NOVEMBER 2021 Volume 2, Issue 6

IMMUNO-ONCOLOGY INSIGHTS

SPOTLIGHT ON:

Bioprocessing/CMC trends, tools and techniques

GUEST EDITOR: **Anurag Khetan,** Executive Director, Biologics Development, Bristol Myers Squibb

IMMUNO-ONCOLOGY INSIGHTS Volume 2, Issue 6

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IMMUNO-ONCOLOGY INSIGHTS

BIOPROCESSING/CMC TRENDS, TOOLS & TECHNIQUES

SPOTLIGHT

FOREWORD

Immuno-oncology CMC aspects

ANURAG KHETAN heads the Global Upstream and Cell Line Development area of Biologics Development in Bristol-Myers Squibb based in New Jersey in USA. His end-to-end team interfaces with Discovery and Manufacturing and carries out process development of biologics and viral gene therapy programs ranging from molecular biology and analytics, cell line development, and upstream process development. Anurag has a BTech in Chemical Engineering from Indian Institute of Technology, Delhi and a PhD from the Department of Chemical engineering at the University of Minnesota. Anurag has had sustained contributions over nearly two decades in the biotechnology industry at Merck, Biogen, Boehringer Ingelheim and BMS in areas ranging from early and late process development, post-approval development, manufacturing support, and GMP manufacturing.

Immuno-Oncology Insights 2021; 2(6), 361–362

DOI: 10.18609/ioi.2021.045

The journal *Science* called immunotherapy the breakthrough of the year in 2013 after the first checkpoint inhibitor-based drug – the anti-CTLA-4 antibody, ipilimumab – was launched. Immunotherapies driven by checkpoint blockade have gone from strength to strength, especially after the launch of anti-PD-1 antibodies, and they have made a marked difference in cancer patients' lives. Immunotherapies are being used alongside conventional therapies like chemotherapy – and in some cases, by themselves – as first-line therapies for cancer. Today, we get to contribute to the innovation and advancements that are currently happening in this exciting area.

The modalities and platforms driving immunotherapy range from cytokines, Fc-fusions, checkpoint inhibitor mAbs, antibody drug conjugates (ADC), bispecifics, gene therapy, cell therapy comprising tumor infiltrating lymphocytes, and the CAR T and NK cell engaging therapies. Additional modalities driven by RNA delivery and gene editing aspects are being explored. Many aspects

remain to be understood to maximize the potency of these therapeutics - particularly in terms of capturing the full complexity of the interaction with the immune system.

CMC challenges range from core platform productivity, lot-to-lot consistency, and scalability, to having a clear understanding of critical quality attributes. Developing assays that will help analyze manufacturing lot-to-lot release consistency – and simultaneously, the critical mechanistic aspects constituting *in vivo* potency – are key for process control. For biologics production, cell lines that can produce enough of the complex proteins with the desired quality attributes are critical. Targeted integration is one technology in cell line development that is enabling speed, predictability, and management of complexity needed to synthesize the molecules. Similarly, gene therapies and cell therapies currently offer a lot of opportunities to innovate to help meet productivity needs. Dose needs for *in vivo* gene therapy are driving lot of innovation to enable commercially viable production platforms. Similarly, realising the promise of allogeneic cell therapy versus autologous therapy is an area of focused efforts.

In this issue, Gregory Zarbis-Papastoitsis of Ankyra Therapeutics gives an overview of challenges in biomanufacturing I-O therapeutics in the 21st Century. Stuart Jamieson *et al.* describe the critical process components making up an ADC, and the challenges that have been overcome over a more than 20-year journey to ensure this modality's molecules are successful in the clinic today. Anurag Rathore and Saxena Nikita shed light on components that are required to make the transition from a batch to a continuous manufacturing system. The ever-increasing volumetric productivities out of continuous systems (6 g/L/day has been reported) are compelling, and this article is timely in bringing out the practical aspects of implementing such processes. Finally, Susan Sharfstein and Scott Tenenbaum outline an interesting technology they are developing based on RNA dependent cell line selection. RNA structures comprising multiple subunits are envisaged to control the translational activity of a messenger RNA.

Happy reading!

AFFILIATION

Anurag Khetan, PhD
Executive Director, Global Upstream and Cell Line Development, Biologics Development, Bristol Myers Squibb, New Jersey, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited.*

Revised manuscript received: *Nov 16 2021;* ***Publication date:*** *Dec 2 2021.*

DOI: 10.18609/ioi.2021.045

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BIOPROCESSING/CMC TRENDS, TOOLS & TECHNIQUES

SPOTLIGHT

EXPERT INSIGHT

Antibody drug conjugates: accelerated approvals targeting oncology

Stuart Jamieson, Daniel Myatt & John Liddell

Monoclonal antibodies (mAbs) have demonstrated great therapeutic potential for the treatment of many conditions with over 100 therapies now approved for use [1]. Critically, a significant proportion of those approved target oncology, an area still in great need for breakthrough treatments. Although mAbs have demonstrated some therapeutic effectiveness in oncology, a mAb treatment may still lack sufficient antibody-dependent cellular cytotoxicity (ADCC) to be effective therapeutically. To overcome this inadequate ADCC activity and trigger a sufficient cell lysis response, the conjugation of cytotoxic small molecules to mAbs has been developed. These molecules, antibody drug conjugate (ADC) therapies, are now amongst the fastest growing drug classes in oncology with 11 approved for use in humans and the active development pipeline promises further products in future. This article describes in detail the important features of ADCs.

Immuno-Oncology Insights 2021; 2(6), 375–384

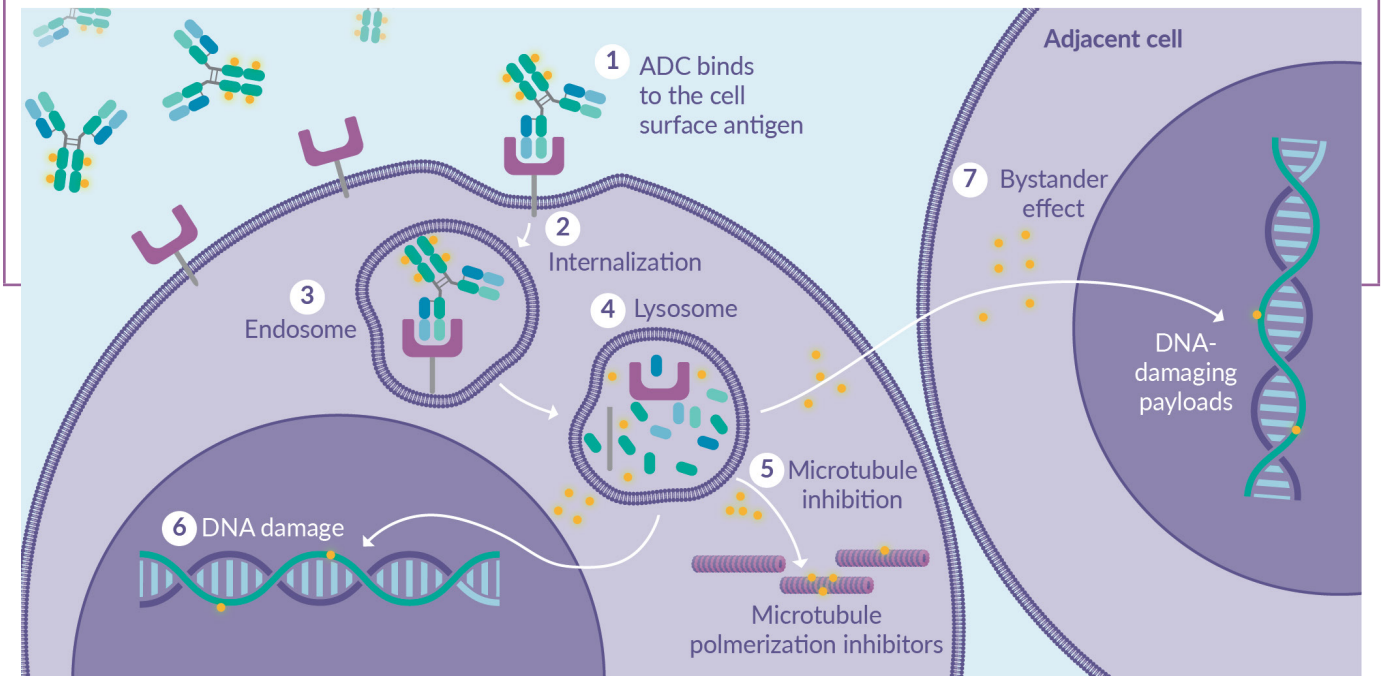
DOI: 10.18609/ioi.2021.042

ANTIBODY DRUG CONJUGATES

The concept of antibody drug conjugates (ADC) is to use an antibody binding to a cell surface marker, which is then internalized within the cell where it releases the cytotoxic payload which kills the cell (Figure 1). This ensures the drug is delivered precisely to the designated cell with minimal collateral damage to the organism.

FIGURE 1

Diagrammatic internalization of an ADC within a target cell.



The first experiments with antibodies linked to toxic payloads occurred over 50 years ago, yet it is only in the last 20 years they have been approved as cancer therapies. There are currently twelve ADCs approved for the treatment of several different cancers with a list of approved ADCs as of April 2021 summarized in Table 1. These molecules are typically made from a full-length monoclonal antibody (mainly IgG1 but also IgG4) with a small molecule cytotoxic payload. The currently approved ADCs also include two molecules based on antibody fragments, specifically scFv – moxetumomab pseudotox, and oportuzumab monatox which are conjugated with a biological toxin, pseudomonas exotoxins.

Principally, an ADC is made up of three main components:

- A mAb targeting a cell surface antigens displayed by the cancer, such as HER2 in the case of breast cancer and CD33 on leukemic blasts
- A cytotoxic payload, typically a small molecule cytotoxic. These payloads should be stable in storage and in the blood stream as well as having non-immunogenic effects
- A linker joining the mAb with the cytotoxin. The linker is attached to the cytotoxin at one end and attached to the mAb through various attachment approaches described later. The linker can be designed to be cleavable or non-cleavable

Figure 2 illustrates this schematically showing the approved ADC Adcetris® (Seattle Genetics) which by now has had over 10 years of clinical administration. Desirable characteristics of an ADC include a good internalization rate, low immunogenicity, high binding specificity and affinity, a potent payload, and a stable linker. Amongst emerging antibody-based therapies, ADCs with these qualities have demonstrated superior effects over standard chemotherapeutics for cancer [2]. Each of these three components presents its own challenges in the development of ADCs, which are described below.

