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REVIEW

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Full-length recombinant antibodies from *Escherichia coli*: production, characterization, effector function (Fc) engineering, and clinical evaluation

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ABSTRACT

Although several antibody fragments and antibody fragment-fusion proteins produced in *Escherichia coli* (*E. coli*) are approved as therapeutics for various human diseases, a full-length monoclonal or a bispecific antibody produced in *E. coli* has not yet been approved. The past decade witnessed substantial progress in expression of full-length antibodies in the *E. coli* cytoplasm and periplasm, as well as in cell-free expression systems. The equivalency of *E. coli*-produced aglycosylated antibodies and their mammalian cell-produced counterparts, with respect to biochemical and biophysical properties, including antigen binding, *in vitro* and *in vivo* serum stability, pharmacokinetics, and *in vivo* serum half-life, has been demonstrated. Extensive engineering of the Fc domain of aglycosylated antibodies enables recruitment of various effector functions, despite the lack of *N*-linked glycans. This review summarizes recent research, preclinical advancements, and clinical development of *E. coli*-produced aglycosylated therapeutic antibodies as monoclonal, bispecific, and antibody-drug conjugates for use in autoimmune, oncology, and immuno-oncology areas.

Abbreviations: ADA Anti-drug antibody; ADCC Antibody-dependent cellular cytotoxicity; ADCP Antibody-dependent cellular phagocytosis; ADC Antibody-drug conjugate; aFc Aglycosylated Fc; AMD Age-related macular degeneration aTTP Acquired thrombotic thrombocytopenic purpura; BCMA B-cell maturation antigen; BLA Biologics license application; BsAb Bispecific antibody; C1q Complement protein C1q; CDC Complement-dependent cytotoxicity; CDCC Complement-dependent cellular cytotoxicity; CDCP Complement-dependent cellular phagocytosis; CEX Cation exchange chromatography; CFPS Cell-free protein expression; CHO Chinese Hamster Ovary; CH1-3 Constant heavy chain 1-3; CL Constant light chain; DLBCL Diffuse large B-cell lymphoma; DAR Drug antibody ratio; DC Dendritic cell; dsFv Disulfide-stabilized Fv; EU European Union; EGFR Epidermal growth factor receptor; E. coli Escherichia coli; EpCAM Epithelial cell adhesion molecule; Fab Fragment antigen binding; FACS Fluorescence activated cell sorting; Fc Fragment crystallizable; FcRn Neonatal Fc receptor; FcγRs Fc gamma receptors; FDA Food and Drug Administration; FL-IgG Full-length immunoglobulin; Fv Fragment variable; FolRα Folate receptor alpha; gFc Glycosylated Fc; GM-CSF Granulocyte macrophage-colony stimulating factor; GPx7 Human peroxidase 7; HCL Hairy cell leukemia; HIV Human immunodeficiency virusl; HER2 Human epidermal growth factor receptor 2; HGF Hepatocyte growth factor; HIC Hydrophobic interaction chromatography; HLA Human leukocyte antigen; IBs Inclusion bodies; IgG1-4 Immunoglobulin 1-4; IP Intraperitoneal; ITC Isothermal titration calorimetry; ITP Immune thrombocytopenia; IV Intravenous; kDa Kilodalton; KiH Knob-into-Hole; mAb Monoclonal antibody; MAC Membrane-attack complex; mCRC Metastatic colorectal cancer; MM Multipl myeloma; MOA Mechanism of action; MS Mass spectrometry; MUC1 Mucin 1; MG Myasthenia gravis; NB Nanobody; NK Natural killer; nsAA Nonstandard amino acid; NSCLC Non-small cell lung cancer; P. aeruginosa Pseudomonas aeruginosa; PD-1 Programmed cell death 1; PD-L1 Programmed cell death-ligand 1; PDI Protein disulfide isomerase; PECS Periplasmic expression cytometric screening; PK Pharmacokinetics; P. pastoris Pichia pastoris; PTM Post-translational modification; Rg Radius of gyration; RA Rheumatoid arthritis; RT-PCR Reverse transcription polymerase chain reaction; SAXS Small angle X-ray scattering; scF Single chain Fv; SCLC Small cell lung cancer; SDS-PAGE Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC Size exclusion chromatography; SEED Strand-exchange engineered domain; sRNA Small regulatory RNA; SRP Signal recognition particle; T1/2 Half-life; Tagg Aggregation temperature; TCR T cell receptor; TDB T cell-dependent bispecific; TF Tissue factor; TIR Translation initiation region; Tm Melting temperature; TNBC Triple-negative breast cancer; TNF Tumor necrosis factor; TPO Thrombopoietin; VEGF Vascular endothelial growth factor; vH Variable heavy chain; vL Variable light chain; vWF von Willebrand factor; WT Wild type

1. Introduction

The market for therapeutic monoclonal antibodies (mAbs) and their derivatives (e.g., fragments, bispecifics, fusion proteins, multi-specifics) has seen substantial growth over the past decade as new drugs have been approved for treating various human diseases, including many cancers, autoimmune, metabolic, and infectious diseases. Globally, at least 600 therapeutic mAbs have been investigated in clinical trials by biopharmaceutical companies, and more than 120 have been approved by the US Food and Drug Administration (FDA) and are currently on the market (www.antibodysociety.org/antibody-therapeutics-product-data/).[1–4] Although most of these therapeutic antibodies are

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produced in mammalian cell lines, such as Chinese hamster ovary (CHO), several antibody fragments have been produced in *Escherichia coli* (*E. coli*).[2,3,5]

mAbs are soluble serum glycoproteins, approximately 150 kilodalton (kDa) in size, and are secreted from terminally differentiated B cells in their natural environments. Each mAb molecule is composed of two identical heavy chains (HCs) and two identical light chains (LCs), with each HC featuring one variable (VH) and three constant (CH1–CH3) domains, and each LC featuring one variable (VL) and one constant (CL) domain (Figure 1a, 1b). The four chains assemble through the formation of intermolecular disulfide bonds (Figure 1b) to produce a tetrameric protein with three functional units – two antigen-binding fragment (Fab) domains and one fragment crystallizable (Fc) domain.[6,7] These structural features require a sophisticated folding apparatus, as well as an oxidizing environment for the formation of disulfide bonds. In addition, glycosylation at a conserved asparagine (Asn, N) residue of the HC is required for immune cell receptors to drive various effector functions (Figure 1a, 1b).[8]

E. coli was the preferred host for the production of first-generation biopharmaceuticals when the sector emerged in the 1980s. There are currently more than 85 approved *E. coli*-produced protein therapeutics in the US/EU,[3,9–11] including more than 25 recombinant hormones, more than 15 recombinant cytokines, 5 recombinant enzymes, and 7 antibody fragments. Because of the lack of complex post-translational modifications (PTMs), such as *N*-linked glycosylation in *E. coli*, to produce complex biologics, such as mAbs, mammalian cell lines, such as CHO, became the hosts of choice despite issues of glycan heterogeneity, long and costly production processes, and the need for viral clearance.

Although initially *E. coli* was not a suitable host for production of full-length antibodies, it was a preferred host for antibody fragments. Currently, seven approved antibody-fragment-based therapeutics are in the market (Table 1).[5,12] Over the years, substantial advances have been made with respect to the optimization of expression plasmids and host engineering that enable the scale of full-length antibody production in *E. coli*.[13–17] This has led to clinical development of several *E. coli*-produced therapeutic mAb and bispecific antibodies (BsAb) in recent years.[18–20]
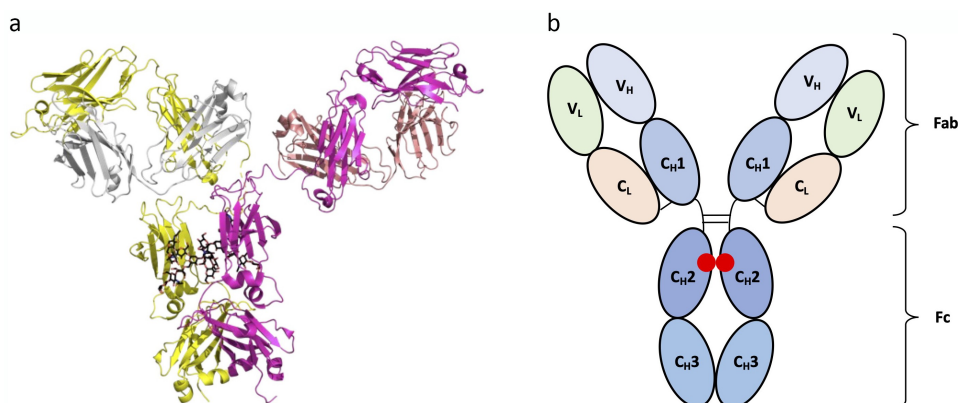

Figure 1. Crystal structure (a) and a simplified diagram (b) of a full-length glycosylated monoclonal IgG1 antibody. a) Crystal structure (PDB:1HZH) showing two Fab domains and one Fc domain with glycans. b) Simplified architecture showing various domains. vH, variable heavy chain; vL, variable light chain; CL, constant light chain; CH1-CH3, constant heavy chain 1–3; Fab, fragment antigen binding; Fc, fragment crystallizable. *N*-linked glycans in the Fc CH2 domain are shown in stick (a) and red dots (b).

Table 1. *Escherichia coli*-produced monoclonal antibody fragments and fragment fusion proteins approved by the US FDA or in Phase 3 studies.

Generic (Brand) names	Molecular format	Compartment	Expression condition	Target	Diseases	Company	Status	References
Certolizumab pegol (Cimzia®)	PEGylated Fab'	Periplasm	Soluble	TNF	Crohn's disease, RA	UCB	Approved in 2008	48
Ranibizumab (Lucentis®)	Fab	Periplasm	Soluble	VEGF	Wet AMD	Genentech/ Roche	Approved in 2006	49
Romiplostim (Nplate®)	Peptide-Fc fusion	Cytoplasm	Inclusion body refolding	TPO receptor	ITP	Amgen	Approved in 2008	50,51
Moxetumab pasudotox (Lumoxiti®)	dsFv-PE38 fusion	Cytoplasm	Inclusion body refolding	CD22	HCL	Astrazeneca	Approved in 2018	52,53
Caplacizumab (Cablivi®)	Tandem NB	Extracellular	Soluble	vWF	aTTP	Ablynx/ Sanofi	Approved in 2019	54–56
Brolucizumab (Beovu®)	scFv	Cytoplasm	Inclusion body refolding	VEGF	Wet AMD	Novartis	Approved in 2019	57
Tebentafusp (Kimmtrak®)	Bispecific TCR-scFv fusion	Cytoplasm	Inclusion body refolding	GP100 x CD3	Metastatic uveal melanoma	Immunocore	Approved in 2022	58,59
Oportuzumab monatox (Vicineum)	scFv-PE38 fusion	Extracellular	Soluble	EpCAM	Bladder cancer	Sesen Bio	Phase 3#	60–62
Bentracimab (PB2452)	Fab	Extracellular	Soluble	Ticagrelor (Brilinta®)	Reversal of Brilinta®	PhaseBio	Phase 3; BLA by late-2022	2,63

#On July 18, 2022, Sesen Bio announced that it made the strategic decision to voluntarily pause further development of Vicineum in the US.

