-canonical amino acid p-azido-phenylalanine (AzF) is introduced at position HC-118 (Figure 6b) using an *Escherichia coli*-based orthogonal system in mammalian cells, as described by other groups (16) (17) (18). Designs A and D differ only by the nature of the non-canonical amino acid. Although AzF has a very different structure than AECK, both designs displayed a similar hydrophobicity (RH = 0.94) (Figure 6c), indicating that design B (RH = 0.46) is also superior to this reference design (16) (17) (18).

Relevance of the conjugation site. Another antibody design, here referred to as design E, was also generated to investigate the introduction of AECK at position HC-119 compared to position HC-118 (Figure 6b). ADCs based on design E were significantly more hydrophobic than those based on design A (RH = 1.32 and 1.32 with mAb2DesignE conjugated with SLAE and LLAE, respectively) (Figure 6c), possibly due to the drug-linker oriented outward from the antibody and being more exposed. This may explain the increased unspecific premature cleavage of the valine-citrulline linker in both mouse and rat plasma matrices observed by Tian

et al. (6) for the position HC-119 compared to HC-118 using AcF, and highlights, even more, the importance of the careful selection of the conjugation site.

Conclusion

Similar to a Trojan Horse design, we conjugated the drug into the Fab cavity of the antibody, via the non-canonical amino acid AECK introduced at position HC-152 using nonsense suppression technology. This approach resulted in a significant increase in the hydrophilicity of the ADCs, far surpassing the current best-in-class designs. The yields of this antibody design (mAb design B) in CHO and HEK systems turned out to be even higher than those of the reference antibody design (mAb design C), a design already described as suitable for industrial application, suggesting that yields of 3 g/L reported by Roy et al. (4) may even be improved. Moreover, the conjugation efficiency and cytotoxic activity on tumor cells were also maintained, while low-antigen cells remained unaffected. Although a shielding effect was observed for our design with all drug-linkers, it was observed that the shorter the linker, the better the shielding effect. This suggests that the design of the auristatin E-based drug-linkers used in this approach may still be optimized to fully exploit the potential of this approach. This includes optimizing or redesigning the linker to allow the drug to be more deeply embedded in the Fab cavity. Also of interest, the investigation of bidentate linkers (30) (31) (32) may be an option to consider for the generation of ADCs with a higher DAR. Although this work focused on the generation and early evaluation of ADC designs, it will remain interesting to measure the impact of the high hydrophilicity of design B on the long-term stability, pharmacokinetic properties, and *in vivo* antitumor activity, according to the ADC candidates and indications.

During the course of this project, Coumans et al. (33) described a similar approach using engineered cysteines. In their approach, amino acid residues of the Fab region were replaced by cysteines, and drugs such as duocarmycin, auristatin E, and auristatin F were successfully conjugated. These designs, with the drug shielded in the Fab cavity, resulted in an outstanding hydrophilicity. An *in vivo* study (33) was performed with mice lacking the carboxylesterase 1c (CES1c), to ensure that differences in ADC efficacy were not due to unspecific premature cleavage of the valine-citrulline linker for designs in which more exposed positions are chosen. It was demonstrated that ADCs based on the design HC-P41C almost led to the complete elimination of the tumor, while ADCs based on a standard design did not show a significant reduction of the tumor volume at the same dose. Analysis of the pharmacokinetics showed a correlation with the antitumor activity.

Although unspecific interactions of drugs with endogenous enzymes, transporters, receptors, or anti-drug antibodies have not been extensively reported in the literature yet, the approaches

described in this article and by Coumans et al. (33) may be considered to address such issues. Unlike current linker-based approaches to reduce hydrophobicity, these antibody-based approaches allow for shielding the drug by surrounding it, while removal of this native shield is simply achieved by lysosomal degradation of the antibody in the targeted cell. Due to the high frequency of optimal rotamers of the residue HC-152 combined with the long linear side chain of AECK, the antibody design described in this article increases the propensity of the drug-linker to be conjugated from the inside of the Fab cavity. Aside from this structural aspect, this design presents the rare advantage of enabling both direct and reliable drug conjugation. By rendering obsolete the processes of functionalization, buffer exchange, or enzymatic conversion, usually associated with extensive handling and characterization, this clickable design promotes reliable assessment and parallelization of candidates for research applications and represents an attractive way to avoid the introduction of additional batch-to-batch variations for industrial production.

Material and methods

3D structures. PDB structures 1HZH (8), 1IGY (9), 6OGE (10), 4HKZ (11), 6B9Z (12), 6MH2 (13), and 1N8Z (14) 5SX4 (15) were used as models. Rcsb.org with viewer = NGL (WebGL) was used for the initial visualization of the backbone and side chains. Rcsb.org, with viewer = Jsmol (JavaScript), was used for visualization of hydrophobic regions, patches, and amino acids. WinCoot (0.9.4.1) was used for single amino acid mutation and rotamers identification.

Antibodies. Amino acids were numbered according to the IgG1-Eu described by Edelman et al. (19). The constant domains of the heavy chain are of m17/-1/-2 allotype, where HC-214 is a lysine (G1m17), HC-356 is a glutamic acid (G1m-1), HC-358 is a methionine (G1m-1), and HC-431 is an alanine (G1m-2). The constant domains of the light chain are of Km3 allotype, where LC-153 is an alanine and LC-191 is a valine. mAb1 heavy chain variable domain is of VH3 humanized type, mAb2 heavy chain variable domain is of VH1 humanized type. mAb1 and mAb2 light chain variable domains are of VKI humanized types. Signal peptides and signal peptide types remain undisclosed. Shiny.bio.lmu.de:3838/iPASSv2/ (29) was used to calculate theoretical iPass scores according to the user guide.

Orthogonal synthetases and tRNAs. Orthogonal Methanosarcina *mazei* AECK-RS is also known as *Methanosarcina mazei* pyrrolysine-RS and was not modified. Orthogonal *Escherichia coli* AzFRS is a modified version of the *Escherichia coli* tyrosine-RS (Y37T-D182S-F183A-D265R, sequential numbering) as described by Chin et al. (34). Orthogonal *Methanosarcina mazei* AECK-tRNA is a modified version of the *Methanosarcina mazei* pyrrolysine-tRNA (a10g-u14g-u16g-u20c-u25c-a52c, sequential numbering) as described by

Serfling et al. (35). Orthogonal *Escherichia coli* AzF-tRNA is a modified version of the *Escherichia coli* tyrosine-tRNA(-gua) (g35c, sequential numbering) as described previously (36) (37). The introduction of a nuclear export signal (NES) to the N-terminus of the orthogonal *Methanosarcina mazei* AECK-RS, supposed to increase its cytosolic localization and suppression in HEK cells (35) (38), did not significantly increase the yields in our HEK system and strongly reduced the overall yields in our CHO system, independently of the suppression efficiency. Therefore, antibody synthesis was performed in all systems without this NES.