DOI: 10.18609/ioi.2021.042

TABLE 1

Summary of the currently approved ADCs (as of April 2021).

ADC name (trade name)	Target	Developer	mAb type	Conjugation method	Cytotoxic	Linker
Gemtuzumab ozogamicin (Mylotarg)	CD33	Pfizer	IgG4	Lysine conjugation	Calicheamicin (ozogamicin)	Acid labile hydrozone bifunctional linker
Brentumab vedotin (Adcetris)	CD30	Seattle Genetics	IgG	Interchain disulphide bonds	MMAE	Cathepsin cleavable valine citruline
Trastuzumab emtansine (Kadcyla)	HER2	Roche	IgG1	Lysine conjugation (activation using SMCC)	DM1	Non cleavable thioether linker
Inotuzamab ozogamicin (Besponsa)	CD22	Pfizer	IgG4	Lysine conjugation	Calcheamicin (CM1/ ozogamicin)	Acid labile hydrozone
Moxetumomab pasudotox (Lumoxiti)	CD22	AZ	VHVL	Fusion protein	Pseudomonas exotoxin (PE38)	Not applicable
Polatuzumab vedotin (Polivy)	CD79b	Roche	IgG1	Engineered cysteines (Thiomab technology)	MMAE	Val-Cit
Trastuzumab deruxtecan (Enhertu)	HER2	Daiichi Sankyo	igG	Interchain disulphide bonds	Deruxtecan (topoisomerase I inhibitor)	Cleavable tetrapeptide linker
Enfortumab vedotin-ejfv (Padcev)	Nectin-4	Astellas/ Seattle Genetics	IgG	Interchain disulphide bonds?	MMAE	Maleimidocaproyl-val-cit
Sacituzumab govitecan (Trodelvy)	Trop-2	Immunomedics/ Gilead Sciences	IgG	Interchain disulphide bonds	topoisomerase I inhibitor - irinotecan (SN38)	CL2A pH sensitive linker
Belantamab mafodotin	Multiple myeloma	GSK	IgG1	Interchain disulphide bonds	Monomethyl auristatin F (MMEF)	Protease resistant maleimidocaproyl (MC) linker
Oportuzumab monatox	EpCAM	Sesen Bio	scFv	Fusion protein	Pseudomonas exotoxin A	Not applicable
Loncastuximab tesirine	CD19	ADC Therapeutics	IgG1	Interchain disulphide bonds	pyrrolobenzodiazepine (PBD)-dimer toxin	Val-ala maleimide linker
Tisotumab vedotin-tftv	TF/ CD142	Genmab/ Seagen	IgG	Interchain disulphide bonds	MMAE	mc-vc-PABC (see Adcetris, Polivy, and Padcev)

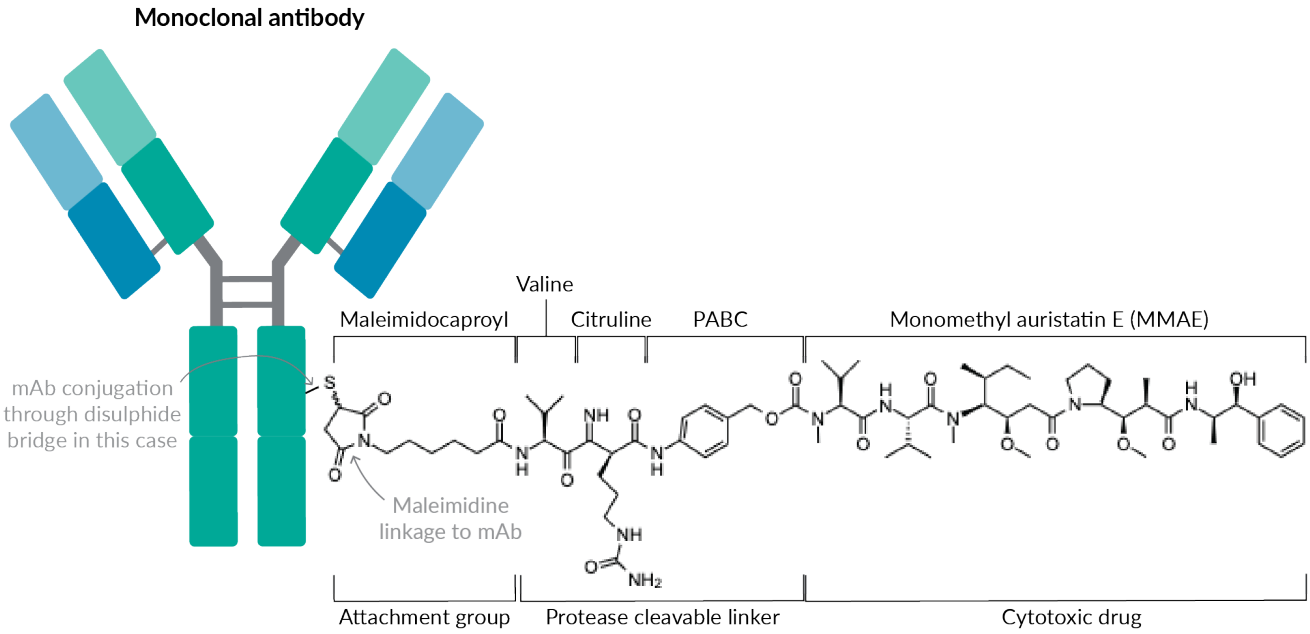
CYTOTOXIC PAYLOADS

The payloads used in ADCs are selected small molecules (with the biological toxin exceptions noted above), with high potency and sufficient hydrophilicity to minimize issues with solubility. Certain conjugation chemistries involve utilize surface lysines, which reduces the overall net surface charge on the mAb impacting the solubility of the conjugate compared to the unconjugated antibody. The cytotoxic payloads are typically hydrophobic with low water solubility, so the overall hydrophobicity of the conjugate increases. This has implications for molecular behaviors in purification or formulation with an increased hydrophobicity increasing the aggregation propensity. This is also influenced by an important parameter, the drug antibody ratio (DAR) which is the average number of payload molecules attached to a single mAb.

In general, with a constant cytotoxin potency, ADCs with high DARs should be

FIGURE 2

Illustration of ADC components.



more potent *in vivo*. However, some high DAR ADCs can be rapidly cleared from the bloodstream by the liver, resulting in similar activity to ADCs with lower DARs in preclinical models [3] indicating the complexity of selection of DAR for an ADC product. Additionally, some payloads provide a 'bystander effect' where cell permeable payloads from within cells harbouring the target antigen diffuse into surrounding cells, on which the drug can exert a cytotoxic effect, irrespective of the target antigen expression [4]. Overall, the ideal level of DAR is often considered between 3 and 4 [5].

The payloads commonly used in ADCs can be divided into three main categories: microtubule inhibitors, DNA-damaging agents and tropisomerase inhibitors [6]. Examples of the structures of ADC cytotoxic payloads are described in Table 2.

Microtubule inhibitors

Microtubules are important in several cellular processes in forming the cell cytoskeleton and thus maintaining the structure of the cell. They are involved in cell division (mitosis and meiosis) and are the major constituents of mitotic spindles, which are used to pull eukaryotic chromosomes apart. Inhibiting microtubule formation therefore ultimately resulting in cell death. Two currently used microtubule inhibitors are maytansinoids and auristatins.

Maytansinoids are natural products initially derived from maytansine, a natural macrolide discovered in the plant Maytenus ovatus. Two maytansinoids derivatives used in ADCs are emtansine and ravtansine referred to as DM1 and DM4 respectively. DM1 is used in trastuzumab emtansine (Kadcyla®).

Auristatins are natural products extracted from the sea hare *Dolabella auricularia*. Two auristatin derivatives commonly used in ADCs are monomethyl auristatin E (MMAE,) and monomethyl auristatin F (MMAF,). Unlike MMAF, MMAE is toxic to neighboring cells through the bystander effect due to its neutral charge which allows diffusion across cell membranes. Auristatins are important ADC payloads with MMAE present in two licensed drugs, brentuximab vedotin (Adcetris®) and polatuzumab vedotin (Polivy®), and with over 10 ADCs incorporating auristatins such as MMAE or MMAF as payload in clinical trials.

DOI: 10.18609/ioi.2021.042

TABLE 2

ADC cytotoxic structures.

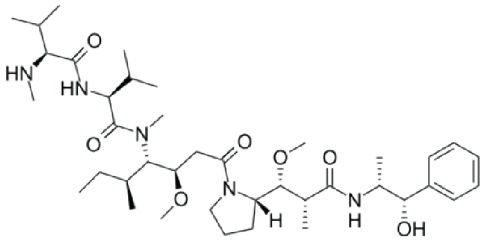
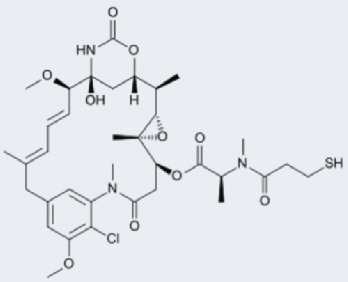
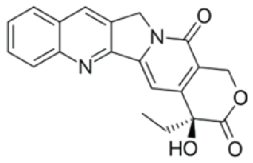
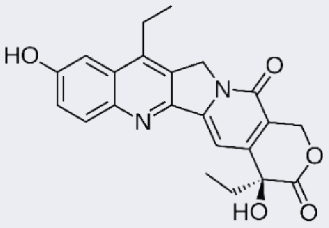
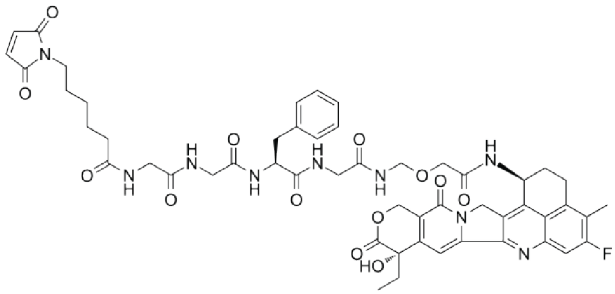
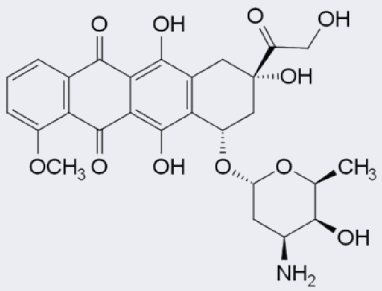
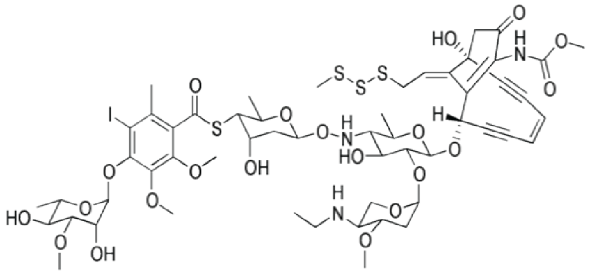
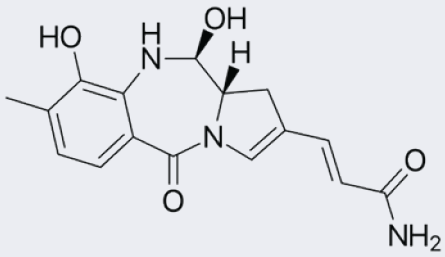
Tubulin inhibition	
Auristatin	
Maytansinoid	
DNA intercalation/Topo1 DNA complex binding	
Camptothecin	
SN38/ 7-Ethyl-10-hydroxycamptothecin	
Deruxtecan (attached to maleimide linker)	
Doxorubicin	

TABLE 2

ADC cytotoxic structures.