Glycosylation was thought to be necessary for improved biophysical properties and serum stability of antibodies in addition to their Fc-mediated effector functions.[21–29] However, comparison of glycosylated versus aglycosylated antibodies, made either in mammalian cell lines or in *E. coli*, demonstrated nearly identical properties *in vitro* and *in vivo*, including serum half-life ($t_{1/2}$), except for effector functions.[21,30,31] Thus, aglycosylated antibodies could become the default format of therapeutics where various effector functions are either unnecessary or detrimental.

Another line of extensive Fc engineering work on *E. coli*-made antibodies demonstrated that various effector functions could be effectively recruited even in the absence of glycosylation.[18,19] In addition, novel mechanisms of action (MOAs) of well-known effector functions, as well as new effector functions were discovered in these studies.[32–34] Considering these findings, it seems likely that *E. coli* could become preferred over CHO as a host for antibody production due to inherent advantages (e.g., speed, cost of production, no viral safety concerns) associated with the former.

Reviews have recently been published on *E. coli* as a host for production of recombinant proteins in general[10,35–40] and for antibody fragment in particular.[5,12,41–43] This review, however, is focused exclusively on the production of full-length antibodies in *E. coli* and their characterization. The potential of *E. coli* remaining a host of choice for production of antibody-based drugs in the future is also discussed.

2. Production in *Escherichia coli*

Escherichia coli as a host for recombinant protein production, in general, and antibody fragment production, in particular

E. coli was the logical choice as the host for recombinant protein production when the biopharmaceutical sector emerged in the 1980s and when the first biopharmaceutical, Humulin (recombinant human insulin), was approved in 1982.[9–11] It remains a popular host as evidenced by its use in the production of more than 85 approved drugs, and many more protein therapeutics in clinical and preclinical development.[3,4] This popularity stems from its fast growth, low cost of production, easy handling, versatile genetic manipulation, high productivity, and simple fermentation process development for manufacturing.[35,37–40,44,45]

E. coli has unique features compared to most other production hosts. While recombinant proteins are usually secreted into the culture media in other hosts (e.g., mammalian or fungal systems), in *E. coli*, they are expressed in the cytoplasm, targeted to the periplasmic space, or secreted into the culture media[41] (Figure 2). Each cellular compartment has unique properties and, based on the protein to be produced, a strategic decision can be made as to where to direct the recombinant protein. In the reducing cytoplasm, proteins can be produced in soluble form or as inclusion bodies (IBs), which can be resolubilized and refolded into functional forms. Proteins that require an oxidizing environment, for disulfide bond formation, can be secreted into the oxidizing periplasmic compartment in soluble and active forms. In some instances, proteins can also be secreted into the culture media for ease of downstream processing. In addition, cell-free protein synthesis (CFPS) systems using *E. coli* cell extracts or systems using purified components (the PURE system) have seen significant improvements, and are now competing with *E. coli* cell-based production systems.[46,47]

The seven approved antibody fragments or derived products (Table 1) are produced in various cellular compartments in *E. coli* (Figure 2). Certolizumab pegol (Cimzia®) is a PEGylated Fab′ of a humanized anti-tumor necrosis factor (TNF) antibody that was developed by UCB Pharma and was approved by the US FDA in 2008 for treating Crohn's disease and rheumatoid arthritis.[48] Ranibizumab (Lucentis®) is a Fab
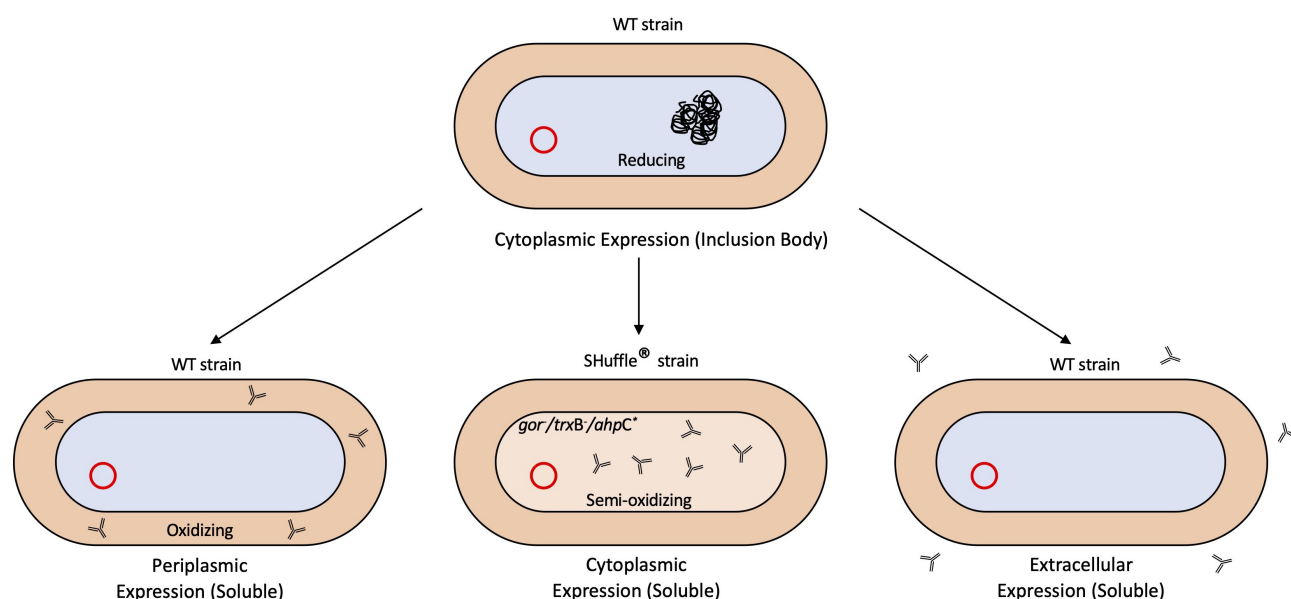

Figure 2. Options for antibody expression, unique to *Escherichia coli*. While oxidizing periplasmic compartment initially was the rational choice for soluble expression of antibodies, antibodies are now routinely produced either in engineered semi-oxidizing cytoplasm or are excreted into the culture medium; in addition, a cell-free expression option is also available, as discussed in the text.

derived from bevacizumab, a mAb against the vascular endothelial growth factor A (VEGF-A) antigen developed by Genentech/Roche and approved by the US FDA in 2006 for the treatment of age-related macular degeneration (wet AMD).[49] Both antibodies were produced as soluble active proteins inside the oxidizing periplasmic compartment of *E. coli*. Romiplastim (Nplate®) is an IgG1 Fc-peptide fusion protein (peptibody) produced in *E. coli* cytoplasm as inclusion body (IB); it was developed by Amgen and approved by the US FDA in 2008 to treat immune thrombocytopenia purpura (ITP).[50,51] It is an agonist of the thrombopoietin receptor and stimulates platelet production. Moxetumab pasudotox (Lumoxiti®), developed by AstraZeneca, is a disulfide-stabilized variable fragment (dsFv) fused with a *Pseudomonas aeruginosa* (*P. aeruginosa*) exotoxin A fragment, PE38. Derived from an anti-CD22 mAb, RFB4, the PE38 fragment is fused with the VH domain of its HC. Moxetumab pasudotox is produced as IBs in *E. coli* cytoplasm.[52,53] The US FDA approved it in 2018 to treat hairy cell leukemia. Caplacizumab (Cablivi®) is a humanized tandem nanobody against blood factor von Willebrand Factor (vWF) that was approved for treating acquired thrombotic thrombocytopenic purpura (aTTP) by the US FDA in 2019.[54–56] Developed by Sanofi/Ablynx, it is produced in *E. coli* as soluble protein and is secreted into the culture media. Brolucizumab (Beovu®), developed by Novartis, is a humanized single-chain variable fragment (scFv) that acts as a VEGF inhibitor. Approved by the US FDA in 2019 to treat neovascular wet AMD,[57] brolucizumab is produced in *E. coli* cytoplasm as IBs. Developed by Immunocore, tebentafusp (Kimmtrak®) is a bispecific gp100 peptide-human leukocyte antigen (HLA)-directed T cell receptor fusion with the scFv of an anti-CD3 antibody to redirect T cells to tumor cells that was approved by the US FDA in 2022 for the treatment of metastatic uveal melanoma.[58,59] It is produced in *E. coli* cytoplasm as IB. Oportuzumab monatox (Vicineum), which is composed of the scFv of an anti-epithelial cell adhesion molecule (EpCAM) antibody fused with *P. aeruginosa* exotoxin A fragment PE38, is currently in a Phase 3 clinical trial for the treatment of bladder cancer.[60–62] Developed by Sesen Bio, it is produced in *E. coli* as a soluble extracellular protein. Bentracimab (PB2452), being developed by PhaseBio as a reversal agent for the anti-blood clotting drug Brilinta® (ticagrelor), is a Fab of an anti-ticagrelor mAb.[63] It is in a Phase 3 clinical trial, and a biologics license application (BLA) is expected to be submitted by mid to late 2022.[2] It is currently produced in *E. coli* as an extracellular protein.