Plasmids and DNA sequences. pcDNA3.4 was used as a final vector for HC and LC and was initially ordered at ProteoGenix S.A.S. pcDNA3.1_Zeo(+) was used as a final vector for orthogonal aa-RSs and was initially ordered at BioCat GmbH. pcDNA3.1_Hygro(+) was used as a final vector for orthogonal tRNAs and was initially ordered at Life Technologies GmbH. DNA sequences for heavy chain constant domains, i.e. from HC-A118 to HC-K447, and DNA sequences for light chain constant domains, i.e. from LC-R108 to LC-C214, were optimized for CHO systems using the GeneOptimizer Algorithm (Geneart AG) and were initially ordered in pcDNA3.1(+) vectors at BioCat GmbH. DNA sequences for heavy chain variable domains and light chain variable domains as well as signal peptides for mAb1 and mAb2 (optimization undisclosed) were initially ordered at BioCat GmbH in pUC57-1.8k vectors. Orthogonal *Methanosarcina mazei* AECK-RS and orthogonal *Escherichia coli* AzF-tRNA synthetase (AzF-RS) were not DNA-optimized and were initially ordered in a pcDNA3.1(+) vector at BioCat GmbH. The DNA sequence for the NES was optimized as described by Serfling et al. (35). For *Methanosarcina mazei* AECK-tRNA and orthogonal *Escherichia coli* AzFtRNA, the U6 promoter, as in BLOCK-iT™ U6 RNAi Entry Vector (Invitrogen, reference K494500), was used as tRNA promoter, following the CMV promoter, and followed by the orthogonal tRNA, a cca-tail, and a tttttt-terminator, in 8 copies, each separated by a *Hin*dIII site. The sequences were ordered at Life Technologies GmbH. For all constructs, no tag was introduced. For all constructs intended to be translated, the aug codon of the Kozak sequence present in the vector was used as a start codon.

Cloning. Gibson assembly technology was mainly used to modify vectors, signal peptides, transcription, and translation elements, as well as to generate amber mutants and final plasmids. For Gibson assembly, polymerase chain reaction (PCR) primers (melting temperature (Tm) annealing part = 60-64 °C; Tm non-annealing part = 48-52 °C) were ordered at Integrated DNA Technologies, BV., and used to generate DNA fragments using a Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs GmbH, reference M0493L). The generated DNA fragments were extracted and purified from agarose gel electrophoresis using the Zymoclean™

Gel DNA Recovery Kit (Zymo Research Europe GmbH, reference D4008) according to the manufacturer's protocol. Purified DNA fragments were then mixed between 1:1 and 1:10 molar ratio for Gibson assembly using NEBuilder® HiFi DNA Assembly, Master Mix (New England Biolabs GmbH, reference M5520AA) at 50 °C for 30 min. After purification using the DNA Clean & Concentrator™-5 (Zymo Research Europe GmbH, reference D4014) according to the manufacturer's protocol, 20-40 ng of the generated product was used to transform *Escherichia coli* JM109 electrocompetent cells (prepared in-house) using a GenePulser Cell™ electroporation system (Bio-Rad Laboratories GmbH). Clones were selected from Luria-Bertani-agar plates containing 100 µg/ml ampicillin, and grown in 10 ml Luria-Bertani Broth (Scharlau, reference 02-406-500) medium containing 100 µg/ml ampicillin for 16 hours. Plasmids were extracted and purified using the QIAprep® Spin Miniprep Kit (Qiagen GmbH, reference 27106) according to the manufacturer's protocol, analyzed by agarose gel electrophoresis, and sequenced by LGC Genomics Berlin.

Plasmid preparation. Transformed *Escherichia coli* JM109 cells were grown, respectively for midi- or maxi-preparation, in 100 or 250 ml Luria-Bertani Broth (Scharlau, reference 02-406-500) medium containing 100 µg/ml ampicillin for 16 hours. Plasmid was extracted and purified using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen / Thermo Fisher Scientific, K210005) or the PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen / Thermo Fisher Scientific, K210007) according to the manufacturer‘s protocol. Eluate was incubated with isopropanol (0.7 volumes per 1 volume eluate) at 25 °C for 2 min, before centrifugation (15000 x g, 4 °C, 60 min). The supernatant was discarded, before new centrifugation (15000 x g, 4 °C, 5 min). The supernatant was discarded, and 70% cold ethanol was gently introduced, before new centrifugation (10000 x g, 4 °C, 5 min). The supernatant was discarded, before new centrifugation (15000 x g, 4 °C, 5 min). The supernatant was carefully removed and discarded, and the pellet was dried (25 °C, 5-10 min). For midiprep or maxiprep, respectively 50 µl or 150 µl ultrapure water were then introduced directly on the pellet, and incubated at 37 °C for 5 min. The resuspended pellet was shaken on a Vibrax® VXR basic (IKA) at 1500 revolutions per minute, 25 °C for 30 min. After the final centrifugation (15000 x g, 4 °C, 30 min), the supernatant was collected.

Antibody synthesis by transient transfection. Although not as optimal as the Expi293™ and ExpiCHO™ systems, HEK and CHO systems with established in-house protocols for further investigation by cell-free protein synthesis were used for antibody synthesis. For FreeStyle™ 293-F cells (Gibco / Thermo Fisher Scientific, reference R79007), HEK TF medium (Xell AG, reference 861-0001) supplemented with 6 mM UltraGlutamine™ (Lonza, reference BE17-