DNA minor groove binding	
Calicheamicin	
Pyrrolobenzodiazepines	

DNA damaging agents

DNA-damaging cytotoxic agents include calicheamicin, pyrrolobenzodiazepines (PBD), indolinobenzodiazepines, duocarmycins and doxorubicin. These payloads operate by binding irreversibly to DNA which thus inhibits translation and transcription and ultimately the damage caused to the DNA results in cell death. Examples of ADC containing calicheamicin are inotuzumab ozogamicin (Besponsa®) and gemtuzumab ozogamicin (Mylotarg®).

Topoisomerase inhibitors

Topoisomerase plays important roles in cellular reproduction and DNA organization, mediating the cleavage of single and double stranded DNA to relax supercoils, untangle catenanes, and condense chromosomes in eukaryotic cells. Topoisomerase inhibitors influence these essential cellular processes with some preventing topoisomerases from performing DNA strand breaks while others (topoisomerase poisons) associate with topoisomerase-DNA complexes and prevent the re-ligation step of the topoisomerase mechanism. These topoisomerase-DNA-inhibitor complexes are cytotoxic agents with the un-repaired single and double stranded DNA breaks resulting leading to apoptosis and cell death. Because of this ability to induce apoptosis, topoisomerase inhibitors have gained interest as therapeutics against infectious and cancerous cells. Examples of topoisomerase containing ADCs are Enhertu® and Trodelvy®.

LINKER TECHNOLOGY

A key part of the drug design is the linker which is the structure making the covalent connection between the antibody and the small molecule payload [7]. The linker should be selected such that it does not induce aggregation, it ensures acceptable pharmacokinetic properties of the construct whilst limiting premature release of the payload in plasma (stability), and finally by its ability to efficiently release of the active molecule at the targeted site of action. These linkers are divided into two categories: non-cleavable and cleavable.

Non cleavable linkers

Non-cleavable linker-based ADCs have high bloodstream stability but must be

DOI: 10.18609/ioi.2021.042

internalized, and the release of the active molecule requires degradation of the antibody by lysosomal proteases. Many non-cleavable linkers have been explored in ADC development, the most representative being SMCC (N-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate), present in trastuzumab emtansine (Kadcyla®). Drugs attached in this manner usually cannot exert bystander effects since the catabolites released have poor permeability [8]. Most ADC activities are now focused on cleavable linkers to achieve the efficient localized release of well-defined, characterized payloads.

Cleavable linkers

Cleavable linkers are appropriate for the design of internalizing ADCs as the release of the active molecule is triggered by the nature of the cleavage site (e.g. lysosome and/or tumor environment). These linkers can be separated into two major categories of chemically labile or enzymatically labile. Hydrazone linkers chemically liable, being stable at neutral pH (e.g. bloodstream) and hydrolyzed in acidic media (e.g. pH <6 for endosomes, pH <5 for lysosomes). The release is triggered by a two-step activation process in which the acid-sensitive hydrazone is hydrolyzed followed by glutathione reduction of the disulphide-bond. Although two ADCs with this linker have been approved (the first approved ADC Mylotarg®, and Besponsa®), hydrazine-based linkers are less stable in plasma, and thus less attractive, than other cleavable linkers [9].

Enzymatically cleavable linkers are desirable to limit release of the payload before internalization, with the action of several classes of enzymes such as cysteine proteases, phosphatases, glycosidases, β-galactosidase and sulfatases of interest for ADC degradation. As an example, cathepsin B is a cysteine protease present in late endosome and lysosome compartments in mammals and is also overexpressed in many cancer cells. Cathepsin B cleavable linker peptides have therefore been developed which require a hydrophilic residue at position P1 of the cleavage site (citrulline, lysine orarginine), whereas a lipophilic residue at position P2 enhances plasma stability (phenylalanine, valine or alanine). The Val-Cit dipeptide is used in the approved ADCs Adcetris® and Polivy® and commonly used with others in development due to its good plasma stability, release behavior, and chemical tractability.

CONJUGATION APPROACHES

The conjugation site has significant impact on ADC stability and its pharmacokinetics yet all the currently approved ADCs display variety in the number of active payloads carried and attachment sites. As mentioned previously, high DAR loading often leads to rapid bloodstream clearance and ADCs with low DAR demonstrate weak activity [10]. The naked mAb also presents a potent competitive inhibitor if the payload is cleaved early. Therefore, new conjugation strategies have been developed aiming to control the position and the number of payloads present [11].

All ADCs currently approved by the FDA exploit endogenous amino acids for conjugation with amino acids such as lysine, histidine, tyrosine and cysteines engaged in interchain disulphide bridges, providing attractive attachment sites. One common method uses the antibody's lysine residues and a simple reaction in which the nucleophilic NH2-group of the amino acid reacts with an electrophilic *N*-hydroxysuccinimide (NHS) function on the linker-payload. This method is used in the production of approved ADCs such as Kadcyla®. However, the high abundance of accessible lysine residues leads to the formation of a heterogeneous mixture of numerous ADC species so tight control of the mAb and payload ratio is required during conjugation to control the DAR.

An alternative strategy is to use disulphide rebridging since IgG1 antibodies contain

four interchain disulphide bonds, two connecting the light and heavy chains, and two located in the hinge region bridging the two heavy chains. These cysteines can instead be used as payload-linker attachment points to the mAb with the reduction of the four disulphide bridges generating eight thiol groups that can each react with maleimide functionalized linkers [12]. This conjugation method is better controlled than lysine conjugation and provides a reliable way of attaching payloads at defined positions on the mAb. However, higher drug loading can increase the risk of aggregation leading to high bloodstream clearance rates and decreased *in vivo* efficacy [13].

Site-specific conjugation of genetically engineered mAbs can also be performed using specific amino acid sequences inserted into the antibody sequence. These sequences are recognized by an associated enzyme capable of performing site specific conjugation, such as formylglycine generating enzyme (FGE), microbial transglutaminase (MTG) or sortase [4]. Alternatively, in the case of THIOMABTM technology, reactive cysteines are introduced into the mAb allowing desired site selective and homogeneous modifications on the antibody [14]. This approach leads to homogeneous ADCs with defined DAR and payload attachment positions.

The incorporation of non-natural amino acids (NNAA) provides another approach for site specific conjugation. Promising results have been achieved with this technique which allows the incorporation of amino acids having a unique chemical structure, which in-turn enables the attachment of linker-payload conjugates in a selective manner [15–18]. This approach therefore also leads to homogeneous ADCs with defined DAR and payload attachment positions.

Although homogenous ADCs have repeatedly demonstrated superior overall pharmacological profiles in compared to their heterogeneous counterparts, engineered antibodies for site- specific conjugation have not yet been used in any FDA approved ADCs.

CMC CONSIDERATIONS

Accelerated time to market is required to deliver urgently needed therapeutics to patients, as well as to reduce commercial risks and costs during development. However, due to their composition, ADCs are more complex to manufacture than a single modality biologic as production requires validated manufacturing processes for the mAb, linker, payload, ADC drug substance (DS) and the finished drug product (DP). Therefore, expedited readiness requires careful planning of process and analytical development, characterization, scale-up and validation to reduce the probability of late-phase changes that place the Chemistry, Manufacturing and Control (CMC) activities on the critical path. Several challenges are commonly associated with ADC production, namely:

- Complex supply chain requiring multiple consistent and robust manufacturing processes and the associated production facilities to produce the mAb, linker, ADC and final DP;
- Comprehensive safe handling practices to protect personnel and thereby allow the development, analysis, and production of the highly potent payload;
- Product heterogeneity concerns for the individual components and conjugated ADC particularly regarding the DAR. Suitable analytical methods are therefore required that can resolve different product variant populations, alongside manufacturing processes with the capability to reliably control them;
- Free-drug related impurities (FDRIs) arising during the manufacture or storage, such as free drug, free drug-linker or any other forms of free cytotoxic drug that are not conjugated to the mAb. Robust processes and analytics are therefore required to understand, control and detect the FDRIs to enable the production of the ADC that meets specifications;

DOI: 10.18609/ioi.2021.042

- Pressure on the ADC developer to commence commercial production as soon as possible due to targeting unmet oncology needs.

CONCLUSIONS

Although the first ADC was approved more than 20 years ago, the pharmaceutical industry needed to go through a long learning process involving improved mechanistic understanding to achieve a steady approval of ADCs and a strong pipeline with therapeutic potential in clinical development. Currently, it's estimated that there are approximately 250 ADCs in development with over 80 of these in clinical development and trials [19]. Recent market analysis suggests the global sales of currently marketed ADCs will exceed US$16.4 billion in 2026 with sales of one product (Enhertu®) expected to achieve global sales of $6.2 billion [20].

Chemistry has enriched of the pool of available payloads by establishing a collection of linking methods to connect drug payloads. Systematic study of the relationship between the chemical nature of the link between the antibody and the drug, and the properties of the ADC, has led to improved understanding and design of new ADCs. Developments in selective modifications of monoclonal antibodies have also resulted in an improved control of the ADC composition, which in turn ensures a better control of ADC properties. Given the modular nature of ADCs (antibody/linker/payload) there may also be scope to apply more bioinformatics involving machine learning/artificial intelligence to increase the efficiency and accuracy of ADC component design from the competing units of the three-part ADC system [21].

The recent and steady approval of new candidates has driven confidence in ADCs at a time of increased interest resulting from a growing focus on immuno-oncology treatments. Together with scientific advances and understanding in the field, the versatility of antibodies, the research into new antigens and cytotoxic payloads, and improved production methods have made ADCs a frontier of research in the search for the next generation of therapeutic treatments for oncology.