Production of full-length immunoglobulin in E. coli periplasm

The expression of full-length immunoglobulins (FL-IgGs) (Figure 3a) in *E. coli* traces back to two studies published in 1984 in which researchers expressed these molecules in the reducing cytoplasm as IBs followed by solubilization and refolding, but very little antigen-binding activity was observed.[64,65] Subsequently, attention shifted from the reducing cytoplasmic compartment toward the oxidizing periplasmic compartment, and functional expression of a FL-IgG was first reported in 2002.[66] By carefully balancing the expression and secretion of HCs and LCs in the periplasm using a monocistronic operon under the control of a phosphate-inducible alkaline phosphatase (*phoA*) promoter and STII signal sequence, Simmons *et al.* expressed several FL-IgGs, including an anti-tissue factor (TF) IgG1 at levels up to ~150 mg/L after 72 h in a 10 L bioreactor. By overexpressing two periplasmic disulfide bond oxidizing and isomerizing chaperones, DsbA and DsbC, respectively, together with other engineering approaches, Reilly and Yansura achieved IgG1 titers of ~1.3 g/L in a high cell-density fermentor (Table 2).[c]

Other groups have also pursued the production of FL-IgGs in *E. coli*. By lowering inducer concentration and delaying induction, Chan *et al.* modulated the translation rate in a common *E. coli* strain and demonstrated that FL-IgGs can be produced in small-scale shake-flask cultures.[67] Georgiou and colleagues developed a periplasmic full-length IgG display system (PECS) combined with a fluorescence-activated cell sorting (FACS) method and isolated high-affinity binders against several antigens.[68–71] Makino *et al.* used this system and adopted a comprehensive and systematic approach encompassing modification of the expression system, including promoter, signal peptide, translation initiation region (TIR),
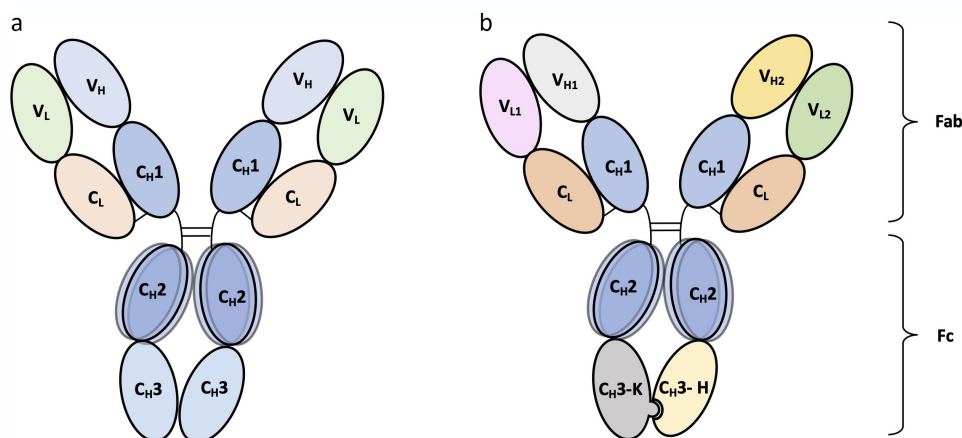

Figure 3. Schematic of *Escherichia coli*-produced aglycosylated monoclonal (mAb; a) and bispecific (BsAb; b) antibodies, shown with Fc domain flexibility. a) Aglycosylated mAb. b) Aglycosylated BsAb with Knob-into-Hole (KiH) mutations in the CH3 domain for heavy chain (HC) heterodimerization.

Table 2. Recent advances in the production of full-length antibodies in *Escherichia coli*.

Format	Antibody	Compartment/ System	Titer (mg/ L)	Strategy	Significance	References
IgG	Anti-TF	Periplasm	150	Two cistrons with a phoA promoter, with balanced HC and LC translational levels	First successful report of IgG production	66
IgG	Anti-TF	Periplasm	1300	Two cistrons with a phoA promoter, with optimized TIRs for both HC and LCs; co-expression of DsbA and DsbC chaperones	Highest reported yield of IgG production in cell-based system	13
IgG	Anti-HER2 (Trastuzumab) variants	Periplasm	40–50	IgG1 Fc variants isolated using novel periplasmic display system and FACS sorting; two cistrons with a ptac promoter and a pelB signal peptide	First report of isolation and production of IgG1 effector function variants	32
IgG	Anti-PA (YMF10)	Periplasm	65	Coexpression of DsbC and SRP-dependent Ffh factor, with an SRP-dependent DsbA signal peptide	Use of engineered and minor SRP secretion pathway to prevent cytoplasmic aggregation	73
IgG	Anti-PA (YMF10)	Periplasm	362	Coexpression of DsbC and the use of sec-dependent pelB signal peptide, with optimized TIRs	High volumetric productivity with major sec secretion pathway	74
IgG	Anti-PA (YMF10)	Periplasm	400	Engineered strain with a 16s rRNA gene rrsE mutant, combined with minor SRP-dependent secretion pathway	Secretion limitation of the SRP pathway was relieved, enabling a higher titer	75
IgG	Anti-EGFR (Cetuximab)	Periplasm	200	First report on sRNA-based screening of chromosomal gene targets utilizing systems-based synthetic biology approach	First successful report of IgG production using systems and synthetic biology approaches	79
IgG	Anti-EGFR (Cetuximab)	Periplasm	150	Second report on sRNA-based screening of chromosomal gene targets utilizing systems-based synthetic biology approach	Second successful report of IgG production using systems and synthetic biology approaches	80
BsAb	Fc€RIxFc RIIb	Periplasm	350	Use of Knob-into-Hole (KiH) mutations for Fc HC heterodimerization in a single cell line with a common light chain	First successful report of the production of full-length BsAb using KiH mutations and a common light chain	83
BsAb	Various BsAbs, including CD3 BsAbs	Periplasm	39–850	Production of full-length BsAb, with natural architecture by coculture instead of separate culture with two HC and two LCs	First report of the production of FL BsAb, with natural antibody architecture	85
IgG-PE38 fusion	Anti-CD30 (T427)	WT Cytoplasm	50	Inclusion body refolding and purification from shake-flask culture	First successful demonstration of the refolding of full-length IgG and IgG-fusion protein into their active forms	90
IgG	Anti-Dig, -MBP, -Gcn4-bZIP, -gpD, -HAG, -PA63	SHuffle® Cytoplasm	1–25	Full-length IgG expression in soluble and active forms in semi-oxidizing cytoplasm, with cytoplasmic DsbC coexpression	First successful report of the production of soluble and active IgG in semi-oxidizing SHuffle cytoplasm in a shake-flask culture	101
IgG	Anti-TNF (Humira®)	SHuffle® Cytoplasm	168–427	Coexpression of human PDI-GPx7 fusion protein to use H_2O_2 as an oxidative agent for increased expression	Use of alternate oxidative agent in SHuffle cytoplasm in shake-flask culture	106
IgG	Anti-CK (MAK33)	CFPS	500	Supplementation with either PDI or DsbC in CFPS reaction mixture	First successful report of the production of soluble IgG in the CFPS system	108
IgG	Anti-HER2 (Trastuzumab), Anti-CD30 (Brentuximab), Germline IgGs (VH3-7xVK3-20 and VH3-23xVK3-20)	CFPS	1000–1500	Supplementation with DsbC and FkpA with optimized TIRs for both HC and LC	Highest titer of full-length IgG in the CFPS system	109,110
BsAb	Various CD3xHER2 & CD3xEpCAM formats	CFPS	200–1000	BsAb production using KiH mutations by manipulating knob and hole plasmid ratio, and further improvement by addition of prefabricated knob or hole	First successful production of BsAb with various formats using KiH mutations in the CFPS system	111
IgG	IgG1 (Trastuzumab, Adalimumab, Cetuximab), IgG2 (Panitumumab) and IgG4 (Nivolumab)	PURE	33–125	Optimization of DsbC, DnaK, and its cofactors, GSH/GSSH ratio, incubation time and temperature in addition to HC and LC DNA ratio	First successful production of full-length IgGs of various isotypes in the PURE system	113

Table note: Only high cell density fermentation and/or purification titers are included. For shake-flask expression and titers, refer to text in section 2, Production in *Escherichia coli*.

and coexpression of chaperones, and isolation of a novel host strain.[72] By integrating these strategies, they achieved a 3–6-fold higher production of several antibodies compared with

their starting strain (up to 4 mg/L titer) in small-scale shake-flask cultures. In two separate studies, Jung *et al.* used the same PECS to isolate several IgG1 Fc mutants.[32,33] They expressed

these IgG1 Fc variants in high cell density fermentation using a bicistronic operon with the *pel*B signal sequence under ptac promoter, and achieved a titer of 40–50 mg/L (Table 2).

Lee *et al.* used a signal recognition particle (SRP)-dependent cotranslational secretion signal sequence (DsbA) instead of a sec-dependent post-translational signal sequence (e.g., STII, *pel*B) to prevent aggregation in the cytoplasm and to improve secretion across the inner membrane.[73] They achieved a titer of ~65 mg/L of a FL-IgG1 by simultaneous coexpression of Ffh (a component of the SRP pathway) and DsbC foldase and isomerase. In a separate study, they used a *sec*-dependent *pel*B signal sequence in conjunction with TIR optimization and DsbC overexpression and achieved a titer of ~362 mg/L IgG in a 5.5 L bioreactor after 22 h.[74] More recently, in 2016, employing a host engineering approach that included PECS, the same team isolated mutants of the 16S rRNA gene, *rrs*E, and demonstrated its positive effect on SRP-dependent protein expression by achieving a titer of 0.4 g/L for IgG in high cell density fermentation (Table 2).[75]

Zhou and colleagues[76] used a comprehensive signal peptide engineering approach to enhance the expression of FL-IgG in shake-flask cultures. They identified inefficient HC secretion across the inner membrane as a rate-limiting step and showed that increasing the hydrophobicity of the hydrophobic core (H-region) of the signal peptide enhanced the secretion of HC and increased the FL-IgG titer by approximately 2.5–3-fold in shake-flask cultures.[76] McKenna *et al.*[77] used a global transcription machinery engineering (gTME) approach with high-throughput screening, using their bacterial antibody display system,[78] to further increase the expression of FL-IgG in a shake-flask culture. In this work, the global housekeeping sigma factor RpoD mutants were isolated and used in conjunction with chaperone coexpression (DsbA, DsbC, and FkpA) to achieve correctly folded IgG titers of ~140 mg/L in shake-flask culture.[77]

In two reports published in 2020,[79,80] Zhang et al. described a system-based synthetic biology approach using small regulatory RNAs to knock down gene expression in a modular fashion utilizing cetuximab as a target FL-IgG for the improvement of expression. In the first report, they took a systematic metabolic engineering approach, using three modules, glycolytic module 1, tricarboxylic acid cycle module 2, and amino acid biosynthesis module 3. They identified the pyruvate dehydrogenase (*ace*F) gene in module 1, citrate synthase (*glt*A) and aconitate hydratase A (*acn*A) genes in module 2, and phosphoserine phosphatase (*ser*B) gene in module 3 as being beneficial. By combining all four mutations into a single strain with optimized fermentation conditions, they achieved a titer of ~5 mg/L in shake-flask and ~200 mg/L in high cell density fermentation.[79] In the second report, a similar three-module approach (pyruvate metabolism in module 1, protease deletion in module 2, and chaperone coexpression in module 3), led to the identification of phosphate acetyltransferase (*pta*), acetyl-CoA synthetase (*acs*), and phosphoenolpyruvate synthetase (*pps*) genes in module 1, serine endoproteases *deg*S and *deg*Q genes in module 2, and disulfide oxidoreductase *dsb*A gene in module 3 as being beneficial for the expression of cetuximab. Combining all the genetic manipulations into a single strain (i.e., repression of *pta, pps*A, *deg*S, and *deg*Q genes, and overexpression of *acs* and *dsb*A genes) with optimized inducer concentration resulted in a titer of 4 mg/L in shake-flask and ~150 mg/L in fed-batch culture.[80] It is currently unknown whether the effects of combining all the beneficial modifications described in these two studies into a single strain would be additive or not.