605E/U1) was used. Cells were maintained between $0.5x10^6$ and $3x10^6$ cells/ml at 37 °C, 5% CO_2, 90 rpm (50 mm shaking diameters) in non-baffled flasks without vent cap (cap not tightly closed). For CHO-K1 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, reference ACC 110), adapted in-house for serum-free and suspension culture, ProCHO™5 medium (Lonza, reference BELN12-766Q) supplemented with 6 mM UltraGlutamine™ (Lonza, reference BE17-605E/U1) was used. Cells were maintained between $0.5x10^6$ and $4x10^6$ cells/ml at 37 °C, 5% CO_2, 90 rpm (50 mm shaking diameters) in baffled flasks without vent cap (cap not tightly closed). For transfection, CHO and HEK cells were centrifuged (300 x g, 25 °C, 5 min), resuspended in fresh pre-equilibrated medium into a concentration of $4x10^6$ cells/ml, and incubated at 37 °C for 2 hours. For 10^6 cells, 625 ng of plasmids were introduced in a 150 mM sodium chloride solution (50 ng/µl final). For 10^6 cells, 2500 ng of polyethylenimine (PEI) linear molecular weight 25 000 (Polysciences, Inc, reference 23966-2) (1 mg/ml) were introduced in a 150 mM sodium chloride solution (200 ng/µl final). The PEI-based mix was introduced into the plasmid mix, and incubated at 25 °C for 10 min. After complexation, the mix was introduced drop by drop on the cells while gently swirling. Cells were incubated for 5 hours at 37 °C, 5% CO_2, 55 rpm (50 mm shaking diameters) for HEK cells, and 50 rpm (50 mm shaking diameters) for CHO cells. An equivalent volume (1:1) of fresh pre-equilibrated medium containing 4 mM of azido-ethoxy-carbonyl-lysine (AECK) (Iris Biotech GmbH, reference HAA2080) or p-azido-phenylalanine (AzF) (Bachem, reference 4020250.0001) and 2.5 mM valproic acid (VPA) (Sigma, reference P4543-10G) (39) was introduced on the cells, followed by an incubation of 9 days at 31 °C (39) (40), 70 rpm (50 mm shaking diameters) for HEK cells and 60 rpm (50 mm shaking diameters) for CHO cells. For both HEK and CHO cells, 20 ml of 300 g/l glucose and 30 ml of 200 mM UltraGlutamine™ (Lonza, reference BE17-605E/U1) per liter of medium were supplemented 4 days after transfection. All samples and solutions were filtered or centrifuged (16000 x g, 4 °C, 60 min) with supernatant collection.

Antibody purification. After centrifugation (10000 x g, 4 °C, 30 min), the pH of the cell culture supernatant was controlled and if necessary adjusted (pH 6.0-7.0) for optimal binding to protein A. Before introduction in the FPLC system, the sample was filtered at 0.2 µm (Sarstedt, reference 92.3940.001, reference 83.1826.102, or reference 83.3940.001 according to the sample volume) with filtrate collection, or was centrifuged (16000 x g, 4 °C, 30 min) with supernatant collection. The sample was introduced at 2.5 ml/min in a HiTrap MabSelect SuRe™ 5 ml column (Cytiva, reference 11003494), pre-equilibrated with 5 column volumes (CV) of 20 mM sodium phosphate 150 mM sodium chloride pH 6.9 solution in an ÄKTA

pure™ 25 L (Cytiva) at 4 °C. After a 20 CV-wash at 5 ml/min, the antibody was eluted with a linear gradient of 50 mM sodium citrate pH 3.0 solution at 2.5 ml/min over 8 CV, and eluates were immediately neutralized with 15% of 1 M Tris pH 9.0 solution (13% final). In the case of isocratic elution, eluates were neutralized with 25% of 1 M Tris pH 9.0 solution (20% final). After neutralization, eluates were formulated in the conjugation buffer, i.e. phosphate-buffered saline (PBS) containing 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM sodium phosphate at pH 7.4 (VWR International GmbH, reference E404-200TABS) at 4-6 mg/ml (Nanodrop quantification based on absorbance at 280 nm) using Amicon® Ultra-4 10 kDa (Millipore, reference UFC8010), followed by sample centrifugation (16000 x g, 4 °C, 60 min) with supernatant collection. For quantification purposes, the elution fractions were directly discarded. All solutions were filtered (Sarstedt, reference 92.3940.001, reference 83.1826.102, or reference 83.3940.001 according to the solution volume) with filtrate collection.

Drug- and fluorophore-linkers. DBCO-C4-PEG3-VC-PABC-AE (MedChemTronica, reference HY-111012) and DBCO-C6-PEG8-PABC-AE (Iris Biotech GmbH) were used as drug-linker models. DBCO-Cyanine5 (BroadPharm, reference BP-23775) was used as a fluorophore-linker model.

Antibody conjugation. The final volume of the conjugation reaction was chosen according to the nature and the concentration of the drug-linker or fluorophore-linker while keeping the final antibody concentration higher than 0.5 mg/ml. The antibody (initially in PBS) was incubated (25 °C, 15 min) in 10%, 15%, or 18% of dimethyl sulfoxide (DMSO) (Sigma, reference D8418-100ML), and the drug-linker or fluorophore-linker (initially in 100% DMSO) was incubated (25 °C, 15 min) in 60%, 50%, or 40% of DMSO before conjugation. Conjugation occurred at 10-20% final DMSO (80-90% final PBS), with 2-4 molar equivalents of drug-linker or fluorophore-linker per site of conjugation, at 25 °C for 16 hours. Conjugates were purified and formulated again in PBS pH 7.4 at 0.5-1.0 mg/ml (Nanodrop quantification based on absorbance at 280 nm) using Amicon® Ultra-0.5 30 kDa (Millipore, reference UFC5030), followed by sample centrifugation (16000 x g, 4 °C, 60 min) with supernatant collection or were introduced at 0.5 ml/min in a HiTrap™ rProtein A FastFlow 1 ml column (Cytiva, reference 17507902), pre-equilibrated with 5 CV of 20 mM sodium phosphate 150 mM sodium chloride pH 6.9 solution in an ÄKTA pure™ 25 L (Cytiva) at 4 °C. After a 10 CV-wash at 1 ml/min, the antibody was isocratically eluted with 3 CV of 50 mM sodium citrate pH 3.0 solution at 0.5 ml/min and immediately neutralized with 25% of 1 M Tris pH 9.0 solution (20% final). After neutralization, eluates were formulated in the conjugation buffer (PBS pH 7.4) at

0.5-1.0 mg/ml (quantification based on absorbance at 280 nm) using Amicon® Ultra-0.5 10 kDa (Millipore, reference UFC5010), followed by sample centrifugation (16000 x g, 4 °C, 60 min) with supernatant collection. All samples and solutions were filtered (Sarstedt, reference 92.3940.001, reference 83.1826.102, or reference 83.3940.001 according to the sample or solution volume) with filtrate collection or centrifuged (16000 x g, 4 °C, 60 min) with supernatant collection.