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AFFILIATIONS

Stuart Jamieson
CPI Biologics
1 Union Square
Darlington DL11GL

Daniel Myatt
CPI Biologics
1 Union Square
Darlington DL11GL

John Liddell
CPI Biologics
1 Union Square
Darlington DL11GL

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 24 2021;* ***Revised manuscript received:*** *Nov 9 2021;* ***Publication date:*** *Dec 2 2021.*

DOI: 10.18609/ioi.2021.042

IMMUNO-ONCOLOGY INSIGHTS

BIOPROCESSING/CMC TRENDS, TOOLS & TECHNIQUES

SPOTLIGHT

EXPERT INSIGHT

Control of continuous processes for production of biopharmaceutical products

Anurag Rathore & Saxena Nikita

With the advent of continuous manufacturing (CM), there is growing interest in development of mechanistic or statistical model-based control strategies. CM has been successfully implemented in the pharmaceutical and food industry owing to the numerous advantages it offers. Over the past decade, there have been considerable developments towards creating technology solutions for performing bioprocess unit operations in continuous mode. Continuous mode has shown superiority in terms of specific productivity and consumable utilization. Hardware for performing continuous processing is available today, often from multiple technology providers. Challenges arise, however, when implementing continuous processing. A major hurdle is process control, as controlling a continuous process is considerably more complex than controlling a batch process. In this article, we focus on recent developments on the topic of control of continuous processing for production of biopharmaceuticals. Hurdles that continue to exist have also been highlighted.

Immuno-Oncology Insights 2021; 2(6), 363–373

DOI: 10.18609/ioi.20210.043

With the advent of continuous manufacturing (CM), there is growing interest in development of mechanistic or statistical model-based control strategies [1,2]. CM has been successfully implemented in the pharmaceutical and food industry owing to the numerous advantages it offers (Box 1) [3–6]. Over the past decade, there have been considerable developments towards creating technology solutions for performing

BOX 1

Advantages of continuous manufacturing [3].

- Shorter process development time as multiple parameters can be estimated at once [4]
- Reduced human intervention and thereby lower operational cost
- Improved product quality [5] and faster product release to market [6]
- Smaller footprint as the equipment size reduces
- Reduced initial capital investment as single use technology is integrated in process line
- Easier to scale up for commercial production

bioprocess unit operations in continuous mode [7]. Continuous mode has shown superiority in terms of specific productivity (up to 10x improvement has been demonstrated) [8] and consumable utilization. As would be evident from the information presented in Table 1 [9], hardware for performing continuous processing is available today, often from multiple technology providers.

Challenges arise, however, when implementing continuous processing [10]. A major hurdle is process control, as controlling a continuous process is considerably more complex than controlling a batch process. In this article, we focus on recent developments on the topic of control of continuous processing for production of biopharmaceuticals. Other challenges have also been highlighted.

PREREQUISITES FOR DESIGNING A ROBUST CONTROL STRATEGY

Any process development involves defining the product profile, understanding the relationship between critical quality attributes (CQAs) and critical process parameters (CPPs) followed by development of appropriate control strategies based on risk assessments and failure mode and effect analysis to handle process variability. In addition, for developing control strategies, analyzing the interaction among the multiple unit operation integrated together to form a continuous platform is also very crucial [11,12]. It is seen that the optimal for the entire continuous platform is often not same as the optimal for an individual unit operation. Thus, it is necessary that development of the process control strategy starts at an early stage of process understanding (Figure 1) [5,13]. The control strategies developed can be either based on the direct measurement of a CQA or on the CQA predicted using first principle/empirical/hybrid models during the manufacturing process. These schemes can be implemented using feedforward or feed backward strategies (Figure 2) [14], such that the CPP for a particular unit operation are manipulated in real time to control the relevant CQA. The correlation between the CQA and the CPP is typically based on a combination of modelling and experimentation. For instance, design of experiment (DOE) is being increasingly used to correlate the system response to the various time invariant factors. Leveraging data to identify process variability and implementing data analytics offers manufacturing intelligence, thereby providing real time control and operation (Figure 3). These process variabilities are directly related to critical material attributes (CMAs). CMAs are associated with the quality of raw material which in turn can be linked to process CQA using statistical techniques. Real time data acquisition, transmission, and analytics are necessary to predict process performance and deal with process variability. Presently, development of integrated self-optimizing processes, a preliminary step of digital manufacturing, is ongoing [15,16]. Continuous unit operations can be coupled with supervisory control for setting reference values to lower-level controllers, thereby ensuring robust control of the CQA.

DOI: 10.18609/ioi.20210.043

CONTROL STRATEGIES FOR CONTINUOUS UNIT OPERATIONS

In this section, the techniques for creating continuous unit operations are briefly discussed. Designing a robust continuous upstream processing is the first step. Owing to the non-linearities and time variant nature of typical bioprocesses, understanding the dynamically changing cell population from exponential growth to producing dead, lysed cells is challenging and advanced inline and online monitoring techniques like spectroscopy (dielectric/Raman/near infrared/Fourier transform infrared), dye-based methods, fluorescence-activated cell sorting and filtration biosensors might be required. Inline monitoring are applications where sensors are mounted directly in the process flow whereas in online measurement, the sample is redirected from the manufacturing platform and may be returned to the unit operation. There are several functions, for example elimination of toxic by-products, reduced and controlled residence time of target protein, and product quality control, that can be fulfilled by producing continuous cell culture. Starting with basic control strategies to mimicking

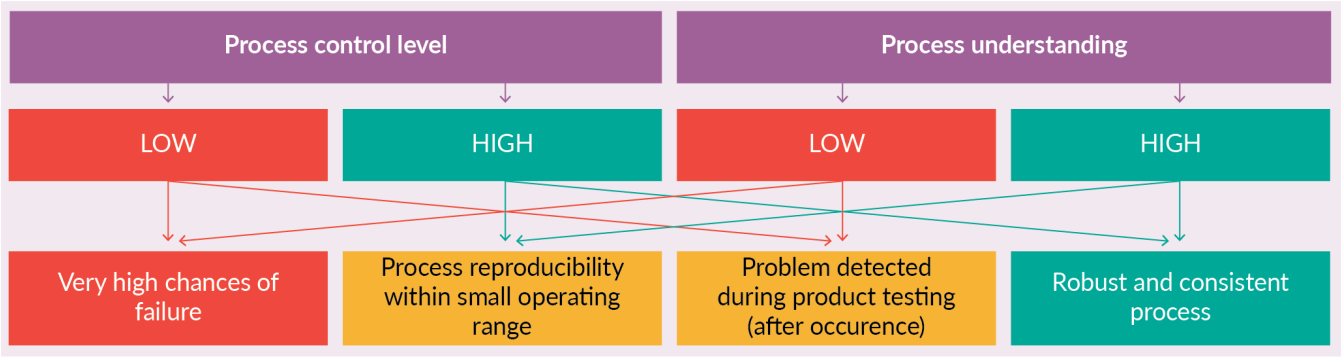
TABLE 1

Current technologies available for designing continuous unit operations with examples of CPPs and CQAs for different unit operations [9].

Unit operation	CPP	CQA	Technology
Upstream processing	Feed flow rate, base flow rate, CO_2 flow rate, air flow rate, O_2 flow rate, antifoaming agent addition, agitation speed	Volumetric mass transfer coefficient (kLa)	Perfusion cell culture systems Alternating tangential flow filtration Single use bioreactors Continuous flow micro bioreactors Acoustic wave separation
Continuous centrifugation	RPM, temperature	Turbidity	Tubular centrifuge Disc stack centrifuge Hybrid centrifuge rotor Continuous tubular bowl centrifuge
Continuous cell lysis	Temperature, shear	Lysis efficiency, aggregation	High pressure homogenizer
Continuous precipitation	Concentration of precipitating agent, incubation period of precipitation process, temperature, pH	HCDNA, HCP, aggregates	Mixed suspension and mixed product removal reactor Tubular reactor
Chromatography	Elution pool volume, control of pump and valves	HCP, HCDNA, yield, charge variants, aggregates	Continuous annular chromatography Periodic counter current chromatography Simulated moving bed chromatography Expanded bed chromatography Counter current tangential chromatography
Continuous refolding	Solubilization and refolding buffer components, temperature, pH	Yield, concentration, oxidized impurity	Refolding in a CSTR coupled to a diafiltration unit CSTR with recycle Tubular reactor Coiled flow inverter On column refolding
Viral inactivation	Flow rate of acid/base, flow rate of CFIR	Aggregates, viral inactivation efficiency	Customized flow reactors and hold tanks
Continuous formulation	Membrane flow rate (feed, retentate), membrane pressure (TMP, ΔP), DF buffer flow rate, DF buffer composition	Concentration of protein, concentration of excipients	Innovative membrane modules Single pass tangential flow filtration
Continuous extraction	pH, conductivity, turbidity of light and heavy phase	DNA, lower molecular weight component	Aqueous 2 phase extraction

FIGURE 1

Relation between process control and process understanding.



cellular dynamic to implementing advanced control strategy, the development in this area has helped in improving product quality and handling upstream variability. Such understanding can help to improve product quality and handle upstream variability. Control strategies that have been successfully demonstrated in continuous upstream operation include:

1. Adjustment of temperature or oxygen uptake rate to control cell density [17, 18]; and

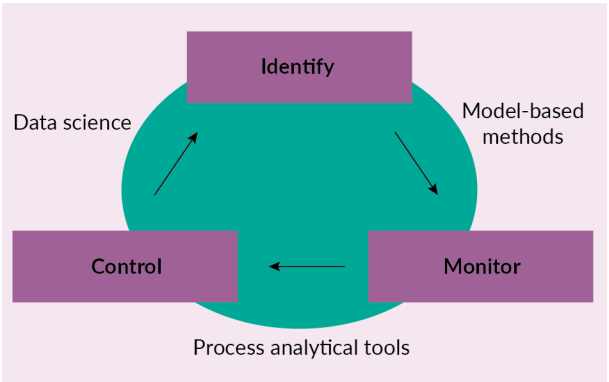
2. Maintaining steady cell bleeding or main substrate/metabolite availability in the medium to adjust perfusion rate [19,20].

Continuous clarification has bridged the gap between continuous upstream and downstream processing. Cell retention devices such as alternating tangential flow filtration (ATF) and tangential flow filtration (TFF) [21,22], continuous centrifugation [23], and acoustophoretic separation [24] have emerged as effective enablers for continuous operation. Researchers have attempted to correlate the cell separation efficiency to the CPPs. An effective control scheme has been proposed for continuous dead-end filtration with multiple filters arranged in parallel with real time monitoring of the CQAs like pressure, turbidity etc. and replacing filter once the threshold is crossed [25].

