Volume 3 Issue 2

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Exosome Therapeutics
Academic Curiosity or Commercial Reality?

Non-clinical Safety Evaluation of Vaccine
Strategic Considerations to Accelerate Clinical Development

Ensuring Reproducibility in Biomedical Research
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2020 PHARMA PUBLICATIONS / Volume 3 Issue 2 – Summer 2020

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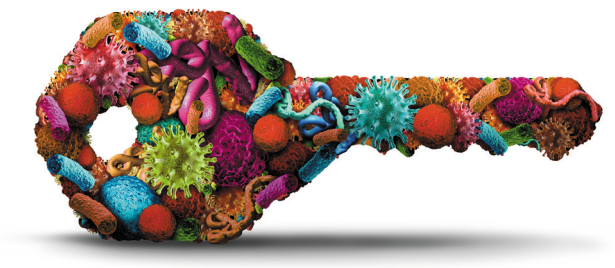
Contents

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Foreword

I wanted to start my note by highlighting a topic which has gained enormous traction in recent years. That is exosomes. Cells continuously secrete a large number of microvesicles, macromolecular complexes, and small molecules into the extracellular space. Of the secreted microvesicles, the nanoparticles called exosomes are currently undergoing intense scrutiny. These are small vesicles (30–120 nm) containing nucleic acid and protein, perceived to be carriers of this cargo between diverse locations in the body. They are distinguished in their genesis by being budded into endosomes to form multivesicular bodies (MVBs) in the cytoplasm. The exosomes are released to extracellular fluids by fusion of these multivesicular bodies with the cell surface, resulting in secretion in bursts. Exosomes are secreted by all types of cells in culture, and also found in abundance in body fluids including blood, saliva, urine, and breast milk.

Currently, the control of exosome formation, the makeup of the "cargo", biological pathways and resulting functions are incompletely understood. One of their most intriguing roles is intercellular communication – exosomes are thought to function as the messengers, delivering various effectors or signalling macromolecules between supposedly very specific cells. Both seasoned and newer investigators of nanovesicles have presented various viewpoints on what exosomes are, with some differences but a large common area. It would be useful to develop a codified definition of exosomes in both descriptive and practical terms. We hope this in turn leads to a consistent set of practices for their isolation, characterisation and manipulation.

Our cover story by David Haylock at VivaZome Therapeutics and Andrew Hill at La Trobe University describes the key aspects of extracellular vesicle biology, opportunities for exploiting this biology, manufacturing challenges in the production of safe and effective exosome therapies, and regulatory considerations for this new class of biological medicines.

Amid this ongoing pandemic, vaccine development is at the forefront of everybody's mind. In vaccine research, where reproducibility and accuracy are of paramount importance, scientists will need to choose the most appropriate technique to examine and characterise carbohydrates that serve as potential vaccine antigens. Within the article titled "Analysing Carbohydrates with Ion Chromatography to Develop Better Vaccines" Jeffrey Rohrer and Wai-Chi Man at Thermo Fisher Scientific discuss how IC can help accelerate vaccine development with robust and sensitive carbohydrate analysis.

One of the other articles I would like to highlight is "Ensuring Reproducibility in Biomedical Research – the Role of Data, Metadata, and Emerging Best Practices" by Martin-Immanuel Bittner and Versha Prakash at Arctoris Ltd, who explore how these foundations are tested by other scientists independently, thereby verifying each other's results.

I thank all our authors and contributors for making this issue an exciting one. We are working relentlessly to bring you the most exciting and relevant topics through our journals and keep the mode of communication ongoing through these difficult times.

I hope you are all keeping safe and healthy, and I look forward to seeing you all soon in person.

Virginia Toteva, Editorial Manager

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Delivering Viable, High-quality Leukapheresis Product via Advanced Cold Storage Solutions

Fresh leukapheresis product (LP) is a critical raw material in the global biopharmaceutical industry, being essential across many primary cell research and therapy development applications. Typical packs of LP comprise many billions of primary cells that closely reflect *in vivo* morphology, metabolism and growth, enabling researchers to advance *in vitro* research into immune behaviour and function.

However, storing and shipping this fresh product is incredibly time-sensitive, as LP must be delivered swiftly to ensure cells remain functional and viable. As a result, it is highly challenging to transport fresh LP on an international scale as, when shipped at ambient temperatures, the product only typically remains of high quality for up to 72 hours before degrading.

Advanced cold storage options now offer a way forward for biopharmaceutical researchers across the globe. Cold storage can maintain and protect product integrity for longer, as proven by a recent collaborative study demonstrating how advanced cold storage workflows can preserve LP viability for up to five days[1]. This extended timeline enables laboratories to access fresh, high-quality LP regardless of location, supporting the acquisition of valuable research results.

The Value of Fresh LP in the Biopharmaceutical Industry

LP is collected from healthy human donors via leukapheresis, a laboratory process by which white blood cells are separated from a peripheral blood sample. This process results in a highly enriched product that contains higher concentrations of certain cells than blood collected or prepared by other methods, including standard venipuncture procedures or buffy coat products (in which samples are centrifuged to separate constituents by density). Researchers turn to LP when they require large quantities of peripheral blood mononuclear cells (PBMCs), or to isolate multiple immune cell types from the same donor (such as mononuclear cells, stem or progenitor cells, dendritic cells, T cells and B cells). LP offers increased convenience and efficiency for researchers and manufacturers, allowing them to refine their processes on demand without needing to rely on actual patient material.

LP is collected and transported via stringent, verified, carefully controlled processes covering donor selection, management, packaging, storage and shipping, ensuring that the product is not compromised at any stage of its production. It is offered by suppliers in the form of 'leukopaks'. Typical leukopak formats include full, half and quarter research-grade leukopaks, with a full pack containing a minimum of 10 billion cells. These packs are excellent resources for use in biopharmaceutical research. Since packs can be ordered as needed, laboratory setups, equipment and personnel can be prepared and scheduled ahead of time, accelerating operations and reducing laboratory downtime.

A Tight Timeline: The Challenges of Shipping LP Worldwide

Despite its potential, LP is only useful if it contains high proportions of functional, viable cells – making appropriate storage and shipping of LP essential to enable its successful use in the laboratory. LP producers and providers must ensure that ordering laboratories receive high-quality product that meets their expectations. However, human tissue product degrades in mere days, making LP transportation a challenging endeavour.