Hydrophobic interaction chromatography. Hydrophobic interaction chromatography was performed on a TSKgel Butyl-NPR, 2.5 µm particle size, 4.6 mm ID × 3.5 cm column (Tosoh, reference 0014947) using an Agilent 1260 Infinity HPLC-DAD system (Agilent Technologies Deutschland GmbH). Mobile phase A consisted of 25 mM sodium phosphate, 1.5 M ammonium sulfate, pH 6.9 and mobile phase B consisted of 80% (v/v) 25 mM sodium phosphate, pH 6.9, and 20% (v/v) isopropanol. The column was pre-equilibrated with 5 CV of mobile phase A and 2 or 5 µg of the sample (0.25 mg/ml in PBS) were subjected to a gradient of 0% to 100% mobile phase B over 20 min at a flow of 0.4 ml/min. Detection was performed at 214 nm, and peaks and area under the curve (AUC) were automatically detected and calculated by the OpenLab CDS 2.14.29 software. For the determination of the relative hydrophobicity, 125 ng of mAb1 wt or mAb2 wt were added to the sample (2 or 5 µg) as internal standard before injection.

***In vitro* cytotoxicity assays.** Samples ($1.5x10^{-12}$ M to $1x10^{-8}$ M) were introduced in wells (Corning, reference Costar 3917) containing 1000 adherent cancer cells (100 µl total volume), seeded 24 hours before, and usually maintained between $15.6x10^3$ and $62.5x10^3$ cells/cm^2 for the high-antigen cancer cell line (undisclosed) and between $28.5x10^3$ and $228.5x10^3$ cells/cm^2 for the low-antigen cancer cell line (undisclosed) in Dulbecco's Modified Eagle's Medium - High glucose with 25 mM HEPES (Sigma, reference D6171-500ML), 10% FBS Supreme (PAN, reference P30-3031), 4 mM Stable Glutamine 200 mM (100X) (PAN, reference P04-82100), 1 mM sodium pyruvate 100 mM (Biowest, reference L0642-100) at 37 °C, 5% CO_2 and 100% humidity. After 120 hours, supernatants (100 µl) were discarded, and 50 µl of a solution composed of 20% (v/v) CellTiter-Glo® 2.0 Cell Viability Assay (Promega GmbH, reference G9243) and 80% (v/v) PBS were introduced in the well, and chilled at 25 °C for at least 20 min protected from light. Luminescence was measured with a FLUOstar Omega plate reader (BMG Labtech).

Authors' contributions

NR originated the concept, outlined the project, designed mAbs, synthetases, tRNAs, and plasmids, established the platforms for cell-based orthogonal translation systems, cell-based

mAb synthesis, purification, and conjugation, prepared the draft manuscript and the figures. NR, YFKD, and MKA generated the data presented in the manuscript. NR and YFKD evaluated project feasibility. YFKD, NR, and MKP set up the methods for mAb/ADC biochemical characterization. MKA, NR, and MS set up the methods for mAb/ADC functional characterization. SKK established the platform for Gibson assembly and related cloning techniques. MKP and MS approved, supported, and supervised the project. YFKD, SKK, MKA, MKP, and MS revised and edited the manuscript. All authors agreed to the final version of the manuscript.

Acknowledgments

The authors acknowledge Dorothea C. Hallier (Fraunhofer-Gesellschaft) for the support with the ÄKTA pure 25 L; Yanan Sun, Jakob Steff, and Felix Bredendiek (Freie Universität Berlin) for the support with the 1260 Infinity; Anika Andersson (Fraunhofer-Gesellschaft) for the support with the culture of adherent cancer cell lines; Anne Zemella (Fraunhofer-Gesellschaft) for the training on orthogonal translation systems; and Sophie Marinoff (Glycotope GmbH) for the factual inputs in respect of ADC design.

Funding

The authors acknowledge the Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e.V., the Freie Universität Berlin, the Bundesministerium für Bildung und Forschung (BMBF), the Ministerium für Wissenschaft, Forschung und Kultur (MWFK), and the Investitionsbank des Landes Brandenburg (ILB) via the Europäischer Fonds für regionale Entwicklung (EFRE) for enabling the work featured in this article.

Conflict of interest statement

NR, SKK, MKA, and MS are employees of the Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e.V.. YFKD and MKP have nothing to disclose.

Animal research statement

Not applicable.

Ethics and consent statement

Not applicable.

Data availability statement

All data are incorporated into the article and its online supplementary material.

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3.2 Publication II (book chapter)

3.2.1 General information

• *Title of the book chapter (digital object identifier)*

Synthesis of fluorescently labeled antibodies using non-canonical amino acids in eukaryotic cell-free systems (doi.org/10.1007/978-1-0716-1406-8_9).

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• *Abstract*

Cell-free protein synthesis (CFPS) enables the development of antibody conjugates, such as fluorophore conjugates and antibody-drug conjugates (ADCs), in a rapid and straightforward manner. In the first part, we describe the cell-free synthesis of antibodies containing fluorescent non-canonical amino acids (ncaa) by using pre-charged tRNA. In the second part, we describe the cell-free synthesis of antibodies containing ncaa by using an orthogonal system, followed by the site-specific conjugation of the fluorescent dye DyLight 650-phosphine. The expression of the antibodies containing ncaa was analyzed by SDS-PAGE, followed by autoradiography and the labeling by in-gel fluorescence. Two different fluorescently labeled antibodies could be generated.

• *Keywords*

cell-free protein synthesis. antibody. antibody conjugates. IgG1. non-canonical amino acid. conjugation.

• *Book / Chapter / Date*

Structural Proteomics: High-Throughput Methods (Springer) / Chapter 9 / 2021.

https://doi.org/10.1007/978-1-0716-1406-8_9

3.3 Publication III (research article)

3.3.1 General information

• *Title of the research article (digital object identifier)*

A CHO-based cell-free dual fluorescence reporter system for the straightforward assessment of amber suppression and scFv functionality (doi.org/10.3389/fbioe.2022.873906).

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• *Abstract*

Incorporation of non-canonical amino acids (ncAAs) with bioorthogonal reactive groups by amber suppression allows the generation of synthetic proteins with desired, novel properties. Such modified molecules are in high demand for basic research and therapeutic applications such as cancer treatment and *in vivo* imaging. The positioning of the ncAA-responsive codon within the protein's coding sequence is critical in order to maintain protein function, achieve high yields of ncAA-containing protein and allow effective conjugation. Cell-free ncAA incorporation is of particular interest due to the open nature of cell-free systems and their concurrent ease of manipulation. In this study, we report a straight-forward workflow to inquire ncAA positions in regard to incorporation efficiency and protein functionality in a Chinese hamster ovary (CHO) cell-free system. As a model the well-established orthogonal translation components *Escherichia coli* tyrosyl-tRNA synthetase (TyrRS) and $tRNA^{Tyr}_{CUA}$ were used to site-specifically incorporate–the ncAA p-azido-L-phenylalanine (AzF) in response to UAG codons. Seven ncAA sites within an anti-Epidermal Growth Factor Receptor (EGFR) single-chain variable fragment (scFv) N-terminally fused to the red

fluorescent protein mRFP1 and C-terminally fused the green fluorescent protein sfGFP were investigated for ncAA incorporation efficiency and impact on antigen binding. The characterized cell-free dual fluorescence reporter system allows screening for ncAA incorporation sites with high incorporation efficiency that maintain protein activity. It is parallelizable, scalable and easy to operate. We propose that the established CHO-based cell-free dual fluorescence reporter system can be of particular interest for the development of antibody-drug conjugates (ADCs).