Multiple researchers have used high-pressure liquid chromatography (HPLC) combined with analytical Protein A columns and multi/single wavelength UV/near infrared spectroscopy in combination with data analysis for enabling real time control of process chromatography [26,27]. Additionally, analytical tools and models have been used to monitor loading on chromatography columns to determine the percentage breakthrough and to asses column health [28,29]. However, due to the high cost associated with protein A capture chromatography along with the low binding capacity and ligand leaching, alternate options are being explored for CM [30]. And for polishing chromatography, a major challenge is in controlling elution pooling in real time. Here, at line HPLC, with its ability to quantify components in load and elution streams, has emerged as a possible enabler [31,32]. Considering these issues, a combination of coiled flow inverter reactor with cation exchange chromatography for capture and multimodal chromatography

FIGURE 3

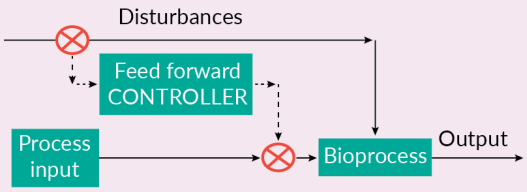
Dealing with process variability using model-based methods, process analytical tools, and data science.



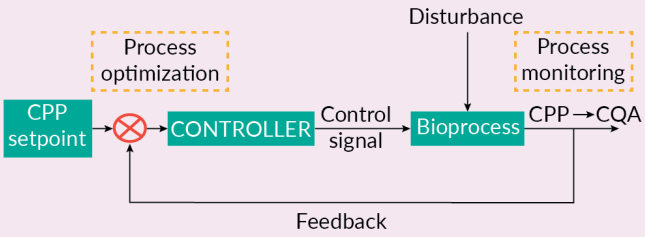
DOI: 10.18609/ioi.20210.043

FIGURE 2

Feedforward and feed backward control schemes.



Feedforward control:
Disturbances are measured and process parameters are manipulated
Controller does not force CQA towards any specific target value
Control structure helps to keep CQA within limit



Feedback control:
Process parameters are manipulated in response to CQA measured/predicted using CPP
CQA is forced towards a target value
Control structure can handle process variability and scale up is easier

with anion exchange membrane for polishing have been successfully demonstrated as an alternative platform for continuous purification of mAb [30]. Further, a control strategy has been proposed for pH adjustment during viral inactivation utilizing sensors for monitoring real time pH along with scheduling of pumps and valves to adjust flowrate [33,34]. In some cases, kinetic models of aggregation have been used to predict the impact of deviation and implement suitable control action [35]. Real time sensors are required for tight control of CQA's in ultrafiltration and diafiltration unit operations that are often utilized for converting the drug substance to drug product [36,37]. For this operation, control strategy should be able to compensate the flux decline and discard/recycle the waste product. Here, the flux decline model can help in predicting the process mechanistic parameters followed by employing real time control decision to handle process variability [38]. One of the recently published studies demonstrates the use of in-line NIR flow cells to determine the process stream mAb concentration. This helps in integrated control and scheduling in continuous mode [39].

Moreover, enabling technologies like the coiled flow inversion reactor, inline concentrator and counter-current chromatography can be implemented for developing integrated continuous PEGylation process [40]. PEGylation is a post-production modifying method to improve the pharmacokinetic properties of protein wherein the chemical link between polyethylene glycol chains to a therapeutic protein/peptide is established. This innovation assists in the development of accessible manufacturability platform for PEGylated therapeutic proteins. Integrated continuous processing requires integrated control methodology. One such control technique involves use of surge tank to enable steady state operation during continuous processing [41]. Placement of surge tanks and their sizing is critical for robust control. Another study demonstrated a customized assembly of coiled flow inversion reactor (for refolding) in combination with 3 column periodic counter current chromatography (for capture) and con current chromatography (for polishing). This assembly offered smaller footprint and higher productivity, equipment and resin utilization [42].

A major challenge in continuous processing is that of bioburden control. During batch processing, bioburden can be controlled relatively easily. However, with considerably longer runtimes, the chances for bioburden build up increases. Potential control strategies to mitigate this issue involve gamma irradiation [43], monitoring the performance of the filter membrane and the chromatography efficiency to determine the filter/column life time [44].

Recently, digital twin-based control has emerged as an enabler for smart manufacturing [45–48]. The digital twin comprises of physical components like equipment, control modules, and information communication, in combination with virtual components like model and system analytics, thereby forming an *in silico* replica of an existing physical system which can then be used for predicting the process behavior under different conditions (Box 2).

Two case studies for real time control of monoclonal antibody (mAb) manufacturing platform are shown in Figure 4. It shows an end-to-end integrated process wherein surge tanks are used for handling the deviations and periodicity of unit operations. Figure 4A shows the control strategy implemented in case of acoustic wave separator failure whereas Figure 4B depicts the control strategy for BioSMB column failure during capture or polishing chromatography. Similar actions can be implemented for other deviations involving reduction in binding capacity of Protein A resin, error in BioSMB sensors (UV/pH/Conductivity) affecting capture chromatography elution pool volume or breakdown in ILC or ILD [41].

PATH FORWARD: EMERGING APPROACHES FOR BIOPROCESS MONITORING & CONTROL

In previous section, different techniques for developing continuous unit operations were discussed. It is evident that comprehensive process knowledge, expertise, and definitive objectives are prerequisites to design a control system. In addition, process models and tools such as analyzers, sensors, pumps, control elements are required for implementing the control strategies. However, considering the complexity of the biopharmaceutical manufacturing and the time variant behavior, advanced computation tools (artificial intelligence, machine learning, statistical process control) for process control have emerged as effective solutions for handling process perturbations and delivering consistent product quality rather than the mechanistic process models. These tools leverage the big datasets produced during continuous processing. Studies show that neural network for 2 level control of fed batch by controlling the combination of the amount of secreted protein on a unit culture volume basis, culture glucose concentration and volume [49], fuzzy control system for controlling pH and temperature [50], for controlling core temperature [51], for controlling carbon dioxide [52], and non-linear model predictive control (MPC) with dynamic models for controlling specific growth rate/poly(hydroxyalkanoates) productions [53] can be implemented for bioreactor operations. Successful implementation of supervised machine learning tools to determine the pooling criteria in polishing chromatography is seen in literature [54].

Statistical process control (SPC) tool is another powerful tool for optimizing process performance and controlling product quality. It works on the process historical data to

BOX 2

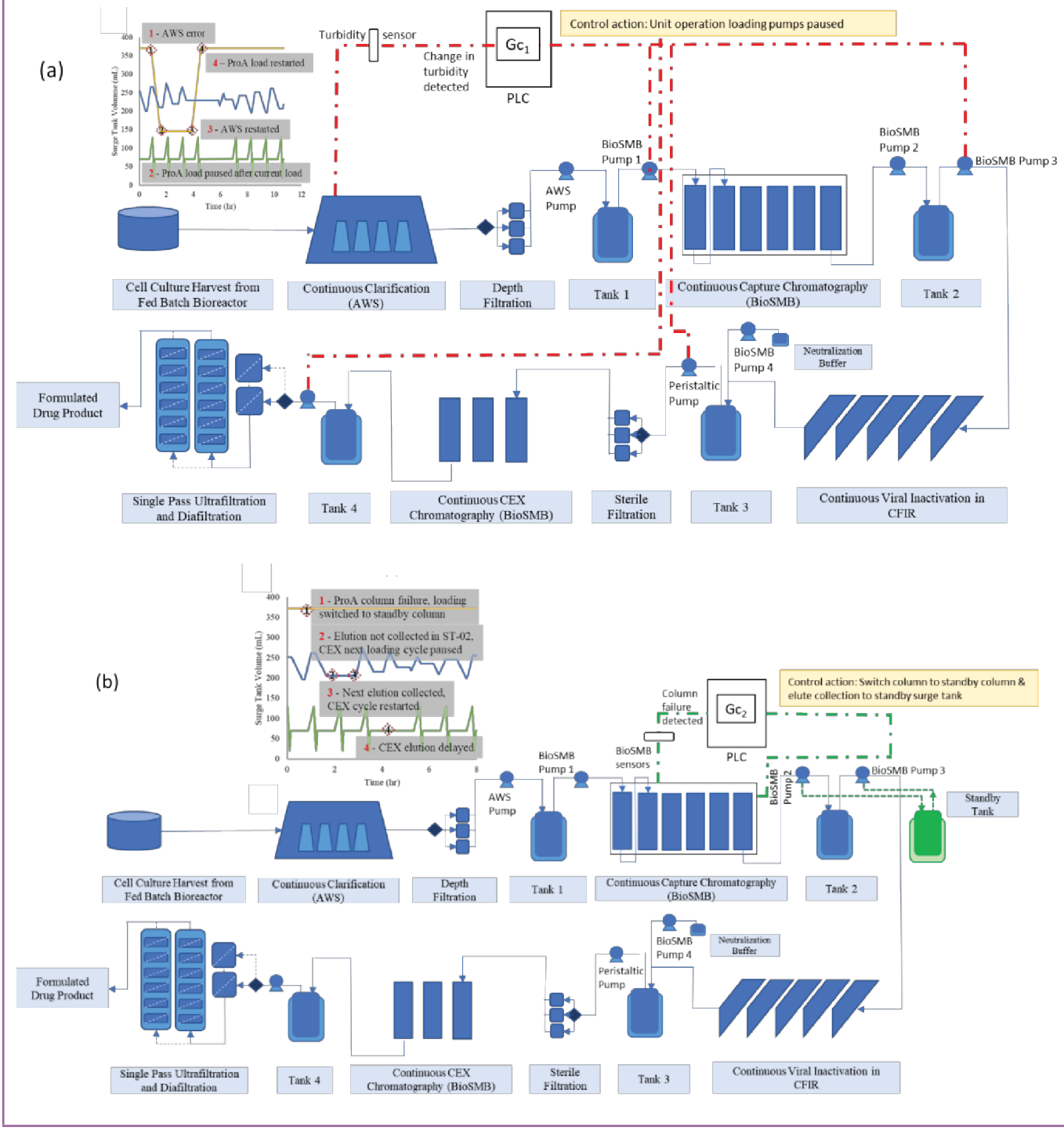
Challenges and opportunities in development of process control for continuous manufacturing.