LP is typically transported at ambient temperatures, ranging from 15–25°C depending on the supplier. At such temperatures, LP remains viable and functional for a maximum of 72 hours, with timelines often being shorter depending on donor characteristics, shipping processes and inherent cell variability. These time constraints limit the geographical range over which LP can effectively and reliably be transported – cells that arrive too late will have degraded, resulting in compromised product integrity, loss of cell viability, and suboptimal cell functionality.

While this short time window for LP viability is a critical consideration for international shipping, it can also affect domestic shipping. Whereas domestic orders of LP remove some of the hurdles associated with transporting the product longer distances or overseas, timing issues are still prevalent. Shipping schedules can be impacted by unexpected delays, and do not always align with those of the receiving laboratory. If a researcher receives LP late in the working day, for example, it will likely not be used until sometime the following day, bringing potential delays of a further 12 hours or more. If a product is already nearing the end of its viability, this can be the difference between high-quality LP that is appropriate for use in valuable research, and LP that contains excessive proportions of unviable cells and has degraded in usefulness and functionality.

The Benefits of Going Cold: Keeping Cells Viable for Five Days

A leading alternative to ambient shipping of LP is cold storage, which keeps product some 10 to 20°C colder than controlled room temperature (CRT) conditions throughout storage and shipping. This is distinct from cryopreservation, which results in frozen leukopaks – while these exist in the market and offer long viability timelines, they are significantly more expensive than fresh leukopaks, and require a complicated thawing process that increases the risk of losing or damaging cells once the product has thawed and returned to the desired temperature.

By keeping leukopaks fresh rather than frozen, cold storage and shipping methods help to avoid the issues of cost and potential cell damage experienced by cryostorage, while still exploiting the benefits of low temperatures to handle and transport LP. Importantly, cold storage offers an array of benefits over methods of ambient shipping of fresh LP, as proven by research including a recent study into cell viability in LP stored and transported at 2–8°C[1]. The study found that cold storage methods expand shipping capabilities significantly by adding days to the cell viability timeline. This reduces the risk of product loss or

degradation, which translates to a higher chance of research success.

In the study, fresh LP was collected from six healthy donors, stored at either ambient or cold temperatures, and sampled daily over a period of five days (with defined setpoints to represent worst case international shipping durations). Each day, the researchers assessed levels of cell functionality and viability across numerous cell types by analysing cell count and viability, cell metabolic status and cell distribution. Studied cells included pan T cells, Natural Killer cells, B cells, cytotoxic T cells, helper T cells, lymphocytes, granulocytes and monocytes. Assessment of T cell activity is often a critical requirement for those working with LP. For this reason, during this study, researchers also isolated and stimulated a number of T cells, exploring all the aforementioned properties alongside additional characteristics of cytokine release, proliferation and activation marker expression.

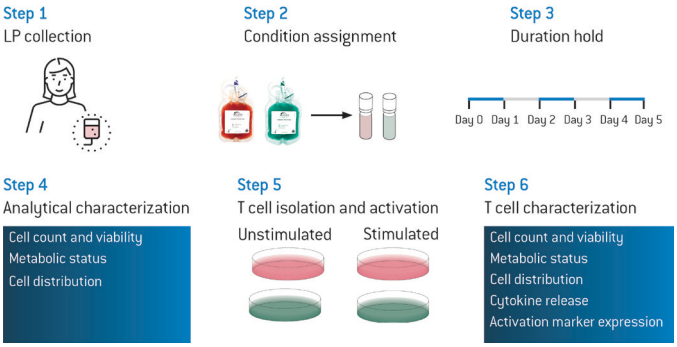

Figure 1. Experimental outline for each leukopak product LP that was collected

The results showed that cold shipping of fresh LP at 2–8°C maintained LP stability for up to five days – far superior to LPs maintained at ambient temperature, which began to sharply decline in viability and metabolic activity on the second day. Isolated T cell functionality was also maintained during the five days in cold storage, but dropped dramatically at day two when stored at ambient conditions.

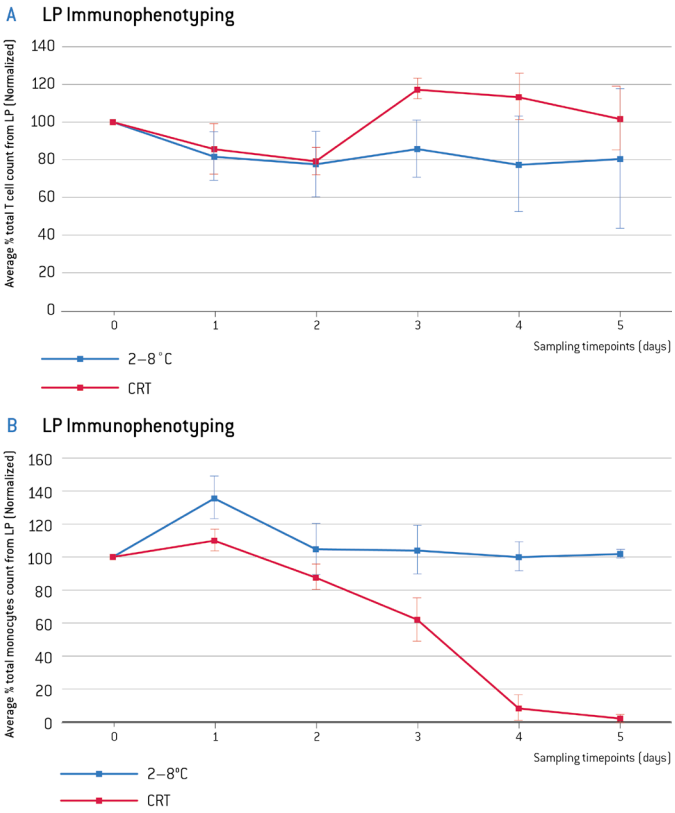

Figure 3. Effect of hold time and temperature (CRT vs 2–8°C) on total viable cell count of A) pan T cells and B) monocytes. Data is represented as the average per cent cells in the LP and has been normalised to Day 0.