Production of full-length bispecific antibody in *E. coli* periplasm

BsAbs[81] can simultaneously engage two distinct targets, thereby broadening the utility of antibody-based therapeutics (Figure 3b). Genentech has developed a BsAb expression system in *E. coli* periplasm using their Knobs-into-Holes (KiH) technology[82] for Fc domain HC heterodimerization. The first such report was on a two-part strategy to develop a BsAb that inhibits immunoglobulin E (IgE) receptor signaling.[83] The proof-of-concept stage involved the production of hinge-less half-antibodies with two different LCs in two separate cell lines with either Knob or Hole mutations, followed by *in vitro* Fc HC heterodimerization during purification. Building upon this work, a clinical candidate was generated with a common LC and *in vivo* Fc heterodimerization into a single cell line containing a single plasmid expressing two HCs and one LC. They reported a BsAb titer of ~350 mg/L in a 10 L bioreactor for this common LC-containing BsAb (Table 2). Using the same approach, they also reported production of a monovalent mAb against the MET antigen using Fc KiH mutations for Fc-heterodimerization in a single cell line. Although no titer information was provided, they reported a 4-fold improvement in the production titer at fermentation scale with the overexpression of DsbA and DsbC.[84]

Genentech has further improved its BsAb production process in *E. coli* for BsAb molecules composed of two unique HCs and two unique LCs either using a two-culture or a coculture approach. It has shown improvements in the coculture method over the two-culture method by eliminating the need for *in vitro* annealing of HCs during the purification process. Using a coculture approach, successful production of 27 unique BsAbs was reported. In shake-flask cultures, 21 BsAbs were produced with titers ranging from 0.3 to 12 mg/L and in fermentors, 6 BsAbs were produced with titers of 39–850 mg/L (Table 2).[85] Genentech has further expanded this technology to successfully produce an IgG4 BsAb, targeting both IL-4 and IL-13, for applications in asthma and allergy.[86] It also reported the production of at least two anti-CD3 BsAbs using this technology, a CD3-HER2 BsAb (~5 mg/L titer in shake flask) and a CD3-CD20 BsAb (no titer reported).[87,88] Furthermore, the company reported the production of a BsAb targeting IL-13 and IL-17 by expressing half antibodies in two separate cell lines followed by assembly during purification, although no titer information was provided.[89]

Production of full-length immunoglobulin in wild-type cytoplasm

The *E. coli* periplasm may not be an optimal compartment for high-level production of recombinant proteins, including FL-IgGs, owing to its small volume, inner membrane translocation

bottleneck, outer membrane leakiness, and lack of ATP and ATP-dependent folding chaperones. For these reasons, initial attempts to produce FL-IgG in *E. coli* leveraged cytoplasmic expression. The first two attempts to express FL-IgG and FL-IgM antibodies inside *E. coli* cytoplasm were reported by Genentech and Celltech, respectively, in 1984. In these reports, proteins accumulated in the insoluble fractions (i.e., IBs), and were resolubilized and refolded to achieve antigen-binding activities (Figure 2).[64,65] This accumulation in the insoluble fraction was due to the reducing redox environment of the cytoplasm where stable disulfide bonds could not be formed *in vivo*. Subsequent efforts[90,91] focused on optimizing the refolding of IgG and IgG-fusion proteins from IBs, and yields of up to 50 mg/L were achieved in shake-flask culture, with >90% purity (Table 2). In one study, production of an FL-IgG against CD30 antigen and its fusion proteins with *P. aeruginosa* exotoxin A fragment, PE38, and their refolding and activity were demonstrated.[90] An anti-ErbB2 FL-IgG fused with superfolder green fluorescent protein (GFP) was refolded, and its activity was demonstrated by antigen binding using flow cytometry and confocal microscopy, leveraging the fluorescence of the attached GFP molecule.[91] Taken together, these reports demonstrate the feasibility of a refolding method for rapid expression of IgG and IgG-fusion proteins, including the bispecific formats and immunotoxin fusion proteins, from *E. coli* cytoplasm.

Production of full-length immunoglobulin in semi-oxidized cytoplasm

As mentioned earlier, the major obstacle in the expression of FL-IgG in a soluble and functional form in the *E. coli* cytoplasm was the inability to form stable and correctly paired disulfide bonds because of the reducing environment. To overcome this limitation, researchers have engineered *E. coli* strains with a more oxidizing environment in the cytoplasm to enable the production of disulfide-bonded proteins, including FL-IgGs (Figure 2). There are two reducing pathways – the thioredoxin and glutaredoxin pathways – in the cytoplasm that reduce disulfide bonds, formed transiently either in some essential genes for catalytic functions or as a result of oxidative stress.[92] While removing these two pathways by deleting the respective reductase genes, *gor* and *trxB*, rendered the strain nonviable, a suppressor strain was isolated with a mutation in the peroxidase gene, *ahpC*, that provided enough reducing power (via GrxA) to support growth.[93–96] This strain, commercially available under the product name Origami™, exhibited the ability to produce many disulfide-bonded proteins.[94] This strain was further engineered to express a cytoplasmic version of the bifunctional chaperone and disulfide bond isomerase, DsbC, and was commercialized as the SHuffle® strain.[97–100] Many FL-IgGs have been expressed in soluble and functional forms in the cytoplasm of this strain.[16]

In 2015, Robinson *et al.* reported the first successful cytoplasmic expression of several FL-IgGs, with correct disulfide bonds, in the *E. coli* SHuffle® strain, achieving approximately 1–25 mg/L production titer in shake-flask cultures for several Fab domain swapping mutants as well as Fc domain mutants.[101] Reddy *et al.* used SHuffle® cells for heavy-atom (2 H, 13C, 15 N) isotope labeling of FL-IgGs for nuclear magnetic resonance studies

facilitating quick and in-depth structural characterization of mAbs. In this study, they used a mAb from the National Institute of Standards and Technology (NIST), named NIST RM 8671, (also known as the NISTmAb), for use as a reference standard to characterize mAbs.[102] By optimizing the culture and induction conditions, two mAbs against HER2 and VEGF were successfully produced in the cytoplasm of SHuffle® cells.[103] Recently, *E. coli* codon-optimized mAb genes for NISTmAb and adalimumab were reported to contain internal translation initiation sites that resulted in truncated HCs, and elimination of these sites led to higher antibody expression and homogeneity.[104] Expression of adalimumab was increased 4-fold in both shake-flask and high cell density fermentation by overexpressing a fusion of the human protein disulfide isomerase (PDI) and peroxidase 7 genes (PDI-GPx7 fusion), combined with the oxidizing power of H_2O_2 accumulated in SHuffle® cells[105] due to the inability of the mutated *ahp*C gene to reduce H_2O_2.[106] In shake-flask cultures, the final adalimumab titer was 168 mg/L vs. 427 mg/L for SHuffle® and SHuffle2® hosts, respectively. In fermentors, adalimumab titer was 137 $ng/mL/OD_{600}$ cells vs. 475 $ng/mL/OD_{600}$ cells for SHuffle® and SHuffle2® hosts, respectively.

Production of full-length immunoglobulins and bispecific antibodies in cell-free protein expression system

In addition to the cell-based, *in vivo* protein expression systems described above, FL-IgGs can also be produced in *in vitro* cell-free protein expression system (CFPS).[107] The first report in this regard was published in 2008, and it described the production of an active antibody (titer, approximately 500 mg/L) in an *E. coli* cell-free expression system supplemented with either PDI or DsbC to assist molecule maturation.[108] Sutro Biopharma further improved the CFPS approach by optimizing TIRs of both HC and LC genes and by adding purified DsbC and FkpA chaperones to the reaction mix, achieving >1 g/L IgG titers (Table 2).[109,110] In addition, the company also successfully expressed several BsAbs with KiH mutations for Fc heterodimerization and reported expression titers of 0.2–1 g/L for different BsAb formats.[111] Furthermore, integrating DsbC and FkpA genes into the chromosome of the strain from which it derives CFPS extracts, the company developed a continuous fermentation-based cell-extract preparation method for manufacturing antibody-drug conjugates (ADCs).[112]

Instead of using crude cell extract, GeneFrontier Corporation took a different approach to reconstitute its CFPS system, termed the PURE system, by combining purified components of the protein translation apparatus into the reaction mix.[113] The company identified DsbC, GSH/GSSH ratio, the chaperone DnaK and its cofactors, incubation time, HC and LC DNA ratio, and reaction temperature as critical factors affecting the production of properly formed FL-IgG molecules. It has successfully produced IgG1, IgG2, and IgG4 antibodies with titers of 125, 33, and 73 mg/L, respectively, under optimal conditions for each antibody.

The CFPS strategy has also been used to facilitate the rapid cloning and expression of antibodies from single B cells.[114,115] In this method, HC and LC variable genes are initially cloned by reverse transcriptase PCR (RT-PCR) and expressed as modified Fabs (Zipbodies) with leucine zipper peptides fused at the

C-termini of the Fab regions during the screening phase. The Zipbodies are then produced in *E. coli* SHuffle® strain cytoplasm as IBs (in shake flasks) and refolded to achieve purified antibody yields of 8.5 mg/L.

3. Biochemical, biophysical, and biological characterization of *E. coli*-produced monoclonal and bispecific antibodies

E. coli-produced antibodies are naturally aglycosylated without any amino acid changes

All human IgG antibodies contain an invariant *N*-linked glycosylation site at the Asn297 (N297) residue in the CH2 domain of the Fc region (Figure 1a, 1b). Antibody properties commonly assumed to be associated with the Fc glycans include solubility, stability, and susceptibility to aggregation and proteolysis, in addition to important biological properties, such as various Fc effector functions, and pharmacokinetic/pharmacodynamic (PK/PD) behavior.[23–29,116] To understand the impact of glycosylation on the aforementioned antibody properties, aglycosylated antibodies have been produced in mammalian expression systems in one of the following two ways: 1) by enzymatic deglycosylation of antibodies (which results in conversion of the N297 residue from asparagine to aspartate [N297D]),[21,23,25,26,117] and 2) by modifying the amino acid sequence at the N297 site to, for example, N297A/Q/G/H, to prevent the attachment of glycans.[27,29–31,116,118,119] However, both these approaches change the protein backbone sequence. These amino acid changes could be completely avoided when antibodies are produced in *E. coli* because it lacks the glycosylation machinery. Thus, *E. coli*-produced mAbs and BsAbs could be considered "authentically aglycosylated" antibodies and are the most relevant molecules to address the importance of glycosylation in comparative studies of glycosylated versus aglycosylated molecules.

In addition to the aforementioned physicochemical and biological properties, the structural consequences of the lack of glycans were studied initially on deglycosylated and aglycosylated Fc fragments produced in mammalian cells[120,121] until the structure of the "authentically aglycosylated" Fc fragment from *E. coli* was determined recently.[122–124] These structural differences are discussed in the next section with respect to Fc engineering of "authentically aglycosylated" antibodies to restore various Fc effector functions. In this section, we review the biochemical, biophysical, and biological properties of *E. coli*-produced mAbs and BsAbs.

Biochemical characterization

Antigen-binding activity of an FL-IgG molecule is conferred by the dimeric Fab domain. This binding activity can be measured using a variety of techniques *in vitro* with purified antigens, with cell lines expressing target antigens on the cell surface, or in cell-based potency assays by inhibition of cell signaling and growth in appropriate cell lines.