• *Keywords*

antibody. cell-free protein synthesis. expanded genetic code. mRFP1. noncanonical amino acid. orthogonal system. sfGFP. unnatural amino acid.

• *Journal / Volume / Year*

Frontiers in Bioengineering and Biotechnology (Frontiers) / Volume 10 / 2022.

https://doi.org/10.3389/fbioe.2022.873906

3.4 Declaration of own contribution

3.4.1 Publication I

1st authorship. "***NR*** *originated the concept, outlined the project, designed mAbs, synthetases, tRNAs, and plasmids, established the platforms for cell-based orthogonal translation systems, cell-based mAb synthesis, purification, and conjugation, prepared the draft manuscript and the figures.* ***NR****, YFKD, and MKA generated the data presented in the manuscript.* ***NR*** *and YFKD evaluated project feasibility. YFKD,* ***NR****, and MKP set up the methods for mAb/ADC biochemical characterization. MKA,* ***NR****, and MS set up the methods for mAb/ADC functional characterization. SKK established the platform for Gibson assembly and related cloning techniques. MKP and MS approved, supported, and supervised the project. YFKD, SKK, MKA, MKP, and MS revised and edited the manuscript. All authors agreed to the final version of the manuscript.*"

3.4.2 Publication II

2nd authorship. The authors' contributions were not documented in the manuscript.

I, Nathanaël Rakotoarinoro (**NR**), designed and performed the experiments, and analyzed the data related to the IgG1 synthesized using the orthogonal system (orthogonal components preparation, IgG1 synthesis, extraction, quantitation, analysis, conjugation). Together with TT, AZ, LT, and SK, I (**NR**) revised the draft manuscript prepared by MS.

3.4.3 Publication III

Shared 1st authorship. "*SKK and* ***NR*** *contributed equally to this work and share first authorship. SKK originated and conceived the project. MS and AZ provided the technological basis for the project. SKK and AZ discussed feasibility. SKK and* ***NR*** *planned the project in detail. SKK designed and generated the DNA templates with ncAA locations based on panitumumab structure analysis by* ***NR****. SKK designed and performed experiments for validation of fluorescence recordings in qPCR cycler and scFv binding.* ***NR*** *prepared orthogonal components and designed and performed experiments involving amber suppression. SKK and* ***NR*** *analyzed the data and conceptualized the manuscript. SKK wrote the manuscript and created the figures. SKK,* ***NR****, MS, AZ, and SK revised the manuscript. MS and SK constituted funding.*"

4 Significance and impact of the work

A new and unconventional clickable antibody design for the generation of ADCs. The manuscript I describes a design and, more broadly, a disruptive and innovative approach for generating antibody-drug conjugates (ADCs). So far, current approaches involve conjugating the drug-linker to a surface-exposed part of the antibody. The aim is to ensure efficient conjugation, which is a key point in research and development as well as in industrial production. The idea in this work was to take an opposite approach. Admittedly, targeting the drug-linker to a buried part of the antibody has the drawback of making conjugation more challenging or reducing linker cleavage and, therefore, drug release in the tumor cell. However, within each drawback, an advantage can be found. Hiding the drug-linker within the antibody (in a Trojan-horse manner) might help to reduce undesirable effects associated with their exposure, such as the drug-induced hydrophobicity of the ADCs, non-specific cleavage of the linkers, and non-specific interaction of drugs or linkers with endogenous elements. In this work, the position HC-152 in combination with the non-canonical amino acid AECK was chosen (referred to as mAbDesignB in manuscript I). The purpose was to conjugate the drug-linker within the Fab cavity. The Fab cavity was preferred over the Fc cavity to circumvent the issue associated with the cleavage of the lower hinge region of IgG1 by proteases in the tumor microenvironment. This antibody design proved to be producible at synthesis yields superior to mAbDesignC (HC-K274AECK)[10,51], a design that has demonstrated its suitability for industrial production[51]. After drug-linker conjugation, the hydrophobicity of the ADCs generated from this mAbDesignB was significantly reduced compared to those of the reference designs mAbDesignA (HC-S118AECK) and mAbDesignC, already described as the best in terms of hydrophilicity[10] and stability[43]. Although the conjugation site is difficult to access in this new antibody design, the conjugation efficiency was as high as that of the reference designs. Although the linker is buried within the Fab cavity, it did not hinder drug release in tumor cells. Conjugation of this design with other drug-linkers demonstrated that the shielding effect was maintained, proving the versatility of this design.

Compared to conventional designs where the drug is conjugated in a surface-exposed part of the antibody, the significant gain in hydrophilicity achieved with this design has the potential to offer better stability, lower propensity for aggregation, and, from a purely therapeutic perspective, to reduce hepatic uptake by Kupffer cells or sinusoidal endothelial cells and thus improve patient outcome. Compared to linker-based approaches to reduce hydrophobicity[8,81–86], this design may protect drug-linkers from possible unspecific interactions. Compared to antibody designs based on conjugation within the Fc cavity[39–41,57,59], this design overcomes the

challenge of ADC cleavage in the hinge region and is also applicable in the case of ADC formats based on Fab fused to half-life extenders such as albumin binding domains[87–89]. Unlike other antibody formats (single-chain variable fragment, diabody, single-domain antibody), the Fab has a hydrophobic cavity capable of containing a drug and therefore ensures the stability of the conjugates. Compared to antibody designs based on the use of engineered cysteines within the Fab cavity[90], this design, through its direct and highly efficient conjugation, avoids significant handling, quantification, and characterization steps. Therefore, this design promotes reliable assessment and parallelization of candidates for research applications, and represents an attractive way to avoid the introduction of additional batch-to-batch variations for industrial production. Beyond its production aspect, this design also offers a structural advantage. The long AECK side chain, combined with the orientation of the α-carbon/β-carbon bond at position HC-152, ensures the drug conjugation from the inside of the cavity. All these points make this new design one of the best-known to date for the generation of ADCs. However, there are still interesting investigations that have not been explored in this work. One of these is the conjugation of other hydrophobic drugs than the standard auristatin and cyanine derivatives described in this work, such as pyrrolobenzodiazepines, calicheamicins, and duocarmycins. Another would be the conjugation of drug-linkers containing two drugs covalently attached to a single linker to generate double-loaded ADCs using the same antibody design (DAR4, instead of the DAR2 described in this work).