This study supports previous findings[2] and indicates that the stability extension from 2–8°C shipment extends geographic feasibility for fresh LPs for cell isolation and other primary cell applications. By utilising cold storage, laboratories can be more confident in formulating their research plans, as they know that they are highly likely to receive functional, viable, high-quality product that is suitable for clinical processing and research use. Cold storage timelines also

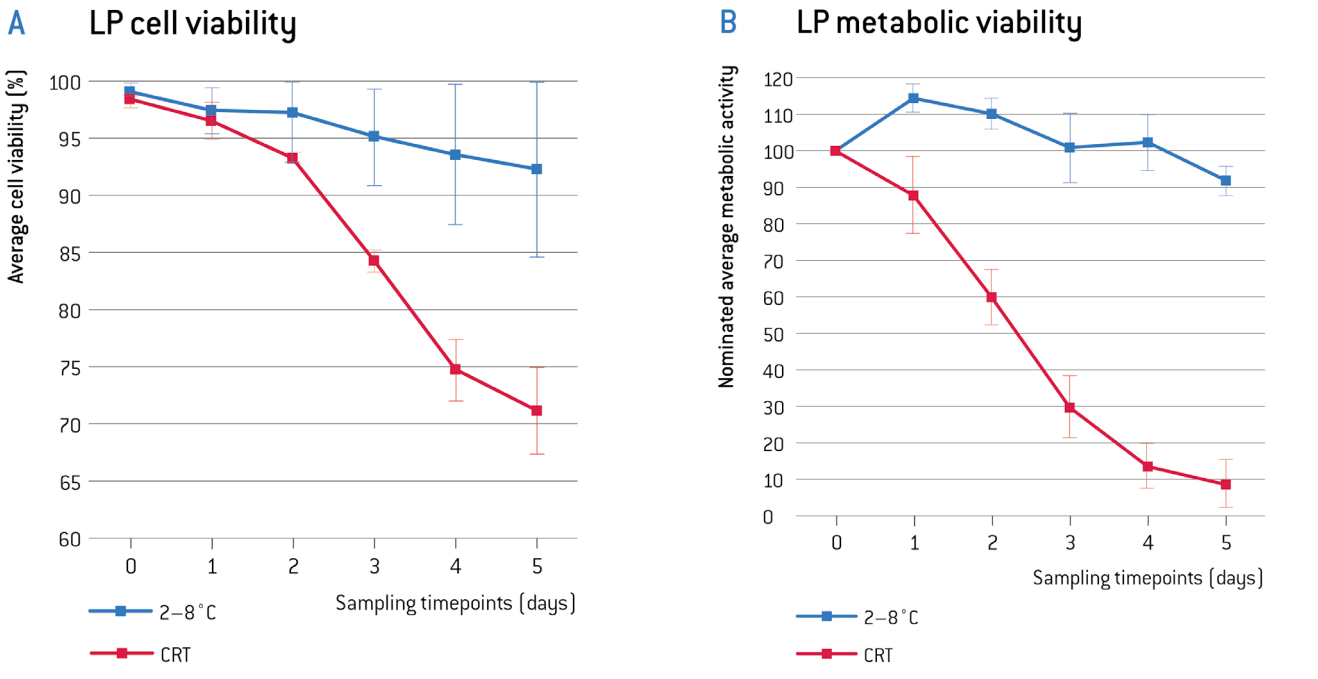

Figure 2. Effect of hold time and temperature (CRT vs 2–8°C) on cell viability in fresh leukapheresis material assessed with A) AO/PI and B) luminescent cell viability assay.

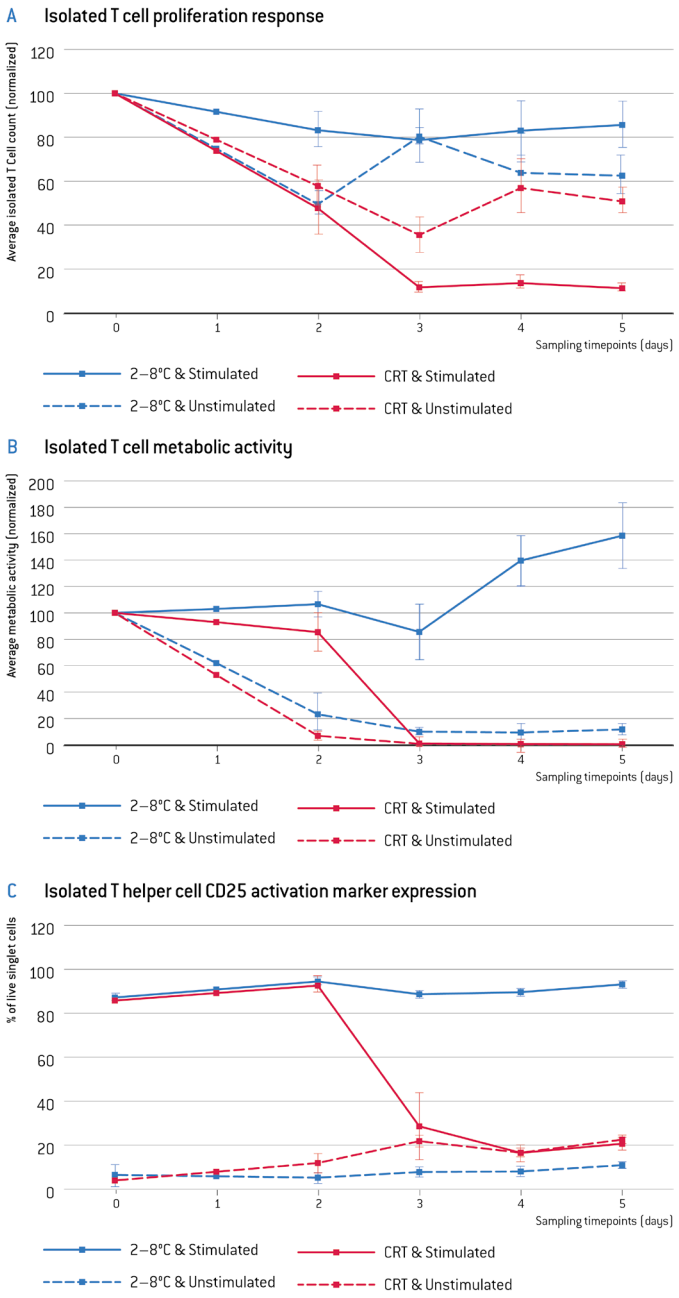

Figure 4. Effect of hold time and temperature (CRT vs 2–8°C) on functional T cell activation. A) proliferative response measured by T cell counts, B) cell metabolism and C) CD25 (IL2R) activation marker expression

mitigate the challenges of misaligned shipping and laboratory schedules, enabling researchers to introduce a greater level of flexibility and adaptability into their plans. If LP arrives at the end of the day, for example, laboratories can rest assured that