1. Requirement of high level and sophisticated automation tools, advanced technologies, suitable software platform, robust historian system, and technique to access data in real time
2. Development of control strategies involving CQA control, real time data analytics and PAT applications is required instead of recipe or volumetric control strategies
3. Development of digital twins for entire end-to-end continuous platform
4. Strategies required to handle inherent periodic nature of downstream unit operations and provide constant flow for subsequent unit operation
5. Understanding interactions among unit operations

DOI: 10.18609/ioi.20210.043

FIGURE 4

Real time control strategy for end-to-end mAb production platform for (a) acoustic wave separator failure (b) BioSMB column failure; inset graph depicts the change in surge tank level that was observed from the time deviation occurred [41].



identify and rectify the process deviations. Histograms, scatter plots and control charts are used to summarize the data distribution and to understand the relationship between variables as well as to monitor product quality. These charts help in visualizing the abnormality in the process and take corrective action at the appropriate time. Techniques likes principal component analysis (PCA), independent component analysis (ICA), partial least square (PLS) regression, and neural network (NN) are helpful in this aspect. SPC tools can also handle process uncertainty while keeping the process within control limits [55].

In the present era of digitization, bioprocessing 4.0 has gained lot of interest from researchers. Bioprocessing 4.0 mainly involves driving manufacturing forward by creating an end-to-end integrated platform with the help of concepts revolving around connectivity, intelligence, and automation [56]. Here, the connectivity refers to integrating unit operations and monitoring tools with machine learning and statistical approaches. Intelligence refers to data analysis and control logics developed for monitoring and controlling the continuous processes whereas automation help to establish communication between different unit operations across the platform. The control strategies can be implemented with the help of programmable logic controllers and supervisory control systems. A common example here can be integrating unit operations to a centralized control system with a user-friendly interface and data analytical techniques in place to optimize the process [10]. This kind of holistic approach is used to convert process from analogue to digital and gain process insight by employing various analytical techniques. Once the strategy is devised, the system provide feedback to the controller which in turn manipulate variable in the unit operation.

CONCLUSIONS

Continuous manufacturing aligns well with the concepts of bioprocessing 4.0. Process analytical tools (PAT) and sensors for monitoring, data analytical techniques for identifying cause of deviations and control strategies to take appropriate actions are key element for its successful implementation. Due to low availability of sensors, the control schemes have been restricted to open loop design space in the past. However, with the advent of soft sensors and PAT, closed loop feedback and advanced control strategies based on machine learning techniques are being explored. The topic of process control is sure to continue to garner interest to researchers worldwide, in particular of those working on continuous processing for production of biopharmaceuticals.

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AFFILIATIONS

Anurag Rathore
Professor
Department of Chemical Engineering,
Indian Institute of Technology Delhi

Saxena Nikita
Research Associate
Department of Chemical Engineering,
Indian Institute of Technology Delhi

DOI: 10.18609/ioi.20210.043

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Aug 31 2021;* ***Revised manuscript received:*** *Nov 3 2021;* ***Publication date:*** *Nov 29 2021.*

INTERVIEW

Exploring the cutting edge in RNA-based cell line selection

SUSAN SHARFSTEIN is a Professor of Nanobioscience at SUNY Polytechnic Institute in Albany, New York. Professor Sharfstein received her BS in chemical engineering with honors from Caltech in 1987 and her PhD in chemical engineering from UC Berkeley in 1993. She received an NIH fellowship to pursue postdoctoral studies, initially at UC Berkeley and subsequently at UCLA Medical School. Dr Sharfstein joined the faculty at the University of Toledo in Bioengineering in 1996. In 2001, she joined the Department of Chemical and Biological Engineering at Rensselaer Polytechnic Institute. In 2010, she joined the faculty at SUNY Polytechnic Institute. Professor Sharfstein's research interests include mammalian and microbial cell bioprocessing, control of protein glycosylation, metabolic engineering, biosensing, and development of systems for high-throughput screening. She is the author of over 70 papers and book chapters in the fields of biotechnology and bioprocessing.

SCOTT TENENBAUM is the Head of Nanobioscience and the Laboratory of RNA Nanotechnology at the College of Nanoscale Science and Engineering at the SUNY-Polytechnic Institute. He received his PhD in Microbiology and Immunology from Tulane University Medical Center and did his post-doctoral training at Duke University Medical Center, where he helped pioneer RIP-Chip/Seq technology and the field of 'Ribonomics', which is the genomic-scale study of post-transcriptional gene regulation. Dr Tenenbaum holds 19 issued or pending patents which have served as the basis for 4 biotechnology start-up companies including his most recent, sxRNA Technologies, Inc. in the NY Capital Region. He has been awarded numerous honors, including The Judith Graham Pool Fellowship, The James A. Wilson, M.D. Fellow

in Cancer Award; The Robert M. and Barbara R. Bell Basic Science of Cancer Award; The SUNY Golden Apple Teaching Award; The SUNY-Research Foundation Rising Star Award; and most recently, The SUNY Excellence in Research Award. Dr Tenenbaum has had numerous NIH and NSF grants, maintaining continuous federal funding since 2004. The focus of his research lab is RNA and RNA-binding proteins and he is currently developing an RNA 'nano-switch' platform technology called sxRNA to be used as a molecular tool, a diagnostic and as a therapeutic.

Immuno-Oncology Insights 2021; 2(6), 349–359

DOI: 10.18609/ioi.2021.044

Q What are you working on right now?

SS: Right now, we have a funded grant with a small business called TEGA Therapeutics to make a recombinant or bioengineered heparin. Heparin is the most widely used anti-coagulant drug in the world. However, it is still a slaughterhouse product, which is almost entirely sourced from pig intestines in China. This creates a lot of problems in terms of contamination and supply chain. TEGA therapeutics has engineered mastocytoma cell lines to make a heparin-like drug.

Heparin is challenging because it's a carbohydrate. Proteins are easy to engineer as they are template based, i.e., transcribed from DNA. As carbohydrates are not templated, they are harder to engineer; this involves metabolically engineering the enzymes. Currently, we are working on the bioprocessing of the newly developed cell lines. Also, heparin is a commodity drug, which means it costs about US$25 per gram. These costs are an added challenge when you are working on a recombinant molecule.

We also have a couple of grants starting this month. One of the challenges in immuno-oncology for CAR T cells is cell expansion and one of our grants is for working with another small business to use their bioreactors to grow T cells. Merck and Genentech are also partners on the grant, and if things go well and we accomplish what we set out to do, then the small company will send their bioreactor to Merck and Genentech to test with their proprietary cell lines.

The third project being done in collaboration with some of my other colleagues at SUNY Poly is called Future Manufacturing. The idea is to figure out how to manufacture things that don't exist yet – in this case, bio-hybrid devices.

This involves linking together electronic and photonic devices with tissues. There's huge potential in this field, but a lot of what you see in the lab isn't manufacturable. One of our greatest strengths is semiconductor processing, so we are trying to leverage those skills plus all our expertise in cell culture to make these future products.

ST: My lab is focused on RNA biology so I work with RNA and things that interact with RNA – for example, RNA-binding proteins. Most recently, my group and I have been focused on RNA structures and how we can manipulate them.

RNA is unique in nature in that it can fold up elegantly like a protein but can simultaneously also carry genetic information. Several years ago, we found that you can take some of these rather impressive RNA structures and turn them into structures that are made of multiple pieces. In other words, instead of one piece of RNA folding up into an RNA structure, we can use multiple pieces. In this way, we can dictate whether a specific RNA structure occurs, because it now requires both pieces of RNA to be present.

We use this approach to make nano-RNA switches, in which one of the two components comes from the cell, while we supply the second piece. This forms the basis for what we call structurally interacting RNA (sxRNA) and is the primary project in which my lab is involved.

Over the last decade or so, we have been trying to explore where we can best exploit the power of the sxRNA technology, and recently we have been focused on integrating it into the biomanufacturing pipeline. This is the basis of the collaboration with Susan and we think it represents a very viable, strong commercial opportunity.

The RNA structures we are focused on, often control the translational activity of messenger RNA (mRNA) that they are naturally found in. We have hijacked some of these translational structures and turned them into two-piece regulatory nodes, enabling us to take a messenger RNA that codes for a protein of interest and can now control its expression, based on the RNA signature of the cell we want to make the protein in.

Typically, we've targeted micro (mi)RNAs because we know a lot about them and they are expressed at sufficient levels to allow us to tailor and tune the activity of our sxRNA switches based on the miRNA profiles of the cells. This also forms the basis of some of our biomanufacturing projects.

Using sxRNA as a therapeutic, we can produce a potential mRNA cancer drug and make it cell-specific based on the RNA profile of the cancer cell instead of using a specific receptor on the cancer cell as a delivery technology. Theoretically, we could make an RNA molecule active or inactive based on the miRNA signature of the cell.

Q What would you highlight as the key specific applications for nanobioscience and its associated technologies in the immuno-oncology field, currently?

ST: There are two that come to mind. We are going to talk about biomanufacturing, but specifically when trying to produce proteins that are comprised of multiple chains. I think there are some neat applications with what we are envisioning that will allow you to produce something like an antibody with both chains in an equal dose, or even playing with the amount of one piece or the other. This may lend itself to some of the technology we have, as well as with target specificity, which will allow us to approach cancer in a heterogenous manner. I think one of the problems in cancer therapeutics is that they tend to treat tumors as homogenous – some are homogenous, but many are not. Being able to tailor and target treatments based on RNA signatures of just the target cell would be a useful tool to have.

SS: Scott and I have been working together on novel cell line selection, which I think may become very useful. One of the biggest challenges is manufacturing molecules

such as bispecifics. These are molecules that nature never intended, so getting good expression can be very challenging

ST: Specifically, in the I-O area, drug delivery is a huge focus for us. The main idea is finding a surface marker that distinguishes the cell you are trying to target from other cells, then taking advantage of this by coating some capsule or particle with antibodies or proteins specific for that marker.

In some ways, the question is always going to be, 'How specific is it?' With a lot of the cancer drugs, it needs to be very specific indeed. It can be advantageous to have additive layers that might combine to give you exquisite specificity.

Ideally, our work would be combined with more traditional approaches for delivering cargo to a tissue. We would then add our sxRNA activity to the mRNA drug further restricting the activity to the cells of interest.

One excellent example of this is the acyclovir/gancyclovir antiviral drugs. Here you have a prodrug that gets activated only in virally infected cells. This is because a naturally occurring enzyme, produced by the virus, is needed to activate the drug. It would be wonderful if we had many more examples like this, which would allow us to tailor drug activity so exquisitely. Our sxRNA approach is potentially a new way in which we can use something within the cell to activate a prodrug, which by itself isn't functional.