The first CHO-based cell-free orthogonal synthesis of IgG1. The manuscript II describes, among others, a method for the CHO-based cell-free orthogonal synthesis and conjugation of IgG1 containing a non-canonical amino acid. So far, CHO-based cell-free orthogonal synthesis of membrane proteins and CHO-based cell-free synthesis of antibodies have been described. However, the CHO-based cell-free orthogonal synthesis of antibodies has never been described. In manuscript II, the first CHO-based cell-free orthogonal synthesis of antibody (IgG1), as well as its conjugation, have been described. The established orthogonal method has yielded IgG1 with similar yields and characteristics to the IgG1 wild type synthesized without orthogonal system and to IgG1 synthesized using pre-charged suppressor tRNA (instead of the orthogonal system). In all three cases, it was possible to differentiate, using non-reducing SDS-PAGE/autoradiography, the different antibody structural states in the microsome, namely the heavy chain alone (~50 kDa), the heavy chain associated with the light chain (~75 kDa), the two heavy chains associated (~100 kDa), the two heavy chains associated with a light chain (~125 kDa), and the full-length product (~150 kDa). The light chain alone was not visible due to the gel used. In addition to this, a conjugation method has been established. This method

enabled conjugation without even needing to purify the antibody or without optimal conjugation conditions.

This cell-free method opens up new possibilities that were previously non-existent for the analysis of antibodies containing non-canonical amino acids and their synthesis. In cell-based synthesis, it is not possible to specifically detect or quantify the various structural states of the antibody of interest in the ER, or to assess their conjugability. Moreover, since the orthogonal system also targets nonsense codons of endogenous proteins, non-canonical amino acids are also introduced into endogenous proteins, which are then prone to conjugation as well. In the cell-free system, the digestion of endogenous mRNA during lysate preparation, combined with the introduction of radioactive amino acids, allows for the specific synthesis, conjugation, detection (autoradiography), and quantification (scintillation) of the antibody of interest. With this method, it is now possible to analyze the folding and assembly of these antibodies, especially if the non-canonical amino acid is located in a critical region, or to assess the antibody conjugability and to identify the cause of a suboptimal conjugation.

The first CHO-based cell-free dual fluorescence technology. The manuscript III describes, for the first time, a CHO-based cell-free dual fluorescence technology. So far, dual fluorescence technologies have been used in CHO cells to enable the pre-selection of clones containing an orthogonal system[48,51], and orthogonal synthesis has been described in a CHO-based cell-free system[77]. However, the combination of dual fluorescence technology and CHO-based cell-free system has never been described. In manuscript III, the first CHO-based cell-free dual technology is described. In this technology, monomeric red fluorescent protein 1 (mRFP1) is fused to superfolder green fluorescent protein (sfGFP) by a linker containing a non-canonical amino acid incorporation site. This cell-free technology has proven to be extremely reliable. mRFP1 did not emit fluorescence in the sfGFP range, and vice versa. At all tested concentrations, volumes, and fusion proteins, fluorescence was correlated with the protein amount in a linear manner. This is the first time this observation has been made with a dual fluorescence technology.

This cell-free technology opens up new possibilities in the analysis of orthogonal antibody synthesis at the translational level in the CHO expression system. In CHO cell-based dual fluorescence technology, variations due to a lower/higher transcription or translation of a reporter protein variant compared to another one can lead to incorrect conclusions when the reported reporter protein is transiently co-transfected and in competition (at the transcriptional and translation level) with the orthogonal synthetase. Indeed, if the reporter protein variant ends up being expressed much more than the orthogonal synthetase (limiting), this will lead to a

higher concentration of termination product. Conversely, if the reporter protein variant ends up being expressed much less than the orthogonal synthetase (in excess), this will lead to a higher concentration of suppression product. Therefore, comparing codon contexts between variants can lead to incorrect conclusions. In the CHO-based cell-free dual fluorescence technology, the open nature of the system allows for the uniform distribution of the translation machinery in all reactions. Indeed, a master-mix containing the orthogonal components (synthetase, tRNA, non-canonical amino acids) is prepared and then split into several reactions where templates for reporter proteins (e.g., codon context variants for a position) are added. As a result, the concentration of orthogonal components available for translation is controlled and consistent in each reaction, ensuring reliable comparisons. Compared to other cell-free technologies, this dual fluorescence technology brings a significant benefit. So far, the analysis of suppression efficiency in cell-free systems was carried out by using a single reporter protein (e.g. GFP, nanoluciferase). Thus, the suppression product is indicated by the fluorescence or luminescence. Zimmerman et al.[50] evaluated 1760 mutants of a *Methanocaldococcus Jannaschii* synthetase by *Escherichia coli*-based cell-free protein synthesis to incorporate the non-canonical amino acid AMF, using GFP as a reporter protein. The selection of the best mutants was done by calculating the ratio between the GFP signal in the presence of AMF and the GFP signal without AMF. Hence, a high ratio indicates an excellent mutant. However, it was not controlled whether the GFP signals were influenced or not by overall synthesis yields. Thus, an increase or decrease in the GFP signal due to lower or higher overall synthesis yields (pipetting error, synthesis not occurring or not being optimal, etc.) would affect the final calculated ratio, leading to the incorrect inclusion or exclusion of a mutant. To ensure that this increase or decrease in signal is not actually due to an increase or decrease in overall synthesis, it is necessary to control synthesis yields by radioactivity for each reaction, as done for example by Zemella et al.[77]. However, adding this quantification step makes high-throughput screening not suitable. In the new CHO-based cell-free dual fluorescence technology, the analysis of the suppression efficiency does not require post-synthesis handling anymore. Moreover, the unique feature of the linear fluorescence in this technology enables the direct and absolute quantification of the suppression product without the need to separate it from the termination product. With this technology, it is now possible to analyze the suppression efficiency in a direct, precise and reliable manner, and therefore the orthogonal synthesis of antibodies in CHO expression systems.