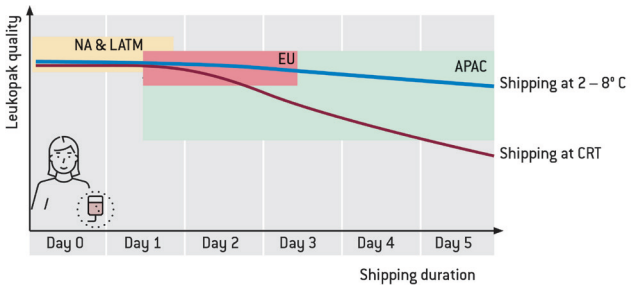

Figure 5. The difference in leukopak quality between CRT and cold temperature storage/shipping (APAC: Asia Pacific, EU: European Union, NA: North America, LATM: Latin America) (Figure adapted[3])

it will remain usable until morning without degradation, rather than worrying that any additional delay will compromise their results.

Conclusion

The use of high-quality fresh LP underpins many applications across biopharmaceutical research and manufacturing – but only if the integrity of this sensitive material can be successfully preserved throughout collection, storage and shipping. To support the acquisition of results of the best possible quality and usefulness in novel cell research and therapy development, LP must be handled in a way that retains cell functionality and viability reliably and for as long as possible.

Cold storage solutions can facilitate this by preserving cell viability for up to five days. This extends shipping timelines and removes geographical limitations on the location of shipper and receiver. As a result, customers can order leukopaks from suppliers across the globe, plan their experiments more reliably in advance, and rest assured that their LP will retain optimal integrity, quality, stability and viability so that it can confidently be used upon arrival at the laboratory.

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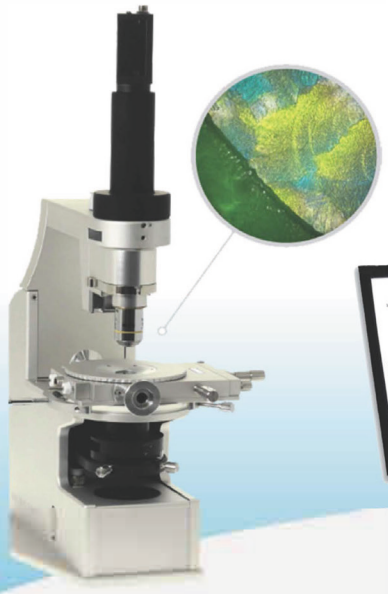

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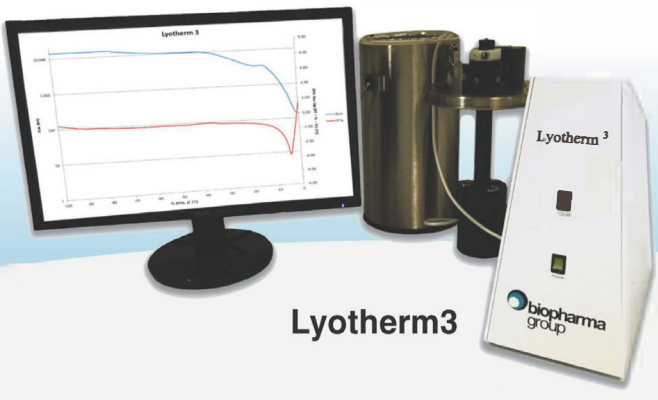

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Exosome Therapeutics: Academic Curiosity or Commercial Reality?

Cell and gene therapies have been called the emerging fourth pillar of healthcare systems. However, recent research suggests that the cell therapy pillar may, in fact, be underpinned by exosomes (also referred to as small extracellular vesicles; sEVs). It is now evident that exosomes are potent cell-to-cell communication vehicles and play essential roles in normal physiology and disease processes. This new knowledge has driven an explosion of academic research and growing interest in using exosomes for therapy (Figure 1). However, this raises two major questions: Can exosome manufacture be commercially viable; and will exosomes really work as therapeutics? Here we describe the key aspects of extracellular vesicle biology, opportunities for exploiting this biology, manufacturing challenges in the production of safe and effective exosome therapies, and regulatory considerations for this new class of biological medicines.

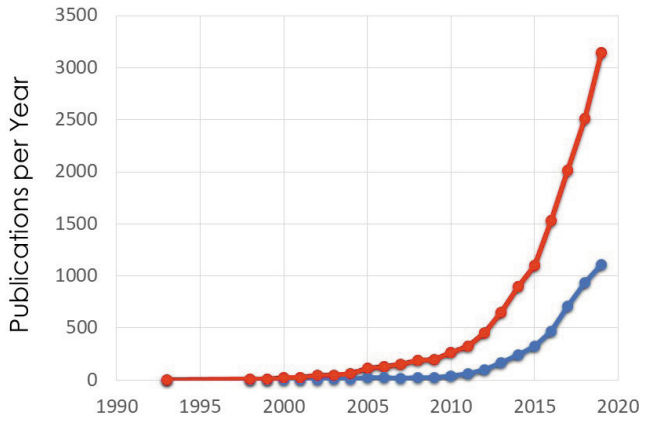


Figure 1. Publications listed in PubMed where the title contains "exosome" (red) or "exosome therapy" (blue). The number of exosome publications per year has risen from 266 in 2010 to 3150 in 2019.

Eukaryotic cells secrete a heterogeneous range of extracellular vesicles, including exosomes. Initially, exosomes were viewed as a form of "cellular waste". It is now clear that their functions go way beyond this. These vesicles of 50–150nm diameter are bound by a lipid bilayer and are laden with a mix of molecular cargo that includes protein, genetic material and lipids. Exosomes bind to recipient cells and release their cargo as a powerful mode of cell-to-cell communication. In doing so, they play important roles in normal physiological processes, in tissue response to injury and in disease processes such as cancer. Exosomes are a vehicle for cells to modify their environment by changing the phenotype of near or distant neighbours.