Q Molecules seem to become more complex by the day in I-O. Can you detail some of the challenges that this complexity presents to protein therapeutic engineering and bioprocessing?

SS: I think the biggest challenge is understanding how the molecule interacts with cell physiology. Over a decade ago, I wrote a proposal on understanding manufacturability, at a time when we weren't even looking at bispecifics. At the time, the grant was turned down, but now people talk about 'manufacturability' constantly.

Currently, companies are screening for manufacturability, but they're doing it very empirically. For example, they will take 15 candidate molecules, transfect them all into a cell, and see which one expresses well. This method works for them, but from a fundamental standpoint, what we would really like to know is why one expresses well and another one doesn't. Where is it being held up? In the endoplasmic reticulum (ER)? Is the Golgi not folding it correctly? Is it being clipped? Is it being truncated? What's the interaction of that molecule with the chaperones? What are you upregulating? What are you downregulating? Those are the fundamental questions that we need to be asking. However, the challenge is finding companies that are willing to work with you on this because it's hard to get the molecules.

For those of us in academia, it is very difficult to design molecules if you are not a protein engineer. But companies often don't want to make their molecules available, even if they are not good ones. So I'm currently not sure whether the sxRNA technology could be useful for that sort of application, but there might be ways for us to look at upregulation or downregulation of certain genes and maybe we could engineer cells as a one-off.

DOI: 10.18609/ioi.2021.044

For example, if the cell makes a significant amount of an ER stress protein, could we then alter something in real-time to help the translation of the protein?

"If the cell makes a significant amount of an ER stress protein, could we then alter something in real-time to help the translation of the protein?"

Susan Sharfstein

ST: It's a good potential application.

One of the useful aspects of targeting regulatory structure is that is separate from the coding region. So, when we modulate it by targeting the presence or absence of a cellular miRNA, the coding region isn't impacted. The coding region can be described as 'plug-and-play': we can drop in anything we want. We are using biomanufacturing to produce a gene of interest (GOI). But we could potentially produce some other molecule that helps the cell do something that would be beneficial for the manufacturing pipeline.

In the biomanufacturing sector, so many of the real pain points are manufacturing problems. In fact, if there's any domain in which I would say the semiconductor industry is experts in, it's high-throughput manufacturing and cost. Plus, the biomanufacturing field, at least for human biologicals, has an added layer of complexity, because everything must be approved and regulated by regulatory agencies such as the FDA. If problems arise within the manufacturing pipeline, it could mean having to go through the entire approval process again.

This limits what you can and cannot do, but also what companies are interested in exploring. This is something we see as a real challenge for the field. And there is no clear solution, unless regulatory agencies help manufacturers find one. Until then, it's a fundamental roadblock to improving the technology. Implementing an improved method could mean that a drug produced using the new approach may not be considered identical to an existing approved drug, and would require further regulatory approvals. It's the nature of the field.

Q Tell us about your work on novel cell line selection based on co-transcribed non-coding RNA – firstly, how did this project come into being? What were the drivers behind it?

ST: Put simply, the idea is that we take a particular RNA in a cell of interest and reverse engineer our sxRNA switches so they are only functional when bound to that cellular RNA.

Originally, we looked at super-producing CHO cells – the ones that produce the most gene of interest, or that are the high expressers of whatever phenotype you like, in this heterogenous population of cells. If those high expresser cells have different non-coding RNAs being expressed, then we could use that to select or bias the population towards them.

When we started, the field hadn't even sequenced the CHO genome, let alone small RNAs like micros. However, this work has been completed now. The person to whom Susan introduced me, Nicole Borth at BOKU in Austria, is an expert in non-coding RNAs and CHOs.

> "We can de-select the cells we don't want and enrich for the cells we do want ... I think that's where we can potentially make a difference"
>
> Scott Tenenbaum

She had already seen that there are different miRNAs being expressed that clearly correlate with expression levels of the gene of interest.

That was the original idea, which has now morphed into our current thinking around replacing the proxy antibiotic gene with a noncoding RNA (a miRNA precursor). This is how most of our earlier work was done; with a non-coding RNA that we link to the gene of interest.

As well as producing the gene of interest, some non-coding RNA is also produced, which we assume is less stressful to the cell and mitigates us having to make a proxy protein.

This non-coding RNA is directly tethered. It's not a separate gene. It's encoded within the intron of the gene of interest and is located downstream of the coding region of the gene of interest. The idea that you get a much better correlation of the expression of your proxy and expression of your gene of interest is inherent to our thinking. Then, we use that non-coding RNA as a trigger to activate a secondary molecule, which we call our 'bait' RNA – that is a transient molecule we put in.

There are a couple of things we can do. We can drive it in a way that is either negative selection or positive selection. One approach uses more of the traditional sxRNA approach, and the other is using miRNAs in the way they were originally envisioned; to suppress the expression of a gene.

One approach is more straightforward and the other uses a new and slightly more complicated sxRNA idea. However, the fundamental idea is to develop a method that allows you to go in both directions.

We can de-select the cells we don't want and enrich for the cells we do want. Through that combination, we can achieve a population that is optimal. Not only can you optimize for characteristics you want, but potentially expedite the clonal selection process. I think that's where we can potentially make a difference; where we take something that normally takes quite a lot of time and scale that back to maybe a week or two.

SS: The real challenge in much of this process is even when you've got a molecule you like, cell line development takes a long time.

Currently, in industry, they are getting more efficient at screening clones. Now, instead of screening hundreds, they are screening thousands, and sometimes tens of thousands of clones. I recently attended a conference where they described how the automated technologies to help this process have advanced over time. Having said this, it's intellectually unsatisfying, because they still have to screen so many. Even when they do antibiotic selection, the problem is that they can have cells that are quite antibiotic resistant but don't make much of the gene of interest. This means it can take a couple of weeks to get a clonal pool, followed by individual cell line development.

We are hoping to get a better pool and achieve this faster. This was one of the things we recently published on in *Journal of Biotechnology*, where we compared the traditional method with ours. When we looked at the number of clones that were positive solely for the gene of

DOI: 10.18609/ioi.2021.044

interest, the result was quite dramatic. We had between 3x and 10x as many positive clones when we undertook limited dilution cloning.

Now our challenge is to figure out how to obtain high productivity clones. In fact, Scott has started companies around this technology. The first company that he founded, HocusLocus, wrote some small business innovation research grants and it made sense for me to be the PI on some of the business grants.

That is how I got involved in the project. Having biomanufacturing expertise as well as an understanding of what companies want is a strength I bring. As well as having the ability to describe our work to potential industrial customers.

ST: On that point, this is the type of technology that will likely only be developed either through small start-ups and small grants like this, or in-house at larger biological biomanufacturing companies.

As companies are risk averse and because it is difficult for them to change an approach due to the implications that brings, any technology presented to them has to be fairly far along in the process – it needs to have been taken through its paces before a company would consider taking it on.

It's difficult to do that with traditional funding because it's not basic research. Therefore, running this through a small startup is likely the only way it will get to where it needs to be. I think a lot of proof-of-concept work needs to be done before we'd be able to get a major player to invest in it. What more can you tell us about your findings to date and next steps in the technology's development and application?

The paper Susan mentioned in *Journal of Biotechnology* used the negative approach. Here we have a gene of interest. We also have what we call our 'trigger', which is miRNA as a precursor. These two are physically linked together, which means in theory they are made one-to-one co-transcriptionally.

The trigger is going to drive a secondary molecule, which in this case is a CD4 molecule. The miRNA is going to bind to traditional miRNA targeting sites in the CD4 message and turn it off. Ultimately, the more gene of interest is being expressed, the fewer CD4 molecules occur. This is described as a negative selection. It is then run through an anti-CD4 column, and the cells you want pass through. This technology still needs further refinement, but Susan's group have undertaken testing and put it through its paces. I would say proof of concept is there, but we are still trying to optimize and characterize it better.

Unfortunately, negative selection is not ideal as you are passively collecting cells of interest by removing everything you don't want, and there is more risk of contamination. Positive selection, where we turn on the expression of CD4, would be more precise and specific. In this case, we would specifically turn on the expression of CD4 molecules only in cells that make the gene

"any technology presented ... has to be fairly far along in the process – it needs to have been taken through its paces before a company would consider taking it on."

Scott Tenenbaum

> "if you have a bispecific where there are four polypeptide chains all of which are different, we could create a reporter associated with each one of the chains ... there is a lot of potential..."
>
> Susan Sharfstein

of interest. Again, it is likely that we would want to use a combination of both positive and negative selection. Most of my team is focusing on optimizing the positive selection right now.

It all comes down to sensitivity and specificity – how on is 'on' and how off is 'off'? We need our gene of interest not to be expressed in the cells where we don't want it to be present, and we want it expressing highly in those where we do. Fine tuning this involves changing sequence. It's all based on Watson-Crick interactions. We try different sequences and look to optimize and figure out which switches are working the most robustly. Importantly, as we are working in a contrived situation where we are making our own trigger miRNA to run everything in these cells, there is always the potential that endogenous miRNAs may disrupt the system. These are things that exist, but which we cannot control.

Currently, we have found that some of our switches aren't as 'on' as we expect them to be, compared to other switches we have made. We also have some background miRNAs that are botching the system and we must find a way around this.

SS: One of the things we have learned from doing this is that we are able to get improved stability with this technology compared to a lot of the antibiotic selection.

Another thing we are able to do is add fluorescent molecules as reporters, which enables us to do FACS (fluorescence-activated cell sorting) sorting on fluorescent proteins. People also do this with antibodies. However, you have to have either your antibody itself on the surface of your molecule, or something that will bind to it.

With this technology, we can use the fluorescent molecule dTomato as a screening method, so we can see whether all the cells that are making our molecules of interest at relatively high levels are also making a transient dTomato. As it is a transient mRNA, it can be separated by FACS. This method allows us to carry out more precise selection and may become useful when applied to molecules that nature never intended. In particular, if you have a bispecific where there are four polypeptide chains all of which are different, we could create a reporter associated with each one of the chains, then you can look and say 'I'm getting red, green, blue, but not yellow'. This indicates that something is wrong with this particular polypeptide chain in that you are not making it properly. So I think there is a lot of potential, especially when you start looking at unusual molecules.

Q Looking to the future, how and where could this technology have the greatest impact on the I-O field?

SS: Anything that gets molecules into the clinic faster has a huge impact.