The equivalency of antigen-binding activities of two *E. coli*-produced and refolded mAbs to their mammalian-produced versions was first demonstrated in 1984 for anti-carcinoembryonic antigen IgG and anti-4-hydroxy-3-nitrophenyl acetyl IgM antibodies by comparing them with their respective hybridoma-derived antibodies.[64,65] Using an improved refolding process, Hakim and Benhar demonstrated equivalent antigen-binding and cell-killing potencies of anti-CD30 and anti-epidermal growth factor receptor (EGFR) antibodies produced in *E. coli* compared with their mammalian cell-produced counterparts.[90,91] In addition, the same group produced fluorescent FL-aglycosylated IgG antibodies with super-folder GFP and demonstrated that these antibodies retained similar antigen-binding affinities compared with their parental glycosylated antibodies. Furthermore, specific binding to antigens on the cell surface could be analyzed using flow cytometry and confocal microscopy without additional labeling with fluorescent secondary antibodies. More recently, an anti-*Vibrio parahaemolyticus* Zipbody mAb produced in *E. coli* SHuffle® strain cytoplasm and refolded was shown to possess equivalent binding affinity comparable to its rabbit-produced glycosylated mAb counterpart.[114,115] Several soluble and functional FL-IgG antibodies against MBP, Dig, gpD, HAG, PA63, and Gcn4-bZip antigens were successfully produced in *E. coli* SHuffle® cytoplasm and were reported to exhibit antigen-binding activities toward their native antigen.[101,102]

The first thoroughly characterized *E. coli*-produced FL-IgG was an anti-TF antibody produced by Genentech in *E. coli* periplasm for which equivalent antigen binding compared to its glycosylated (CHO-produced) counterpart was demonstrated.[66] Another mAb against the anthrax toxin protective antigen (PA) was purified from *E. coli* periplasm and was shown to have equivalent antigen binding compared to a commercially available IgG produced in mammalian cells.[67]

As discussed in Section 2 and shown in Figure 3b, Genentech has produced many BsAbs in *E. coli* periplasm with their KiH mutations in the CH3 domain for Fc heterodimerization and has characterized them thoroughly. The first such BsAb targeted both FcεRI and FcγRIIb to inhibit IgE-induced activation of mast cells for the treatment of asthma. This BsAb shows efficient and dose-dependent binding to both the antigens and induces the release of histamine in transfected cell lines.[83] Onartuzumab, an 'one-armed' monovalent mAb against MET antigen, generated using the KiH technology, demonstrated potent antigen binding and inhibited cell signaling and proliferation.[84] The anti-MET /anti-EGFR BsAb showed more potent activity in different transfected cell lines compared to a combination of two mAbs.[85] The anti-IL4/anti-IL13 BsAbs were generated for both IgG1 and IgG4 isotypes and showed efficient binding to both the receptors in Biacore and cell-based proliferation assays.[86] Genentech also produced two CD3-based T cell-dependent BsAbs against HER2 (CD3-HER2) and CD20 (CD3-CD20) in *E. coli* periplasm, demonstrating efficient binding of these BsAbs to both the antigens and target-dependent T-cell activation and cytotoxicity.[87,88]

The first reported characterization of an *E. coli* CFPS-derived FL mAb, a murine-derived mAb against human creatine kinase, MAK33, was published in 2008. Compared with the authentic MAK33 antibody produced in a mammalian cell line, the CFPS-derived antibody had indistinguishable affinity for the antigen (Kd of 70 nM vs. 71 nM).[108] Sutro Biopharma compared its

CFPS-produced aglycosylated trastuzumab with CHO-derived trastuzumab, and reported comparable affinities (Kd of 4.4 nM vs. 5.0 nM) of both the antibodies toward HER2 antigen expressed on the surface of SKBR-3 cells.[109,110] In addition, this company has produced ADCs against CD74 antigen using its batch and continuous fermentation cell extracts and demonstrated target-dependent cell-killing potencies of these ADCs.[112] Sutro Biopharma has also produced and characterized two T cell-recruiting BsAbs (CD3-EpCAM and CD3-HER2) using its CFPS system employing the KiH technology. It demonstrated dual binding and targeting of both the antigens in antigen-binding assays, T-cell activation, and subsequent tumor cell lysis.[111] More recently, Gene Frontier Corporation used the PURE system to produce five FDA-approved antibodies of IgG1, IgG2, and IgG4 isotypes and analyzed their binding to respective antigens, providing results that were comparable to the reported values for these antibodies produced in mammalian cell lines.[113] For trastuzumab, this company further characterized the internalization and binding kinetics and showed comparable values with CHO-derived trastuzumab.

In summary, many mAbs, BsAbs, and ADCs have been successfully produced, purified, and characterized from either *E. coli* cytoplasm or periplasm or from *in vitro* cell-free systems, such as the CFPS and PURE systems. In most cases, similar antigen-binding affinities have been reported with purified antigens and/or *in vitro* cell-based binding assays, inhibition of cell signaling pathways, and/or *in vitro* cell killing potencies compared to their mammalian cell-produced (and glycosylated) counterparts.

Biophysical characterization

Biophysical characterization of mAbs includes assessment of their homogeneity, solubility, and aggregation propensity. The *in vitro* stability of mAbs can be assessed with respect to their stability toward temperature, storage conditions, pH, salt, freeze–thaw cycles, and proteases, parameters that in turn might affect *in vivo* stability and serum PK properties. Antibodies lacking their natural glycans, which are commonly presumed to have stabilizing effects, might therefore be expected to exhibit poorer stability toward one or more of the above conditions.[21,23,25–27,117,118]

The first characterization of an *E. coli*-produced mAb was reported in 2002 by Genentech. They purified one anti-TF mAb from *E. coli* periplasm and two anti-TF antibodies from CHO cells using protein A capture, cation exchange (CEX), and size exclusion (SEC) chromatography and compared them using mass spectrometry (MS), amino acid analysis, and N-terminal sequencing; all three antibodies produced equivalent results.[66] The anti-CD30 chimeric mAb T427 was purified by refolding from IBs obtained during cytoplasmic expression and compared with its mammalian cell-produced counterpart using SDS-PAGE, immunoblotting, and gel filtration chromatography; both preparations showed identical properties. Both the antibodies were equally stable in serum and showed no loss of activity over 4 days at 37°C.[90] In another case, the anti-ErbB2 antibody FRP5 was fused with superfolder GFP, and was expressed and purified from *E. coli* IBs; its fluorescence was stable up to 12 days

at 37°C, matching the stability of the parental FRP5 antibody produced in mammalian cells.[91]

The first thorough characterization of a CFPS-derived mAb, MAK33, against human creatine kinase, was published in 2008, wherein the antibody was compared with its mammalian cell-produced counterpart. The authors reported comparable quaternary structure of both the antibodies, as judged from the results of analytical gel filtration, non-reducing SDS-PAGE, and fluorescence spectroscopy.[108] They also reported similar secondary structure and thermal stability based on circular dichroism (CD) spectra for both the antibodies, with a melting point (Tm) difference of only 3°C (63°C for mammalian vs. 60°C for *E. coli*). Sutro Biopharma produced trastuzumab in its CFPS system and purified it via protein A chromatography, hydrophobic interaction chromatography (HIC), and SEC.[109] Based on differential scanning calorimetry (DSC) analysis, they reported a 6°C difference between the CH2 domain unfolding melting temperature (Tm) (Tm1, 62°C vs. 68°C; Tm2, 81°C vs. 83°C). Gene Frontier Corp. purified trastuzumab using its PURE system in a two-step chromatography process via protein A resin and gel filtration chromatography and compared its stability with CHO-produced glycosylated trastuzumab with a thermofluor assay using a fluorescent dye that binds to hydrophobic patches exposed with the unfolding of the protein. The aggregation temperature (Tagg) of aglycosylated trastuzumab was lower than that of glycosylated trastuzumab (Tm1, 65°C vs. 71°C; Tm2, 81°C vs. 84°C), presumably due to the lack of glycans.[113]

A comprehensive comparative analysis of 6 mAbs raised against the same antigen target and recognizing the same epitope was reported for glycosylated, deglycosylated (generated by PNGase treatment to remove glycans, thus, the N297D mutation) and aglycosylated (rendered by N297A mutation) versions.[30] These antibodies were produced and purified from HEK293-6E cells and do not have identical protein backbones (i.e., N297 with glycans vs. N297D with deglycosylation vs. N297A with aglycosylation). Nonetheless, the authors did not find any significant difference in the comparisons of glycosylated vs. deglycosylated vs. aglycosylated versions of these antibodies. All forms were soluble at concentrations up to 30 mg/mL, no differences in terms of structural heterogeneity were observed, and all antibodies were stable in stress tests at 37°C for 3 weeks and at 4°C for 4 weeks, as judged by SDS-PAGE, capillary gel electrophoresis, and analytical SEC. Aglycosylated antibodies bound more strongly in protein A and CEX chromatography, suggesting a change in the conformation of the protein A-binding domain and a slight change in the surface charge. Even though aglycosylated antibodies were more thermolabile when subjected to DSC analysis and were more susceptible to pH-induced aggregation, as expected from previous reports,[22,26,117] the authors concluded that aglycosylated antibodies were functionally equivalent to their glycosylated counterparts.[30]

In summary, many aglycosylated mAbs produced in *E. coli*, either using *in vivo* or *in vitro* production processes, have been biophysically compared with their mammalian cell-produced counterparts. Although the biophysical studies have been somewhat limited, *E. coli*-produced aglycosylated antibodies have been shown to exhibit similar stability profiles under the conditions tested, with slightly reduced (3 to 6°C lower)

melting and/or aggregation temperatures than their mammalian cell-produced glycosylated counterparts.