Exosome biogenesis is a controlled process involving the endosomal sorting complex required for transport. This process results in the incorporation of specific molecular cargo. A number of proteins are commonly found within the cargo or lipid bilayer of exosomes and are considered canonical exosome markers; these include the tumour susceptibility gene 101 protein (TSG101) and the lipid embedded tetraspanin proteins CD9, CD63 and CD81. The most attractive feature of exosomes from a therapeutic perspective is their diverse range of molecular cargo including mRNA, small RNAs (including mi-RNAs), proteins, lipids and peptides[1]. When delivered to cells, this cargo effects change in cell phenotype and function and underpins exosomes' therapeutic potential. Importantly, not all exosomes (as individual entities) contain the same mix or abundance of molecular species. Therefore, in contemplating a therapeutic exosome product, it is preferable to consider cargo from a population of exosomes, as this better reflects what would be manufactured and administered as a therapy. In addition, different cell types isolated from primary human tissues will each secrete exosomes with unique molecular cargo that is fit for its intended purpose.

This uniqueness of molecular cargo from different cell types and tissues is a key consideration for the manufacture of exosomes for therapeutic use. An understanding of the intrinsic properties of populations of exosomes isolated from different cell types and tissues informs how they could be applied therapeutically. Notably, it is widely acknowledged that the efficacy of mesenchymal stem/stromal cell (MSC) therapies for regenerative medicine can be attributed in large part to their secreted exosomes[2]. As a result, many companies developing exosome therapies are using bone marrow or adipose-derived MSC as their preferred cell type for exosome production. Moreover, if MSC-based therapy proves clinically useful, it is likely that exosomes derived from these cells will also be effective in the same clinical settings. As outlined below, the rate of exosome secretion and their molecular cargo can be enhanced or modulated by cell culture conditions. This provides an opportunity to further tune and tailor exosomes for their intended clinical application.

The current status of exosome therapy is reminiscent of the early days of cell-based therapy, where a limited understanding of effective cell dose and mode of delivery made it difficult to predict clinical outcomes, to develop manufacturing processes and to generate sufficient numbers of well characterised cells for clinical trials. However, the manufacturing technology and processes being considered for exosome manufacture are more closely based on those used for the manufacture of antibody and recombinant therapeutic proteins. Therapeutic exosome manufacture involves two linked steps: (a) upstream cell culture to generate the cell-substrate; and (b) downstream exosome concentration and purification. This is followed by a 'fill - finish' step to facilitate storage and transport. For detailed information on the approaches and challenges within the upstream and downstream steps of exosome manufacture, we refer the reader to recent comprehensive reviews by leading academic

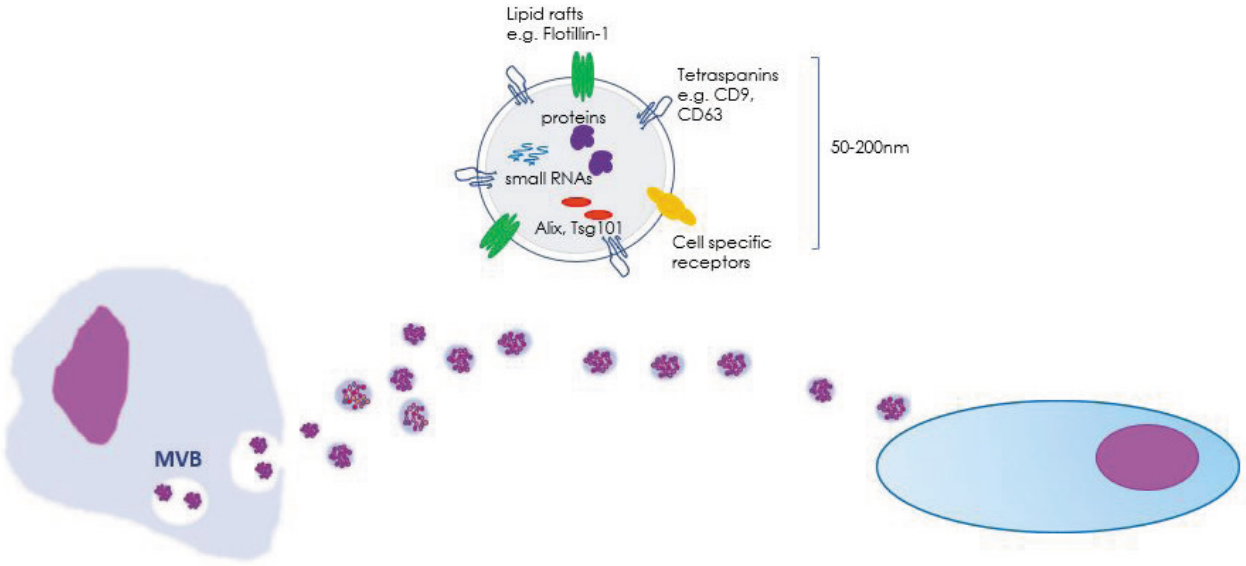


Figure 2. Cell to cell communication and impact on recipient cells as mediated by exosomes and their molecular cargo.

and commercial groups[3,4]. Herein we comment on the key considerations of:

1) upstream processing: cell selection and cell culture,
2) downstream processing: separation and concentration of exosomes,
3) product consistency and safety: in-process controls, exosome analytics,
4) regulatory requirements.

The first key consideration for exosome manufacture, upstream processing, involves selection and culture of cells, at scale, to produce large volumes of cell culture supernatant as a starting substrate for downstream processing. As noted above, cells vary in their ability to produce exosomes and in the molecular cargo contained within their exosomes. Therefore, a prudent cell selection approach would involve screening a diverse range of cell types to identify those that not only have optimal growth properties but also secrete exosomes suitable for the intended clinical application. This requires a suite of analytical tools to quantify and characterise exosomes, annotate their molecular cargo and define their therapeutic potential using potency assays and relevant animal models. As an example of this approach and the associated challenges, we screened many of the MSC types currently used for regenerative medicine and found wide variation in exosome secretion and cargo (unpublished data). None of these MSC types were a suitable starting cell for manufacture of exosomes suitable for our target indication, peripheral arterial disease, where pro-angiogenic factors are likely to be critical for efficacy.

As mentioned above, large volumes of cell culture supernatant need to be produced for exosome therapy to be an effective modality. These volumes may be in the order of hundreds of litres. Fortunately, a number of well-established bioreactor platform technologies and Good Manufacturing Practice- (GMP-) compliant processes have already been used in industrial-scale pharmaceutical processes to produce recombinant proteins, viral vectors and cells for therapy. Optimisation or adaption of these mature technologies is an ideal strategy for exosome manufacture. However, there are several essential aspects of cell culture to consider in the application to exosome manufacture. This includes the cell culture medium: whether it supports long term cell proliferation, high cell viability and ongoing exosome secretion; and whether it contains "contaminating" vesicles and other components that interfere with downstream separation and concentration. Notably, medium supplements such as foetal bovine serum are rich in contaminating vesicles. A number of companies, recognising these issues, now manufacture customised media for exosome production and manufacture.