5 Joint discussion, conclusion, and outlook

Joint discussion. While the manuscript I focuses on product development, the other two manuscripts (II and III) are oriented toward assisting in product development. The design developed in manuscript I has no issues (mAbDesignB), neither in terms of synthesis yields nor conjugability. However, if that had been the case, the cell-free tools developed in manuscripts II and III could have been used to identify the reasons and potentially find solutions.

If the synthesis yields of design of interest (mAbDesignB in manuscript I) had been reduced compared to the reference designs (mAbDesignA and mAbDesignC), the method developed in manuscript II could have been used to determine at the post-translational level if this was due to a suboptimal folding kinetic. An analysis by SDS-PAGE/autoradiography would make possible to determine if there is a decrease in the proportion of heavy chains associated with a light chain compared to the wild type or the reference designs. If the conjugation efficiency of the design of interest had been reduced compared to the reference designs, this method could also have been used to determine whether the non-canonical amino acid is itself suboptimally oriented within the CH1 domain, or if the CL domain prevents access of the drug-linker to the non-canonical amino acid. After incubation of the fluorophore with the design of interest, only the heavy chain not associated with the light chains would be fluorescent in the latter case (CL domain preventing access to the non-canonical amino acids by the drug-linker). In the other case (non-canonical amino acid side chain suboptimally oriented within the CH1 domain), no fluorescence would occur at all, except maybe on poorly or completely unfolded heavy chains.

The technology described in manuscript III can be used in conjunction with the method described in manuscript II to determine what might be the causes of poor yield of a design. If the design of interest in manuscript I had a low yield, this technology (manuscript III) could have been readapted and used to determine at the translational level if this was due to less efficient suppression of the amber codon at position HC-152. Comparing the sfGFP/mRFP1 ratio of the reference design codon context with the sfGFP/mRFP1 ratios of the reference designs codon context would make it possible to determine if suppression is the cause.

Thus, this technology described in manuscript III complements the method described in manuscript II for the evaluation of the orthogonal synthesis of antibodies. While the method described in manuscript II allows for the evaluation of antibodies containing non-canonical amino acids as well as their synthesis at the post-translational level (folding, assembly, conjugation), the technology described in manuscript III enables the evaluation of their synthesis at the translational level (suppression efficiency).

This work has not only enabled the creation of an antibody design based on non-canonical amino acids, but also the creation of complementary tools for their investigation. These tools are indeed the first method and the first technology for the orthogonal synthesis of antibodies in CHO-based cell-free systems. More broadly, this CHO-based approach is of high relevance, since antibodies are produced in CHO cells for eleven of the thirteen ADCs approved to date (Adcetris, Aidixi, Besponsa, Blenrep, Elahere, Enhertu, Kadcyla, Padcev, Polivy, Tivdak, Zynlonta).

Conclusion. To summarize and to conclude, the aims of the work have been achieved. A groundbreaking ADC design based on a disruptive clickable antibody design has been created and characterized. At best, this design has the potential to revolutionize the performances and developability of current ADCs, and "at worst", to reduce the risk of failures in clinical phases, which are quite critical for pharmaceutical groups. On the other hand, a CHO-based cell-free method and a CHO-based cell-free technology have been created. By opening up new perspectives for a broader and deeper investigation of antibodies containing non-canonical amino acids and their synthesis at the translational and post-translational level in CHO systems, these pioneer tools will contribute to a better understanding of clickable antibodies. More than the achieved aims, this work enabled major contributions and breakthroughs in the field of ADCs and CHO-based cell-free protein synthesis, and will raise awareness among ADC stakeholders about this type of design and, more broadly, approach, as well as the new complementary tools available for their characterization.

Outlook. While this pioneer design or more broadly, approach, may become the new standard in the near future, ADCs will have to reach a new level. The use of these non-canonical amino acids, not only for the conjugation of drug-linkers but for the conjugation of paratope-masking peptide-linkers, is probably the approach of the future to generate better ADCs, such as probody-drug conjugates (PDCs)[91,92]. These ADCs, ideally activatable in the tumor microenvironment by proteases[91,92], small molecules[28], lower pH[15–17], or on-demand by stimuli such as near-infrared light irradiation[29,93], would be an interesting option to consider for enhancing specificity and targeting antigens which were not considered as suitable up to date. In this context, CHO-based cell-free methods and technologies will have to be developed accordingly to support the investigation of these clickable antibodies.

6 Summary in English

Antibody-drug conjugates are cancer therapeutics that combine specificity and toxicity through the antibody and its conjugated drug, respectively. Due to their unique structures and chemical functions, non-canonical amino acids enable tailor-made designs of protein conjugates and their generation in a direct and efficient manner. The CHO-based cell-free protein synthesis, based on translationally active CHO lysates and microsomes, is of high relevance since antibodies are produced in CHO cells at the industrial scale. It represents a promising analytical tool for investigating the CHO-translation, ER-related post-translational modifications, folding, and assembly of a protein of interest, as well as their effects on the protein properties. The aim of this doctoral work was to leverage the unique specificities and advantages of these non-canonical amino acids to address recurrent and unresolved challenges in ADCs, thereby enhancing their developability. The second aim of this doctoral work was to harness the unique characteristics of this CHO-based cell-free protein synthesis to support the development of such ADCs. In this context, a disruptive clickable antibody design based on non-canonical amino acids was created and evaluated. In this unconventional design, the drug-linker was buried within the Fab cavity, similar to a Trojan horse-type approach. This allowed for the decrease of the adverse effects associated with the exposition of the drug-linker, such as hydrophobicity. This design, which outperformed conventional and best-in-class designs, has the potential to significantly increase the developability of ADCs. Additionally, the first method for the CHO-based cell-free orthogonal synthesis as well as the conjugation of IgG1 was established. This method opens, for the first time, the possibility of investigating the folding and assembly of IgG1 containing non-canonical amino acids synthesized in CHO systems as well as their conjugability at a post-translational level. Finally, the first CHO-based cell-free dual fluorescence technology was created and characterized. This technology enabled, for the first time, the direct, precise, and reliable analysis of amber suppression, which opens possibilities for investigating the orthogonal synthesis of antibodies containing non-canonical amino acids at a translational level. Aside from the achieved aims, this doctoral work brings major contributions and breakthroughs in the field of ADCs and CHO-based cell-free protein synthesis.