Biological characterization

The therapeutic effects of IgG-based drugs stem from two distinct functions that involve independent mechanisms. First, the IgG can neutralize, inhibit, or activate target antigens by binding to them via the Fab. Aglycosylated mAbs are identical to their glycosylated counterparts in this respect (as discussed above). Second, IgG can elicit Fc domain-mediated immune responses against pathogens or cancer cells. These so-called "Fc effector functions" include complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis (ADCP). All three mechanisms are triggered by the formation of immune complexes, which then recruit complement protein, C1q, and/or various effector cells.[125,126]

The canonical human Fc receptor family consists of six members, namely FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a), and FcγRIIIb (CD16b), all of which are involved in effector functions either through activating or inhibitory mechanisms.[126] An additional IgG-binding receptor, the neonatal Fcγ receptor (FcRn), plays a critical role in IgG homeostasis by mediating antibody recycling via intracellular trafficking, thereby enabling long serum circulation half-lives ($t_{1/2}$) of 3–4 weeks.[127,128]

Effector functions of E. coli-produced IgGs

The first reports comparing the binding of E. coli- and CHO-derived IgG1 molecules to FcγRI, FcγRIIIa, and C1q proteins were published by Genentech,[13,66] wherein they showed that, as expected, E. coli-derived materials failed to bind these receptors due to the lack of glycosylation. Thus, it was concluded that both ADCC and CDC activities would be minimal with the aglycosylated antibodies produced in E. coli. Subsequently, it was demonstrated by others that the binding affinity of E. coli-produced aglycosylated trastuzumab for FcyR1 was reduced by two orders of magnitude, and its binding to FcγRIIa, FcγRIIb, FcγRIIIa, and complement protein C1q, was undetectable, resulting in the complete loss of ADCC, ADCP, and CDC functions.[32–34,129,130]

Pharmacokinetic properties of E. coli-produced IgGs

Several groups have reported that IgGs produced from mammalian cells lacking N-glycans (either due to genetic mutation or enzymatic removal) lost their effector functions while still maintaining prolonged serum circulating half-lives, enabled by the pH-dependent binding to the FcRn receptor.[28,29,116,119] Thus, it was hypothesized that E. coli-produced IgGs might exhibit long serum half-lives without the Fc effector functions.[13,66]

The PK properties of an E. coli-derived anti-TF IgG1 antibody were compared to those of CHO-derived IgG2 and IgG4 antibodies in a single intravenous (IV) bolus dose in chimpanzee, and the half-lives ($T_{1/2}$ in days) were found to be very similar (E. coli-IgG1, 0.94; CHO-IgG4, 0.9; CHO-IgG2, 0.69).[66] The PK properties were also analyzed in rats, where

the clearance of the CHO-derived antibody was ~8.3 ± 0.6 mL/kg/d vs. 12.5 ± 3.1 mL/kg/d for the E. coli-derived antibody.[13] Leabman and colleagues[31] compared the PK properties of an anti-Y antibody produced in E. coli (aglycosylated) to those of the wild-type (WT) (glycosylated) and N297A mutant (aglycosylated) antibodies produced in CHO cells, in cynomolgus monkeys in a single IV dose of 20 mg/kg and reported very similar exposures for all antibodies through day 7 and 14. In addition, they studied the PK properties of WT (glycosylated) and N297G (aglycosylated) versions of an anti-gD antibody derived from CHO cells in cynomolgus monkeys and observed a biphasic disposition, with a rapid distribution phase followed by a slower elimination phase, for both the versions. The clearances (CL) and terminal half-lives ($t_{1/2}$) of the WT and N297G versions of the anti-gD antibodies were 3.68 and 3.93 mL/d/kg and 16.1 and 14.1 d, respectively, following a single IV dose of 10 mg/kg. Lee et al. compared the pH-dependent FcRn binding of their E. coli-produced IgG1 antibody against the anthrax toxin PA63 with its mammalian cell-produced counterpart and reported similar binding of both the antibodies at pH 6.0 and 7.4, with binding at pH 7.4 being lower than that at pH 6.0 for both the molecules, as expected.[74] Additionally, the rat PK properties of HEK293 cell-produced glycosylated versus aglycosylated (due to N297A mutation) IgG1 antibodies were compared and found to be almost identical. The terminal half-lives between 48 and 240 h were 62 h for glycosylated versus 64 h for aglycosylated antibodies, with a plasma clearance rate of 1 mL/kg/h for both the mAbs.[30]

Genentech studied the PK properties of at least six BsAbs produced in E. coli using its KiH technology. The PK properties of an Fc€RI-FcγRIIb BsAb was compared with the parental mAbs (from CHO cells) in mice and found to be very similar. The serum half-lives of the BsAb were 12.1 d as opposed to 13.5 and 21.2 d for the two parental mAbs, with a clearance rate of 5.70 mL/kg/d for BsAb vs. 5.88 and 3.34 mL/kg/d for the mAbs.[83] The PK properties of a monovalent mAb with the Fc domain, onartuzumab, were analyzed in mice. At a 5 mg/kg IV dose, the half-life was 6 d, with a clearance rate of 30 mL/kg/d. The mean half-life of onartuzumab at doses 3, 10, and 30 mg/kg was about 6 d, with a mean clearance rate of 21 mL/kg/d.[84] The PK properties of MET-EGFR BsAb were studied in mice administered single intraperitoneal (IP) doses of 5, 25, and 100 mg/kg, and were found to be linear and dose-proportional, consistent with the findings for other antibodies.[85] The PK properties of IL4-IL13 BsAbs of IgG1 and IgG4 isotypes were evaluated in cynomolgus monkeys at three different doses. Both the antibodies had a relatively slow clearance rate and a long terminal half-life as expected for human IgG4 and IgG1 antibodies in cynomolgus monkeys (mean clearance rate of 5.79–6.70 mL/kg/d for the IgG4 isotype and 3.59–4.09 mL/kg/d for the IgG1 isotype).[86]

The PK properties of T-cell redirecting bispecific antibodies produced in E. coli have also been reported. A CD3-HER2 BsAb's PK properties were compared with those of trastuzumab in rats administered as a single IV dose of 10 mg/kg. The half-lives were 7 ± 1 d for the BsAb vs. 10 ± 1 d for trastuzumab, and the clearance rate was 10 ± 2 mL/kg/d for the BsAb vs. 8 ± 1 mL/

kg/d for trastuzumab, respectively.[87] The PK properties of another T-cell redirecting BsAb, CD3-CD20, were evaluated in both rats and cynomolgus monkeys.[88] In rats, after a single IV dose of 0.5 mg/kg and 5 mg/kg concentration, the half-lives were 5 ± 0.2 d vs. 5 ± 0.4 d, and the clearance rates were 19 ± 0.5 mL/kg/d vs. 13 ± 1 mL/kg/d, for the lower and higher doses respectively, for the CD3-CD20 BsAb, which is typical of a nonbinding human IgG1 mAb in rats. In cynomolgus monkeys, the PK properties were evaluated at both single and four weekly repeat IV doses of 1 mg/kg. The CD3-CD20 BsAb exhibited linear clearance after B cell elimination phase, consistent with expectations for a human IgG1 mAb. The BsAb maintained good exposure throughout the treatment period, with an initial clearance rate of about 17 mL/kg/d, which decreased to 6 mL/kg/d by the fourth dose.[88]

In a separate study, the PK properties of three CD3-EpCAM BsAbs were compared with those of two mAbs at a single 5 mg/kg IV dose in mice. These antibodies were produced using the Sutro Biopharma's CFPS expression system. The reported half-lives were 5.2, 4.9, and 5.4 days for scFv-KiH, BiTE-KiH, and Bite-KiH^r BsAbs compared with 0.2 d for BiTE BsAb alone.[111] Trastuzumab and its scFv-Fc version were also compared and the $t_{1/2}$ was reported to be 25 and 9.1 d, respectively.

In summary, the PK properties of many mAbs and BsAbs produced in *E. coli* and mammalian cells were compared in mice, rat, and non-human primates, including cynomolgus monkey. Similar PK properties of *E. coli*-produced aglycosylated antibodies were observed in the tested animal models compared with their mammalian cell-produced glycosylated counterparts.

4. Fc engineering to recruit various effector functions in *E. coli*-produced aglycosylated antibodies

As discussed before, the Fc domain of mAbs recruits various effector elements, such as natural killer (NK) cells, T cells, macrophages, dendritic cells (DCs), and the complement pathway components, by interaction with various FcγRs on cell surfaces and with the complement protein, C1q, of the complement system. These interactions with FcγRs lead to the activation of immune cells for enhanced ADCC and ADCP activities, resulting in clearance of targeted cancer cells.[8,125,126,131] On the contrary, complement activation results in direct cell killing through the formation of a membrane attack complex (MAC) or through CDC and in the deposition of complement opsonins on pathogenic cell surfaces.[132–134] Subsequently, the recognition of complement opsonins by complement receptors (CRs) on the surface of leukocytes can lead to complement-dependent cellular cytotoxicity (CDCC) and complement-dependent cellular phagocytosis (CDCP) by effector cells. As described earlier, mAbs produced in *E. coli* cells and lacking *N*-glycans do not bind effectively to either FcγRs or C1q protein, resulting in the loss of ADCC, ADCP, and CDC activities. While this lack of effector function is a desirable feature in instances where they are detrimental to the desired therapeutic effect, such as in certain immuno-oncology applications,[135–137] many drugs rely on these functions as part of their MOA. The following section summarizes recent engineering approaches to restore ADCC, ADCP, and

CDC activities of *E. coli*-produced aglycosylated antibodies. Moreover, some novel MOAs of known effector functions as well as the discovery of new effector functions, such as CDCC and CDCP with *E. coli*-produced aglycosylated antibodies are discussed (Table 3).

Compared with their glycosylated counterparts produced in mammalian cells, aglycosylated mAbs expressed in *E. coli* have flexible Fc conformations, resulting in little to no activation of effector immune cells (Figure 3a). Thus, it was hypothesized that greater conformational flexibility may provide engineering opportunities to fine-tune receptor selectivity relative to their glycosylated counterparts.[18] To explore this, Jung *et al.* developed a robust system for the display of combinatorial Fc mutant libraries in the *E. coli* inner membrane, and isolated variants that bind to desired fluorescently labeled FcγRs using flow cytometry. Using this technique, they first isolated a mutant (named Fc5) that selectively binds only to FcγRI, without exhibiting significant binding to other FcγRs (IIa, IIb, and IIIa), whereas glycosylated Fc domains bind to all of them. The aglycosylated trastuzumab-Fc5 with two mutations induced potent DC-mediated ADCC in HER2-overexpressing tumor cells, in sharp contrast to glycosylated trastuzumab produced in HEK293T cells or clinical-grade trastuzumab (where ADCC is induced by NK cells instead of DC cells), without losing pH-dependent FcRn binding or serum resistance.[32] Further, engineering resulted in a new mutant (named Fc701) with five more mutations in addition to the two Fc5 mutations, which exhibited even higher affinity to FcγRI while still showing no binding to other Fcγ receptors and without interfering with the pH-dependent FcRn binding (Table 3).[33]

In another study, an aglycosylated Fc was successfully engineered for selective binding to stimulatory receptor RIIa over inhibitory receptor RIIb despite the 96% sequence identity between these two receptors. The mutant (Fc1004) had a total of five amino acid substitutions that conferred an activation-to-inhibitory ratio of 25 (A/I ratio; RIIa-R131: RIIb). Incorporation of the Fc1004 mutations into aglycosylated trastuzumab resulted in a 75% increase in the ADCP activity of macrophages compared with WT glycosylated trastuzumab for both medium and low HER2-expressing cancer cell lines (Table 3).[138]

Lee *et al.* used a bacterial display system to identify a mutant Fc domain (Fc801), with only two mutations, which selectively binds to C1q protein with concomitant loss of all binding to Fcγ receptors.[34] They used this mutant with rituximab to unravel the role of Fcγ and complement receptors in the killing of tumor cells. They found that tumor cells can still be killed by aglycosylated rituximab variant RA801 by NK or myeloid cells using serum deficient in the complement component, C9, which is critical for the formation of the MAC (i.e., CDC). They further demonstrated that the CDCC- and CDCP-mediated killing of tumor cells occurs through interactions with the complement receptors, CR3 and CR4, on the surface of NK cells and myeloid cells, such as macrophages and granulocytes (Table 3).[34]

FcγRIIIa is predominantly expressed on the surface of NK cells and plays a key role in the ADCC activity. Jo *et al.* engineered an aglycosylated Fc domain and isolated an Fc variant (Fc-MG48) that exhibits higher binding affinities to RIIIa-158 V and RIIIa-158 F (two common human isoforms

Table 3. Engineering of standard and novel effector functions in *Escherichia coli*-produced aglycosylated antibodies.