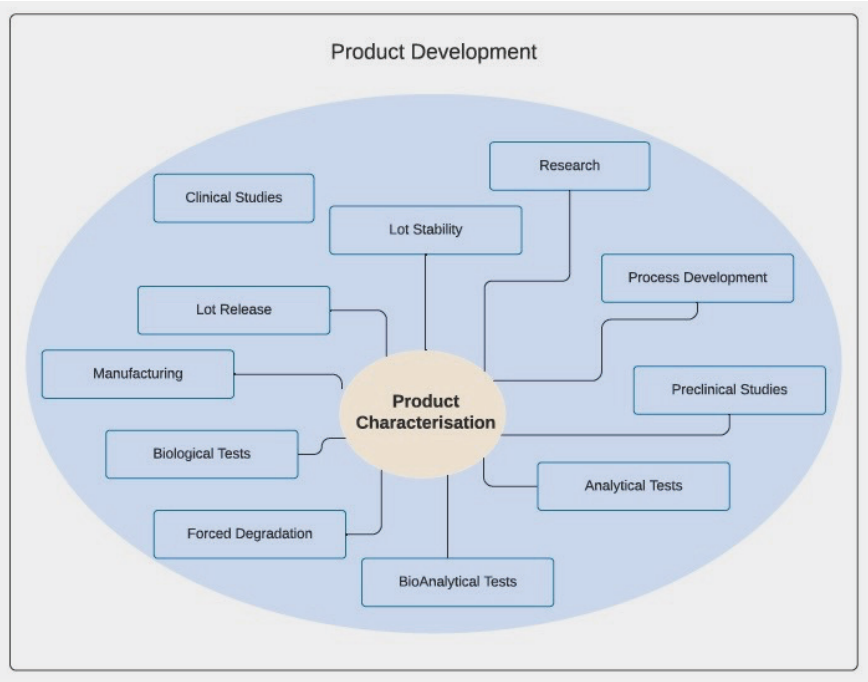


Figure 3. Product characterisation is core to the development of a complex biological product like exosomes.

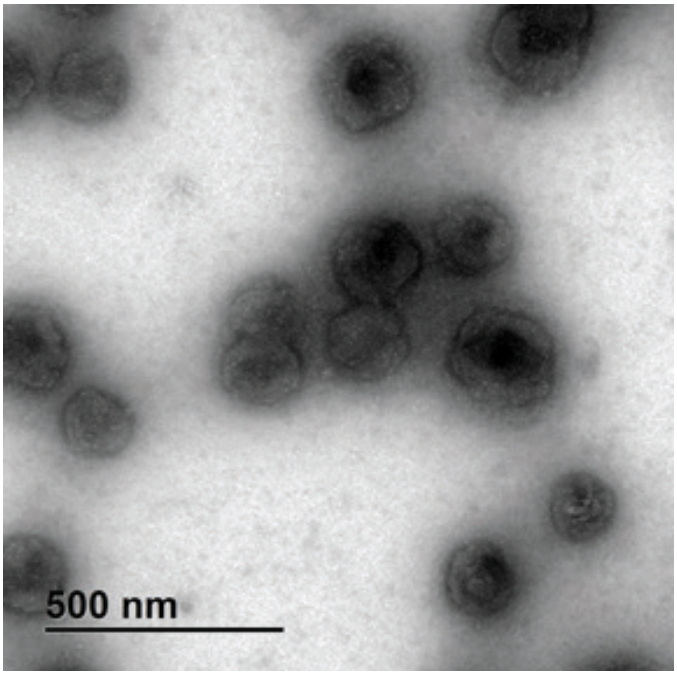
Another important aspect of cell culture is how changes in culture conditions impact on exosome biogenesis. These conditions include pH, oxygen tension, hydrodynamic shear stress, and culture surface topology and chemistry. There is significant opportunity to optimise cell culture conditions to increase exosome secretion and tailor molecular cargo, thereby increasing product yield and clinical efficacy. This underscores an essential aspect of exosome manufacture: the need to assess and monitor the impact of the process on the "product". Upstream processes for cell culture need to be supported by real-time assessment of cell viability, exosome number and exosome characteristics.

The second key consideration for exosome manufacture, the downstream processing, involves separation and concentration of exosomes from cell culture supernatant. This step represents both the major challenge and the major opportunity for the sector. The aim is to yield a highly purified exosome fraction that is free of contaminating biological material which could modulate the biological properties of the exosomes or result in adverse effects. The challenge is to remove proteins, cellular debris, other microvesicles and host cell DNA, while concentrating the exosomes. Although ultracentrifugation and density gradient separation are very useful techniques for the concentration of EVs for research purposes, they are not easily scaled. They can also damage the exosome structure and hence reduce function. As a result, these methods are unsuitable for the manufacture of therapeutic exosomes.

Although there are multiple technologies available for the upstream and downstream steps of exosome manufacture, selection of preferred options should consider the commercial and clinical demands and limit technological and financial risks. Navigation through this maze of considerations is critical for companies. The recent development of a decision support tool and costing model that identifies the cost of consumables, labour and hardware is significant for the sector[5]. Application of this tool identifies large-scale culture and exosome harvesting technologies as the most important considerations in manufacture of sufficient exosomes to meet clinical demand.

The third key consideration facing manufacturers of therapeutic exosomes is ensuring product consistency and safety. This is impossible without a comprehensive toolkit of analytics for both in-process control and final product characterisation. A comprehensive process control strategy is key to achieving process consistency and product quality.

In today's regulated bioprocessing environment, this type of control strategy is known as continuous process verification (CPV). It is the third phase in the Food and Drug Administrations (FDA)'s lifecycle approach to process validation; the first being process design using quality by design principles and the second being process performance qualification. A process control strategy starts with a well characterised product and an understanding of how each product attribute impacts its safety and functionality. While the strategy will evolve throughout product development, it must start early so that the exosomes are sufficiently well characterised with regard to their identity, safety, purity and biological activity prior to a preclinical and first-in-human study. To be effective, a process control strategy requires reliable analytics to evaluate product quality attributes



and extensive online monitoring of process parameters. All aspects of product development are interrelated, with product characterisation being central to understanding of the product. Figure 2 shows the elements necessary for successful product development.