DOI: 10.18609/ioi.2021.044

It is often the case that cell line development is the rate limiting step: this is often what holds everything back on the bioprocess side. The sooner you can get things into clinical trials, the sooner you can figure out if it's going to have potential, and whether or not it's a molecule you can move forward.

ST: I think where I struggle trying to answer this question as accurately as possible is the 'I' in 'I-O': it depends on how you define it. If you are asking specifically in the area where antibodies are being used in oncology, there are some limits. If you move past that, including to the production of the antibody in cells, not in a lab, then there are limits there, too. But I think we may have some traction in being able to modulate the activity of an RNA in a cell-specific manner.

I think it is likely the greatest benefit will be in the ability to potentially manufacture bispecific or even other more complex molecules in a more robust manner, allowing them to get into clinic quicker.

Q Susan, your lab is also involved in the development of novel biosensors for use in bioreactors. Where do you see the greatest needs for innovation in this particular area, and corresponding opportunities to harness nanotechnology to the benefit of the biopharma sector?

SS: Everywhere! What we measure in a bioreactor today – inside, in real-time – is pH, dissolved oxygen, carbon dioxide, and cell density. I believe there are some new technologies that also measure glucose and lactate. However, the amount that we can measure in a bioreactor is very limiting.

My lab also has a lot of interest in protein glycosylation and currently, it is not possible to measure glycans on a protein in a bioreactor. We can't even accurately measure the concentration of antibody well in a bioreactor.

As such, there's a tremendous need for these kinds of technologies. I think some of what we are going to do in terms of future manufacturing isn't necessarily focused on this technology, but is likely to impact it, because we need to start thinking more about how electronic and photonic devices engage in environments that are wet, salty, and proteinaceous, and how to keep measuring them robustly in those environments.

Q Finally, can you each sum up your major goals and priorities in your work over the coming 12–24 months?

ST: Our focus right now is on trying to develop a kit. We believe this may be the best thing we can do. We are currently in the process of getting some reviewer comments.

We've had a number of people say this is something they would use for basic cloning in the lab, and that's a good chance for us to figure out the nuts and bolts of what would go into a

kit like that. For example, what plasmids do we include? What controls do we include? What buffers do we include? What is the actual process?

I'm hoping that in the next year, we will have a physical kit we can put into people's hands and ask them to try it out and tell us what they think. I think that is our next big milestone.

SS: I would echo that. I have been talking about this work at lots of meetings. My job is to go out and expose this technology to the world.

One of the best things about being a professor is doing things that you never thought you would be doing. I am excited that we can try this novel approach, the reverse of what we previously published, and potentially get a better result. The key to being successful commercially is to increase productivity. So far, we have been able to get lab-scale levels of productivity, but that is approximately 1–3 orders of magnitude lower than what we are going to need to be in the biotech industry.

Some of that will come out of bioprocess development, but I think we are going to have to gear-up the technology to increase probably by a factor of 10 to a 100. Ideally, we need to approach 100 mg/L to be in the right ballpark. This is where I'm hoping we can get to.

AFFILIATIONS

Susan Sharfstein
Professor of Nanobioscience, SUNY Polytechnic Institute, Albany, NY, USA

Scott Tenenbaum
Head of Nanobioscience, SUNY-Polytechnic Institute, Albany, NY, USA

DOI: 10.18609/ioi.2021.044

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Dr Sharfstein holds two patents assigned to the Research Foundation for SUNY. She is on the Bioengineering (MDPI) Editorial Board (2020–present) and the BioDrugs Editorial Board (2018–present); and was the Biotechnology Subject Editor, Elsevier Life Science Reference Module (2015–2019; compensated position). Dr Tenenbaum is an inventor on several issued sxRNA related patents and is a Founder and equity holder in sxRNA Technologies, Inc.*

Funding declaration: *Dr Sharfstein has received funding from NIH-STTR, HocusLocus, the Center for Advanced Technology in Nanomaterials and Nanoelectronics Investment Program (NY State money) with sxRNA, NSF, TEGA Therapeutics, Sepragen, NIIMBL, Genentech, Merck and NIH UAlbany. She has received royalties from Glauconix and Informa Life Sciences; consulting fees from BIOMILQ, GLG Research, NIH and NSF; payment for expert testimony from Proskauer-Rose, Fisk and Richards, Cooley, Wilkie Farr and Gallagher; and support for attending meetings and conferences from Cambridge Healthtech Institute Bioprocessing Summit, Festival of Biologics (Terrapin) and PEGS Boston. Dr Tenenbaum has received funding from NIH/NIGMS and sxRNA Technologies, Inc; and support for attending meetings and conferences from NIH/NIGMS.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited.*

Interview conducted: *Sep 14 2021;* ***Publication date:*** *Nov 24 2021.*

IMMUNO-ONCOLOGY INSIGHTS

BIOPROCESSING/CMC TRENDS, TOOLS & TECHNIQUES

SPOTLIGHT

EDITORIAL

Biomanufacturing I-O therapeutics in the 21st century

"...there is a drive to switch from batch to continuous processing where efficient intergradation of the units of operation, monitoring and control of the process and the product are achieved."

GREGORY ZARBIS-PAPASTOITSIS

EVP of Process and Manufacturing Sciences, Ankyra Therapeutics

Immuno-Oncology Insights 2021; 2(6), 345–348

DOI: 10.18609/ioi.2021.041

It has been 40 years since the initial production of the first biotherapeutic (recombinant insulin, 1982), and the massive advance of various modalities from R&D to development, clinical trials, and market authorization. While drug development has been challenged by the pandemic onslaught of 2020 and 2021, it has further demonstrated the

capabilities of the industry, and government agencies to rapidly develop, test, and approve safe and effective biotherapeutics in the form of vaccines and antibodies. The former category includes molecules manufactured with a relatively new technology, mRNA, with two highly effective COVID vaccines approved in record time (less than 2 years), from Moderna (US), and BioNTech/Pfizer (Germany/US).

Cancer immunotherapies are rapidly advancing with several monoclonal antibodies approved targeting inhibitory immune-checkpoint molecules such as PD-L1, PD-1, and CTLA-4, and two CAR-T therapies approved [1]. Other proteins such as interleukin-2 (IL-2), and interferon alpha (IFN-α) are also included in the arsenal of cancer immunotherapies. Oncolytic viruses that infect and kill cancer cells are also employed in treating cancer. One such oncolytic virus, talimogene laherparepvec (T-VEC™, Imlygic), was approved to treat metastatic melanoma. Finally, vaccines have been approved to prevent and/or treat cancer such as vaccines against the human papilloma virus.

Biomanufacturing of these life savings drugs poses some significant challenges due to the nature and diversity of the different modalities. When compared to small molecules, biologics have structural complexity, which results in expensive and time-consuming processes and analytical development. They often require cold chain for supply and distribution (the example of viral therapies that require storage at lower than –70°C), concerns of deleterious immunogenic responses in patients that render the drugs ineffective at best, and difficulties in scaling up production. With newer therapeutic modalities such as cell and gene therapies, challenges remain in aspects of manufacturing such as quality expectations for cells, CAR-T formulation and release criteria, vector production and purification, quality expectations for vectors, and vector delivery.

The workhorse to produce monoclonal antibodies has been CHO cells. CHO cell lines have been highly effective in producing multiple therapeutics including some of the highest commercial successes (i.e., Humira®, Keytruda®, Eylea®). As antibodies are becoming increasingly successful so has the design of these molecules become increasingly complex. Protein engineers are designing bispecific T-cell engager antibody constructs, Fc fusion proteins and antibody fragments. A few of these constructs express poorly in CHO cells, and/or they face challenges in stability and formulation. As a result, alternative expression systems that can produce these molecules in satisfactory quantity and quality are highly desirable. Systems such as human derived cell lines (i.e., PER.C6®) have previously demonstrated the ability to express difficult to make molecules with high yield and the right critical quality attributes [2]. Similarly, other molecules such as cytokines, hormones, and enzymes could benefit from an alternative expression system. In addition to choosing an appropriate expression system, recent transfection technologies further enable robust expression of complex proteins. At Ankyra therapeutics we employ the Leap-In Transposase® technology from Atum to produce high productivity cell lines. The technology enables the insertion of multiple copies of the gene of interest in open chromatin areas of the host genome [3], resulting in high productivity and stability cell lines.

Most biopharmaceuticals are manufactured utilizing batch operations where the various units of operation, from the expression of the molecule in the bioreactor to the final formulation step, are executed sequentially. This mode of production is often attractive in early clinical development where low amounts of the potential therapeutic molecule is required and the understanding of the process performance is still limited. Batch operations are less desirable once the molecules advance to commercial manufacturing since they entail less efficient use of manufacturing space, equipment, and labor, and finally result in increased manufacturing cost. As a result, there is a drive to switch from batch to continuous processing [4] where efficient intergradation of the units of operation, monitoring and control of the process and the product are

DOI: 10.18609/ioi.2021.041

achieved. This drive will further intensify as pressures on drug pricing increase (especially in the US) and more biosimilars are approved in the next few years.

Monoclonal antibodies represent a well understood area of manufacturing with defined units of operation which enabled drug manufacturers to develop 'platform' processes for their production. Protein A is utilized as the first purification step and has been instrumental in the design and implementation of these platform processes, and the drive to employ continuous manufacturing in the future. In addition to Protein A, perfusion processes with intensified cell culture conditions (high cell density), and advances in process analytical technologies have further encouraged the switch from batch to continuous manufacturing. While continuous processes may result in lower cost, there are risks associated with such production mode. These risks include limited experience at large and commercial scale, equipment robustness, maintaining sterility during lengthy production times, and uncertain regulatory reception. In addition, the implementation of a fully integrated continuous process requires well established real-time process analytical technologies (PAT) capable of monitoring key product quality attributes during continuous processing. PAT should be able to maintain a production process within defined specifications, which will require coordination between most if not all unit operations currently limited or absent in existing processes and technologies.

As of October 2021, US FDA has approved over 60 immunotherapies [5], and there is a strong need to develop more soon. This generates tremendous opportunities for drug developers and manufacturing organizations which include developing better drugs and better processes. While the upside can be significant for both people's health and the success of organizations, there are still significant hurdles to overcome. Such hurdles include improvement of the manufacturing technologies and processes for different drug modalities, adequate manufacturing capacity, and availability of critical manufacturing supplies from vendors especially in the times of a pandemic.

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AFFILIATION

Gregory Zarbis-Papastoitsis
EVP of Process and Manufacturing Sciences, Ankyra Therapeutics

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

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Article source: *Invited.*

Revised manuscript received: *Oct 18 2021;* ***Publication date:*** *Nov 18 2021.*

DOI: 10.18609/ioi.2021.041