Fc Variant	Fc mutations	Antibodies tested	Fcγ receptors	Effector functions	Comments	References
Fc5	E382V/M428I	Trastuzumab	67-fold enhanced binding to Fcγ RI	ADCC by dendritic cells (DC)	Novel mechanism	32
Fc701	Q295R/L328W/A330V/ P331A/I332Y/E382V/ M428I	Trastuzumab	120-fold enhanced binding to FcγRI	ADCC by dendritic cells (DC)	Novel mechanism	33
Fc1004	S298G/T299A/E382V/ N390D/M428I	Trastuzumab	160-fold enhanced binding to FcγRIIa; 25-fold enhanced selectivity over Fc RIIb	ADCP by macrophages	Known mechanism	138
Fc801	K320E/Q386R	Rituximab	231-fold increased affinity for C1q	CDCC and CDCP activities through complement receptors by NK and myeloid cells, respectively	Novel mechanism	34
Fc-MG48	V264E/S298G/T299A/K326I/ A327Y/L328G/T350A/ E382V/N390D/M428L	Trastuzumab	16-fold and 25-fold increased affinities for FcγRIIIa-V158 and -F158 alleles, respectively	ADCC by NK cells	Known mechanism	129
Fc-HW86	V264E/S298G/T299A/K326I/ A327Y/L328G/T350A/ E382V/T384A/N390D/ M428L	Trastuzumab	3-fold enhanced binding to FcγRIIIa over glycosylated trastuzumab	2-fold increased ADCC over glycosylated trastuzumab by NK cells	Known mechanism	139
Fc3aV	V264E/V282M/T299A/ L309Q/S329T/A378V/ P428I	Trastuzumab, Rituximab	Exclusive binding to FcγRIIIa-V158	ADCP by GM-CSF macrophages	Novel mechanism	140

of this protein) compared with clinical-grade trastuzumab.[129] The resulting aglycosylated trastuzumab (AglycoT-MG48) displayed potent ADCC activity with peripheral blood mononuclear cells as effector cells in a HER2 receptor density-dependent manner. To further enhance the ADCC activities of aglycosyated mAbs, Yoon *et al.* constructed and screened a library of all mutations previously identified for both the glycosylated and aglycosylated Fc variants using their flow cytometric screening assay.[139] They obtained an aglycosylated Fc variant (Fc-HW86) that exhibited approximately 3–4-fold higher RIIIa binding and 2-fold enhanced ADCC activity compared with glycosylated trastuzumab. All mutations in this variant were derived from previously reported beneficial mutations for engineered aglycosylated Fc variants as opposed to engineered glycosylated Fc variants (Table 3).

In a recent study, Kang *et al.*, engineered an aglycosylated Fc variant, Fc3aV, with exquisite selectivity for FcγRIIIaV158 allele and explored its contribution to the ADCP activity with myeloid-derived effector cells, such as macrophages and monocytes.[140] With Fc3aV formatted rituximab and trastuzumab, they demonstrated ADCP activities toward CD20- and HER2-expressing cancer cell lines, respectively, at a level comparable to those of the respective glycosylated WT antibodies. Earlier reports suggest that the ADCP activity is primarily mediated by hFcγRIIa. Here, the authors showed that potent ADCP activity can also be triggered by selective engagement of FcγRIIIa on the cell surface of granulocyte macrophage-colony stimulating factor (GM-CSF)-differentiated macrophages by engineered aglycosylated mAbs (Table 3).

Indeed, other groups also engineered aglycosylated antibodies that can bind to FcγRs *in vitro* and can confer biological effects *in vivo*. Using yeast surface display and flow cytometric screening, Sazinsky *et al.* isolated an Fc variant (Fc2a) with S298G/T299A mutations that exhibited high binding affinity to RIIa and induced RIIa-mediated platelet clearance in a transgenic mouse model.[141] Chen *et al.* engineered another Fc variant (DTT-IYG) with N297D/S298T/K326I/A327Y/

L328G mutations in their yeast surface display system that bound to RIIIa with higher affinity than WT antibodies and demonstrated comparable ADCP activity by GM-CSF-differentiated macrophages.[130] Furthermore, this aglycosylated antibody demonstrated comparable biophysical and PK properties and reduction of lung metastasis in a transgenic B16F10 mouse model. Additionally, Desjarlais and colleagues obtained two aglycosylated Fc variants with S239D/N297D/I332E and N297D/A330Y/I332E mutations that showed 28% and 43% FcγRIIIa binding activities, respectively, compared with their glycosylated counterparts produced in HEK293T cells.[142]

Recent comparisons of the structures of glycosylated and aglycosylated Fc regions using small-angle X-ray scattering (SAXS) led to the conclusion that both the glycosylated and aglycosylated versions formed a "semi-closed" CH2 domain and the absence of *N*-glycan only affected the orientation of the CH2 domain in solution negligibly.[124] This contrasts with earlier observations[122,123] that the aglycosylated Fc (aFc) forms an "open orientation" in solution due to the lack of *N*-glycans. The earlier conclusion was drawn based on modeling of "solid versus hollow" spheres between the two CH2 domains of "gFc versus aFc".[122,123] It is now assumed that the scattering from the *N*-glycans reduces the radius of gyration (Rg) of glycosylated Fc (gFc) compared with that of aglycosylated Fc (aFc) without any changes in the orientation of CH2 domain.[124] Thus, the Fc mutations conferring various effector functions in aglycosylated antibodies mentioned above might provide a path to retain these important immune functions while sidestepping the common issue of *N*-glycan heterogeneity altogether.[18,19]

5. *E. coli*-produced aglycosylated antibodies in clinical trials

Genentech and Sutro Biopharma have advanced multiple *E. coli*-produced, full-length IgG-based antibody therapeutics, including mAb, BsAb, ADC, and bispecific ADC drug

modalities, into different stages of clinical trials in the areas of oncology, immuno-oncology, and allergy (Table 4).

Genentech

Genentech advanced two *E. coli*-produced (periplasmic) molecules that used its flagship KiH technology[82] for Fc HC heterodimerization into clinical trials. The first such molecule was onartuzumab (OA-5D5, RG3638), a uniquely designed monovalent anti-MET antibody having only one Fab domain. This antibody was designed to block MET signaling in tumor cells by antagonistically binding to the extracellular domain of MET and inhibiting hepatocyte growth factor-mediated activation.[84,143] After obtaining encouraging data on safety, tolerability, and PK/PD in a Phase 1 clinical trial, onartuzumab was tested in several Phase 2 and Phase 3 trials in patients with various advanced solid tumors (glioblastoma, squamous, and non-squamous non-small cell lung cancer (NSCLC), metastatic colorectal cancer [mCRC], triple-negative breast cancer [TNBC], and gastric cancers) to evaluate its efficacy (Table 4).[144–147] In these large placebo-controlled trials, the safety of onartuzumab was found to be adequate, but the Phase 3 trials were terminated due to a "lack of meaningful clinical efficacy." In a retrospective case-study analysis, the failure of onartuzumab was attributed to a false personalized biomarker-guided approach based on Phase 2 trial data.[148] This clinical failure is apparently not related to the design and production process of this founding member of a new class of mAb (monovalent, KiH mutations, aglycosylated because the production host is *E. coli*).

The therapeutic effects of BsAbs are superior to those of mAbs, with broad applications in many disease areas, including allergy and inflammation.[81] The next molecule from the Genentech's *E. coli* platform to enter clinical study was a full-length IgG4 BsAb BITS7201A (RG7990) against IL-13 and IL-17 cytokines for the treatment of asthma. The safety, PK/PD, and immunogenicity of this BsAb were evaluated in a Phase 1 study (Table 4) where it was found to be well tolerated, with an acceptable safety profile, but administration was associated with a high incidence of anti-drug antibody (ADA) formation.[89] However, ADAs generally had a minimal effect on PK, which was linear across all cohorts, consistent with a typical stabilized IgG4 antibody. In an integrated approach that combined *in silico* analysis, *in vitro* assays, and *in vivo* study in non-human primates to characterize this immunogenic response, the immunogenicity was found to be associated with epitopes in the Fab domain of the anti-B arm of this BsAb, and not with the Fc mutations engineered through the KiH process.[149–151]

Sutro Biopharma

ADCs are among the fastest-growing drug modalities for the treatment of various cancers, with 11 ADCs approved so far by the US FDA for the treatment of both liquid and solid tumors (www.antibodysociety.org/antibody-therapeutics-product-data; accessed July 26, 2022).[152] These cancer biopharmaceuticals combine the specificity of mAbs with the antitumor activity of cytotoxic small molecule drugs, and can theoretically widen the therapeutic window of the conjugated cytotoxic

agents. Sutro Biopharma has combined its *E. coli*-based CFPS with nonstandard amino acid incorporation technology to develop next-generation biopharmaceuticals of various modalities.[112,153,154]

As of July 26, 2022, Sutro Biopharma has three monoclonal ADCs and one bispecific ADC in Phase 1 trials (Table 4) (www.sutrobio.com). STRO-001 is a novel ADC composed of an aglycosylated anti-CD74 IgG1 human antibody (SP7219) conjugated to a non-cleavable linker-maytansinoid payload with a drug–antibody ratio (DAR) of 2.[155,156] It is being evaluated in a Phase 1 trial of patients with multiple myeloma (MM) and B cell malignancies, and has already shown signs of improved therapeutic index in hematological cancers compared with other ADCs, with no signs of ocular toxicity.[154] STRO-002 (luveltamab tazevibulin), which contains the anti-FolRα human IgG1 antibody (SP8166), is in development for FolRα-overexpressing platinum-resistant ovarian cancer and other solid tumors. This antibody has been conjugated to a tubulin-targeting 3-aminophenyl hemiasterlin payload with a proprietary protease-cleavable linker, resulting in an ADC with a DAR of 4.[154] It is being investigated in two Phase 1 trials for the treatment of advanced ovarian and endometrial cancers, and has already shown good patient tolerability with no ocular toxicity. Ispectamab debotansine (CC-99712, BMS-986352) is an ADC against B-cell maturation antigen (BCMA) with a maytansinoid payload site-specifically conjugated with a non-cleavable linker, and has a DAR of 4. It is being studied in a Phase 1 trial for MM with partner Bristol Myers Squibb. M1231 is a first-in-class bispecific ADC targeting both mucin 1 (MUC1) and EGFR antigens on cancer cells that is being developed with EMD Serono/Merck KGaA. This BsAb is based on a strand-exchange engineered domain (SEED) technology with a Fab-Fc-scFv bispecific format, and a hemiasterlin-related microtubule inhibitor payload has been conjugated to it with a cleavable linker having a DAR of 4. It is in a Phase 1 trial for NSCLC and esophageal cancers.