More importantly, a process control strategy requires detailed analysis, acquired through carefully controlled experiments, to understand the impact of process parameters and material attributes on the quality and function of the exosomes. The ideal approach is to incorporate real-time analytics as an integral component of the manufacturing process. However, one of the obstacles exosome manufacturers face is the absence of an integrated control system where real-time product test data allows for detection and adaption to process changes. This highlights the importance of online (ideally) and offline process monitoring assays and data, specific to exosomes, that comprise part of the Chemistry, Manufacturing and Contol (CMC) section of the regulatory submission for product approval, including approval of release criteria.

For complex products like exosomes, it is necessary to evaluate several assays that can measure the product attributes related to the mechanism of action (biological activity). During the product development phase, several assays will be explored with the hope that one or more will be robust enough to be validated as a potency assay for lot release. However, it is more likely that one assay will not be sufficient, and that an assay matrix approach will be required. Assays should include critical measures of process reliability and consistency: the amount (content) of exosomes in the biomass (upstream) and the purified drug substance (downstream), and the identity and amount of miRNA and/or protein species in the cargo. Exosomes, mi-RNA and protein cargos must meet defined release specifications to be suitable for clinical use. Regardless of the purpose and the type, assays used in the manufacture of exosomes for clinical or commercial supply must be well controlled. This control is achieved through the use of standard protocols for sample collection, processing, analytical methodology, and

data analysis/interpretation. Assays must be validated to the standards of ICH and relevant US Pharmacopeia.

To assure product safety, multiple issues need to be addressed, including cell bank qualification and product purity. Cell bank safety testing and characterisation are essential steps toward obtaining a uniform final product with lot-to-lot consistency as well as to demonstrate that cell lines are free from adventitious agents and endogenous viruses. Product purity is critical; contaminants such as host cell DNA, extraneous soluble proteins and viruses will lead to adverse side-effects and increase the risk of immunogenic responses.

The fourth consideration concerns regulation of exosome therapeutics. Although exosomes have been used clinically, no exosome-based therapeutic has been approved by a regulatory agency. For exosomes to reach the clinic, and eventually market, numerous regulatory considerations need to be addressed. The use of allogeneic exosomes requires submission and approval of a new drug application (NDA). Several countries offer accelerated approval pathways, which may be influenced by the indication (e.g., orphan, rare disease, unmet need). Despite the possibilities for accelerated approval, exosome-based therapeutics may be defined differently in different countries. However, as they can be considered a subset of cell therapies, exosomes are likely to be regarded as biologicals. Regulators will also need to be assured that exosome manufacturing processes are controlled (i.e., GMP compliant) and all components used in manufacture appropriately qualified to ensure the biological activity of the exosomes. Here a comprehensive characterisation of the cell source is imperative. In addition, donor eligibility criteria must be carefully selected and applied in accordance with the appropriate ethical and regulatory requirements. Donor screening should include a comprehensive medical record review, physical assessment, medical history interview and screening for infectious disease, in compliance with the appropriate regulatory framework.

Finally, manufacturers need to address measures of exosome product potency. Regulators define potency as the products' specific ability or capacity to affect a given result. With no gold standard technique for quantification of exosome potency, assessment of potency for the intended clinical use helps overcome inconsistent preparations and lot-to-lot variation and guides clinical use. Standardisation of exosome preparations remains a challenge for the field. The International Society for Extracellular Vesicles (ISEV) has produced a set of guidelines (MISEV2018) which ascribe current best practice methodologies and parameters for the accurate reporting of EV experiments[6].

Current and future regulatory requirements will drive developers of exosome-based therapeutic products to incorporate robust quality attributes early in the design phase. This will be instrumental to ensure a focus on patient safety by means of a high degree of process understanding. Early and regular dialogue with the regulators through the development programme is strongly encouraged. Exosome therapeutics are a next-generation therapeutic modality that has the potential to treat a diverse number of diseases. The widespread clinical use of and commercial success of exosomes depends on the development of large-scale GMP-compliant processes to deliver quality products of known composition. This requires the resolution of several technological issues and a holistic approach to manufacturing process control. A cautious and strategic approach between regulators and industry is required to ensure patients are treated with safe and effective exosome products.

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Non-clinical Safety Evaluation of Vaccine: Strategic Considerations to Accelerate Clinical Development

Vaccines are medicinal products intended to elicit an immune response(s) that can prevent (prophylactic vaccine) and / or lessen the severity (therapeutic vaccine) of a given disease. Vaccination involves priming the immune system of a host with an infectious agent or components of an infectious agent, modified in a manner to ensure that the vaccine does not cause any harm or disease to the host, but ensures that when the host is confronted with that infectious agent, its immune system can respond adequately to control the invading organism before it causes any ill effect[1].

Like other non-vaccine pharmaceuticals, the development of vaccine is also a step-wise process comprising preclinical proof of concept, non-clinical development (efficacy, quality, and safety), and clinical development; the data generated in the initial stages guides the strategy for the next stage, until it is proven efficacious and safe in the well conducted clinical trials in the target human population.

Special Features of Vaccines from a Safety Perspective

The decision to approve a drug is taken after careful weighing of the potential risk that the product may pose to the patients and the benefits that the products provide in terms of preventing or treating a disease. Therefore, a drug product may get approval even if it is known to cause some harm, provided that the relief that patient gets will far outweigh the risk, taking into consideration other existing medical options.

With respect to safety evaluation of vaccines, especially preventive vaccines, two important things need to be kept in mind:

- Firstly, the safety bar is very high as it is given to healthy people (at least at the time of vaccination) as opposed to patients, and is given to millions of people in a short time-span.

- Secondly, some sections of the general public still hesitate to get vaccinated, partly due to the misconceived notion about their potential to cause an adverse effect.

Therefore, the safety evaluation strategy, both non-clinical and clinical, should be robust enough to identify the potential risk and to show sufficient evidence of safety.

How do I Plan my Non-clinical Safety Evaluation Strategy?