7 Summary in German (Zusammenfassung)

Antikörper-Wirkstoff-Konjugate (Antibody-drug conjugates, ADCs) sind Krebstherapeutika, die Spezifität und Toxizität jeweils durch den Antikörper und den daran konjugierten Wirkstoff kombinieren. Aufgrund ihrer einzigartigen Strukturen und chemischen Funktionen ermöglichen nicht-kanonische Aminosäuren maßgeschneiderte Designs von Protein-Konjugaten und deren Generierung auf direkte und effiziente Weise. Die CHO-basierte zellfreie Proteinsynthese, basierend auf translational aktiven CHO-Lysaten und Mikrosomen, ist von hoher Relevanz, da Antikörper in CHO-Zellen im industriellen Maßstab produziert werden. Sie stellt ein vielversprechendes analytisches Tool dar, um die CHO-Translation, ER-assozierte post-translationale Modifikationen, Faltung und Assemblierung des Proteins von Interesse sowie deren Auswirkungen auf die Proteineigenschaften zu untersuchen. Das Ziel dieser Doktorarbeit war es, die einzigartigen Spezifika und Vorteile dieser nicht-kanonischen Aminosäuren zu nutzen, um wiederkehrende und ungelöste Herausforderungen bei ADCs anzugehen und damit ihre Entwicklung zu verbessern. Das zweite Ziel war es, die einzigartigen Merkmale der CHO-basierten zellfreien Proteinsynthese zu nutzen, um die Entwicklung solcher ADCs zu unterstützen. In diesem Kontext wurde ein disruptives, klickbares Antikörperdesign auf Basis nicht-kanonischer Aminosäuren kreiert und evaluiert. In diesem unkonventionellen Design wurde der Wirkstoff-Linker innerhalb der Fab-Kavität verborgen, ähnlich wie bei einem Trojanischen Pferd-Ansatz. Dies ermöglichte die Verringerung der nachteiligen Effekte, die mit der Exposition des Wirkstoff-Linkers, wie Hydrophobizität, verbunden sind. Dieses Design, das konventionelle und Best-in-Class-Designs übertraf, hat das Potenzial, die Entwickelbarkeit von ADCs signifikant zu erhöhen. Zusätzlich dazu wurde die erste Methode zur CHO-basierten zellfreien orthogonalen Synthese sowie Konjugation von IgG1 etabliert. Diese Methode eröffnet für das erste Mal die Möglichkeit, die Faltung und Assemblierung von IgG1 mit nicht-kanonischen Aminosäuren, die in CHO-Systemen synthetisiert wurden, sowie deren Konjugierbarkeit auf post-translationaler Ebene zu untersuchen. Schließlich wurde die erste CHO-basierte zellfreie Dual-Fluoreszenztechnologie entwickelt und charakterisiert. Diese Technologie ermöglichte erstmalig, die direkte, präzise und zuverlässige Analyse der Amber-Suppression in CHO-Systemen, und eröffnet Möglichkeiten zur Untersuchung der orthogonalen Synthese von Antikörpern mit nicht-kanonischen Aminosäuren auf translationaler Ebene. Neben den erreichten Zielen bringt diese Doktorarbeit bedeutende Beiträge und Durchbrüche auf dem Gebiet der ADCs und der CHO-basierten zellfreien Proteinsynthese.

8 References

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9 Abbreviations

AcF	acetyl-phenylalanine
ADC	antibody-drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
AECK	azido-ethoxy-carbonyl-lysine
AMF	azido-methylphenylalanine
AzF	azido-phenylalanine
C-terminus	carboxy-terminus
C1	complement component 1
C1q	complement component 1q
CDC	complement-dependent cytotoxicity
CH1	heavy chain constant domain 1
CH2	heavy chain constant domain 2
CH3	heavy chain constant domain 3
CHO	chinese hamster ovary
CL	light chain constant domain
CpHK	cyclopentadienyl-ethoxy-carbonyl-lysine
CypK	cyclopropene-lysine
DAR	drug-to-antibody ratio
Db	diabody
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
Fab	fragment antigen-binding
Fc	fragment crystallizable
FcγR	Fcγ receptors
FcRn	neonatal Fc receptor
HC	heavy chain
HEK	human embryonic kidney
IgG	immunoglobulin gamma
LC	light chain
mAb	monoclonal antibody
mRFP1	monomeric red fluorescent protein 1
mRNA	messenger ribonucleic acid

N-terminus	amino-terminus
ncaa	non-canonical amino acids
NK	natural killer
PDC	pro-antibody-drug conjugate
PEI	polyethylenimine
qPCR	real-time polymerase chain reaction
scFv	single-chain variable fragment
sdAb	single-domain antibody
sfGFP	superfolder green fluorescent protein
tRNA	transfer ribonucleic acid
VH	heavy chain variable domain
VL	light chain variable domain

10 Other publication(s)

Other peer-reviewed publication(s):

• Krebs, S. K.; Stech, M.; Jorde, F.; **Rakotoarinoro, N.**; Ramm, F.; Marinoff, S.; Bahrke, S.; Danielczyk, A.; Wüstenhagen, D. A.; Kubick, S. Synthesis of an Anti-CD7 Recombinant Immunotoxin Based on PE24 in CHO and E. Coli Cell-Free Systems. International Journal of Molecular Sciences 2022, 23 (22), 13697. https://doi.org/10.3390/ijms232213697.

Poster(s) at conferences, symposia, workshops, summer schools:

• Krebs, S. K.; **Rakotoarinoro, N.**; Stech, M.; Zemella, A.; Wüstenhagen, D. A.; Thoring, L.; Marinoff, S.; Danielczyk, A.; Zhang, H.; Bruckdorfer, T.; Bahrke, S.; Kubick, S. Cell-free protein synthesis as a platform for streamlined antibody-toxin conjugate development.

- *2nd New and emerging technologies "Biotech meets Medicine"*, 18-20 September 2019, Potsdam-Golm (Brandenburg, Germany). Poster presented by Krebs, S. K. and Rakotoarinoro, N.

• **Rakotoarinoro, N.**; Dyck, Y. F. K.; Schloßhauer, J. L.; Krebs, S. K.; Ramm, F.; Wenzel, D.; Assi, M. K.; Zemella, A.; Parr, M. K.; Stech, M. Design and generation of antibody-drug conjugates using non-canonical amino acids.

- *74th Mosbacher Kolloquium - "Immune Engineering - from Molecules to Therapeutic Approaches"*, 23-25 March 2023, Mosbach (Baden-Württemberg, Germany). Poster presented by Stech, M.
- *1st Annual Research Symposium Potsdam - Regensburg*, 28 September 2023, Potsdam-Golm (Brandenburg, Germany). Poster presented by Stech, M.
- *12th International Biotech Innovation Days (IBID)*, 17-19 October 2023, Senftenberg (Brandenburg, Germany). Poster presented by Stech, M.