Approved and late-stage aglycosylated mAbs and BsAbs produced in other organisms

Two US FDA-approved aglycosylated mAbs and three molecules in late-stage clinical trials are produced in mammalian cell lines with amino acid substitutions at N297 position (N297A/H) to genetically render them aglycosylated. In addition, one aglycosylated CD3-CD20 BsAb (mosunetuzumab) has recently been approved in the European Union (EU), and is currently under review by the US FDA. First approved in 2016, atezolizumab (Tecentriq®) is a humanized aglycosylated IgG1 N297A mAb against programmed cell death-ligand 1 (PD-L1) produced in CHO cells.[157] It has been approved to treat urothelial carcinoma, NSCLC, TNBC, small cell lung cancer (SCLC), and hepatocellular carcinoma. Eptinezumab (Vyepti®), an aglycosylated IgG1 N297A mAb that targets calcitonin gene-related peptides (CGRP) alpha and beta, is produced in *Pichia pastoris*.[158] It was approved in 2020 for the preventive treatment of migraine in adults.

Mosunetuzumab (RG7828, Lunsumio®) is a humanized CD3-CD20 T cell-dependent bispecific (TDB) antibody with N297G mutation constructed using the KiH technology

Table 4. *Escherichia coli*-produced aglycosylated antibodies in clinical trials.

Antibody	Target	Diseases	Molecular format	Antibody modification	Clinical trials	Company	References
Onartuzumab (OA-5D5; RG3638)	HGF	Glioblastoma, NSCLC, mCRC, TNBC, Gastric cancer	One-armed, monovalent mAb	KiH mutations	Phase 3 (NCT02488330, NCT01887886, NCT02031744, NCT01456325, NCT01662869); Phase 2 (NCT01590719, NCT01186991, NCT01418222, NCT01496742, NCT01519804, NCT01632228, NCT01186991, NCT00854308, NCT01418222, NCT02044601); Phase 1 (NCT01897038, NCT02031731, NCT01974258, NCT01068977, NCT02044601, NCT01014936)	Genentech/ Roche	84,143–148
BITS7201A (RG7990)	IL13xIL17	Asthma	BsAb	KiH mutations	Phase 1 (NCT02748642)	Genentech/ Roche	89,149–151
STRO-002	FolRα	Ovarian cancer, Endometrial cancer	ADC	nsAA incorporation	Phase 1 (NCT05200364, NCT03748186)	Sutro Biopharma	154, www. sutrobio. com/
STRO-001	CD74	Lymphomas, MM	ADC	nsAA incorporation	Phase 1 (NCT03424603)	Sutro Biopharma	154–156, www.sutro bio.com/
CC99712 (BMS-986352)	BCMA	MM	ADC	nsAA incorporation	Phase 1 (NCT04036461)	Sutro Biopharma/ BMS	154, www.sutro bio.com/
M1231	MUC1xEGFR	NSCLC, Esophageal cancer	Bispecific ADC	nsAA incorporation; SEED mutations	Phase 1 (NCT04695847)	Sutro Biopharma/ EMD Serono	154, www.sutro bio.com/

and is produced in CHO cells.[88,159] This antibody simultaneously targets CD20 on B cells, and CD3 on T cells and redirects T cells to eliminate malignant B cells while avoiding the destruction of already engaged T cells. The safety and efficacy of mosunetuzumab as a single agent, or in combination with other agents, is being evaluated in 16 clinical trials (2 Phase 3, 9 Phase 2, and 5 Phase 1) for the treatment of various CD20-expressing B cell malignancies (follicular lymphoma, non-Hodgkin's lymphoma, diffuse large B cell lymphoma [DLBCL], chronic lymphocytic leukemia).[159,160] Preliminary results for patients with relapsed or refractory follicular lymphoma showed an overall response rate of 68%, with 31 patients (50%) achieving complete response. Based on these encouraging clinical data, Genentech's marketing authorization application for mosunetuzumab as treatment of (3 L+) for follicular lymphoma in the EU was approved in June 2022 (www.antibodysociety. org/antibody-therapeutics-product-data/ (July 26, 2022)).[2] In July 2022, the US FDA has accepted Genentech's BLA and granted a priority review of mosunetuzumab for the treatment of the same indication.

Clazakizumab (ALD518; BMS-945429) is an aglycosylated humanized mAb designed to block IL-6 that is being developed by Bristol Myers Squibb and Alder Biopharmaceuticals for the treatment of rheumatoid arthritis, psoriatic arthritis, and antibody-mediated kidney transplant rejection.[161–163] It includes the N297A mutation and is produced in *P. pastoris*; clazakizumab is currently in several Phase 3 trials. Nipocalimab (JNJ-80202135; M281) is a fully human, aglycosylated IgG1 N297A mAb designed to selectively bind, saturate, and block the IgG binding site on the endogenous neonatal Fc receptor (FcRn).[164] It is produced in CHO cells and is being developed by Janssen/ Momenta Pharmaceuticals (currently in Phase 3) for the treatment of myasthenia gravis, a rare, heterogeneous, neuromuscular disease, characterized by fluctuating, fatigable muscle weakness. UB-421 is a humanized aglycosylated N297H mAb targeting the CD4 receptor on T cells that is being developed by United Biopharma for use in the treatment of human immunodeficiency virus (HIV) infection. It is produced in CHO cells, and it is currently in a Phase 3 trial.[165]

Thus, numerous aglycosylated mAbs and BsAbs, either produced in *E. coli*-based systems with the native asparagine residue (N297) or in eukaryotic cell lines with the N297 amino acid changes, are already approved drugs or are in late-stage clinical trials. This dispels long-standing assumptions that such antibodies could not be effective therapeutics because of minor biophysical differences due to the lack of glycans.[23–29,116]

Approved glycosylated mAbs with effector function disabled, produced in other organisms

At present, in cases where Fc effector functions are either not needed or are detrimental, Fc mutations are identified through extensive Fc engineering to either reduce or disable various effector functions with glycosylation for IgG1 antibodies, such as the approved anti-PD-L1 checkpoint inhibitor antibody, durvalumab (Imfinzi®), with L234F/L235E/P331S mutations in the Fc region.[136,137,166–174] In some cases, non-IgG1 antibodies, such as the IgG4 isotype, with the hinge-stabilizing S228P mutation, are used to either reduce or avoid effector function-related toxicities.[136,137,166,167] There are several approved IgG4 checkpoint inhibitor antibodies that target the programmed cell death 1 (PD-1) receptor on T cells in oncology and immuno-oncology disease indications, including blockbusters pembrolizumab (Keytruda®) and nivolumab (Opdivo®).[136,137] Producing aglycosylated antibodies in *E. coli*

enables the production of effector-attenuated IgG1 antibodies without requiring Fc mutations to disable the effector functions. It is now well established that the absence of glycans does not affect *in vivo* serum half-life and PK properties of aglycosylated antibodies. Additionally, production and downstream processing of IgG4 antibodies is more difficult than that of typical IgG1 antibodies. Thus, *E. coli*-produced IgG1 antibodies, without any Fc mutations, might be a better choice for therapeutics not requiring the Fc effector functions.

6. Concluding remarks

Full-length IgG antibodies produced in *E. coli*, a prokaryotic system, are aglycosylated, which prompted speculation about their potential as therapeutics due to the lack of Fc-dependent effector functions.[28,29,116] Additionally, concerns were raised about the stability, activity, PK, and immunogenicity of mAbs lacking *N*-glycans.[23–27] However, over the past 20 years, many aglycosylated antibodies have been successfully produced in *E. coli* (Table 2) (or in other systems) and found to be almost identical to their glycosylated counterparts with respect to their binding to target antigens, PK, and biodistribution. Although there are some subtle biophysical differences, such as in the melting temperature (Tm) of gFc and aFc, SAXS analysis revealed minimal perturbation of the orientation of the CH2 domain of aFc in solution.[124] Consistent with this finding, no significant differences were found with respect to "drug-like" properties for *E. coli*-produced antibodies.

Due to the absence of glycans, aglycosylated antibodies lack various Fc-mediated effector functions, such as ADCC, ADCP, and CDC. However, effector functions conferred by glycosylated antibodies have been engineered into *E. coli*-produced mAbs, in some cases resulting in enhanced effector functions (Table 3). Moreover, new and unique effector functions (i.e., CDCC and CDCP) as well as improved Fcγ receptor selectivity have been discovered with *E. coli*-produced aglycosylated antibodies. It will be interesting to see how these new discoveries translate into the clinic. Now, both the WT aglycosylated antibodies and mutant aglycosylated antibodies with these unique sets of mutations without and with effector functions, respectively, could be produced in *E. coli* and can be compared with mammalian cell-produced glycosylated antibodies with any drug modality for treating any disease.

Significant improvements have been made over the past 10 years with respect to the production of mAbs in both the cytoplasmic and periplasmic compartments of *E. coli* as well as in cell-free systems (Figure 2 and Table 2). These improvements initially included optimization of the antibody expression cassette, combined with chaperone coexpression and traditional strain engineering. More recently, antibodies have been produced in semi-oxidized SHuffle cytoplasm as soluble proteins without the need for renaturation and refolding.[16] Recent progress in proteomics and metabolomics combined with systems and synthetic biology approaches could be harnessed in *E. coli* to improve the titer and quality of antibodies and to reduce manufacturing costs compared with that achievable with mammalian cell-based processes. The *E. coli*-produced aglycosylated antibodies should, in fact, be preferable

over their CHO-produced counterparts considering their homogeneity (due to the lack of glycan isoforms), cost effectiveness and simplified downstream processing, and suitability for disease indications where effector functions are either unnecessary or unwanted.

Currently, there are four aglycosylated *E. coli*-produced mono and bispecific ADCs in active clinical trials being conducted by Sutro Biopharma (Table 4), and very encouraging clinical efficacies have already been observed. *E. coli*-produced aglycosylated antibodies are well suited for allergy, immunology, oncology, and immuno-oncology disease areas where effector functions are either not needed or are detrimental. Moreover, *E. coli*-produced aglycosylated antibodies with engineered and finely tuned effector functions could offer better clinical performance than heterogeneously glycosylated antibodies produced in eukaryotic systems. Given the many advantages of *E. coli*-produced aglycosyslated antibodies (fast and economical production and no glycan heterogeneity), without or with the effector functions, more of these molecules are expected to enter clinical studies in the future.

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