Potential safety concerns associated with vaccines include general systemic toxicity, (paradoxical) enhancement of the intended disease, induction of local toxicity, pyrogenicity, adverse immunological effects such as autoimmunity or sensitisation, and in some cases teratogenicity / reproductive effects. There cannot be a single strategy that can meet requirements of every type of vaccine under development. With the advancement in science, new types of vaccines are being developed, for example DNA vaccine, mRNA vaccine, vaccines involving recombinant viral vectors and recombinant proteins, etc. To complicate the matter further, various types of adjuvants, antigen combinations, cytokines, complex excipients, etc. are included, and different delivery methods are explored with an intent of developing more efficacious and safer vaccines. Therefore, vaccines represent the most diverse class of product candidates in the pharmaceutical industry. While the basic principles laid out for the safety evaluation of non-vaccine pharmaceuticals apply to vaccines, there are fundamental differences between vaccine and other pharmaceuticals, which necessitates a careful tailor-made strategy that suits the individual type of vaccine being developed. A checklist-based study conduct using standard study design is unlikely to satisfy either the scientific or the regulatory requirements; rather the strategy should be designed, taking into account the type of vaccine being developed, nature of the antigen, type and duration of intended immune response, potential similarity / dissimilarity in the immune response between non-clinical species and human, route and method of administration, qualitative and quantitative composition of vaccine product, including adjuvants, excipients, and potential impurities.

Regulatory Expectations and the Study Requirements

Guidelines with respect to development of different vaccines are available from agencies such as EMA, US FDA, ICH, and WHO (see Table 1). These guidelines provide a general framework for evaluation, and it is not necessary that all studies included in the guidelines would be needed for the candidate vaccine. It is also true that studies / investigations not listed in these guidelines may also be warranted depending on the issues specific to the vaccine being developed. Regulatory agencies also stress more a case-by-case and science-based approach when it comes to identifying the studies needed, their design and timing. Therefore, engaging the concerned regulatory agency in a timely manner, and seeking their feedback and concurrence on the proposed strategy early in the development, avoids delays in the development / approval due to concerns raised by the agencies that may require additional investigations. Fundamental principles and the studies needed for non-clinical safety evaluation of any pharmaceuticals are laid out in ICH M3[2]. Certain concepts described in ICH M3 do apply to vaccines; however, due to the unique features of vaccines, there are considerable differences with respect to type of studies needed and their design, as summarised in Table 2.

Pharmacodynamic End points are Critical to Enhance the Study Value and Acceptance

As the vaccine is inherently designed to act on the immune system, evaluation of effects on immune organs and / or functions are invariably included as part of any toxicological studies. These evaluations are important from a safety assessment perspective.

Guideline	Scope
Notes for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95)	All vaccines
Guidance for Industry. Consideration for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications (CBER, FDA, 1985)	
WHO guidelines on Non-clinical Evaluation of Vaccines (WHO/BS/03.1969)	
WHO guidelines for assuring the quality and non-clinical safety evaluation of DNA vaccines (WHO/2007)	DNA vaccines
WHO guidelines for assuring the quality, safety, and efficacy of DNA vaccines: draft (WHO/DNA/DRAFT/26 July 2019)	
Guidance for Industry Considerations for Plasmid DNA Vaccines for Infectious Disease Indications (CBER, FDA, 2007)	
ICH Document S6: Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (CPMP/ICH/302/95)	All vaccines (and other biologics)
Guideline on Adjuvants in Vaccines (CPMP/VEG/17/03/2004v5/Consultation)	Adjuvanted vaccines
Notes for Guidance on Pharmaceutical and Biological Aspects of Combined Vaccines (CPMP/BWP/477/98)	Combination vaccines
Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99)	Viral vector and DNA vaccines
Points to Consider on Human Somatic Cell Therapy (CPMP/BWP/41450/98)	Cell-based vaccines
Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006)	Gene therapy medicinal products
Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines (EMA/CHMP/VWP/141697/2009)	Live recombinant viral vectored vaccine
Guidance for Industry. Guidance for Human Somatic Cell Therapy and Gene Therapy (CBER, FDA, 1998)	Viral vector and cell-based vaccines
Guidance for Industry. Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications (CBER, FDA, 2010)	Cell substrates, viral seeds, and other biological materials used for viral vaccines
Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology (CBER, FDA, 1985)	Recombinant protein/peptide vaccines
Guidance for Industry. Considerations for Reproductive Toxicity Studies for Preventative Vaccines for Infectious Disease Indications (CBER, FDA, 2000 (draft)	Vaccines for pregnant women and women of child-bearing potential
Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application (EMEA/CPMP/VEG/4717/2003- Rev.1)	Inactivated influenza vaccines
Guideline on influenza vaccines prepared from viruses with the potential to cause a pandemic and intended for use outside of the core dossier context	Pandemic influenza vaccines
Points to consider on the Development of Live Attenuated Influenza Vaccines (CPMP/BWP/2289/01)	Influenza vaccines
Note for Guidance on the Development of Vaccinia Virus Based Vaccines Against Smallpox (CPMP/1100/02)	Smallpox vaccines
Guidance for Industry. Development and Licensure of Vaccines to Prevent COVID-19 (CBER, FDA, 2020)	Vaccines for the prevention of COVID-19

Table 1: Guidelines relevant to safety testing of vaccines

However, evidence of immune responsiveness as a measure of pharmacodynamic effect in a toxicology study is an important entity that enhances the acceptability and value of the study performed. This is especially important when the tested vaccine has not produced any adverse effects, especially on the immune system. This evidence of immune responsiveness is determined by antibody level and time course or other markers of immune activation (e.g.: specific type of T cell activation). The evidences confirm that the chosen species is relevant to the vaccine under development and therefore the effect profile in the non-clinical studies is likely to be relevant and predictive of response in human.

Novel Additive Requires Extensive Toxicological Assessments

The risk of adverse effects with the additives used in the vaccine, including adjuvants, excipients, and preservatives, should be evaluated and their level in the vaccine should be justified. Additives previously used in medicinal products, for which safety has been well established, may not require additional testing. However, the context in which it is used in the candidate vaccine may be different from the previous products. Therefore, a careful evaluation of all available data, either proprietary or publicly available, should be made use of and a decision should be taken whether any new studies are needed. If there are certain data gaps, it is possible to bridge them by including an additional

Type of study	Non-vaccine pharmaceuticals	Vaccines
Acute toxicity	- Generally, the information obtained from repeated dose studies are adequate (stand-alone acute studies may not be needed) - Lethality is not intended	- Not required - No need to establish lethal dose
Repeated dose toxicity	- Two species - Rodent up to 6 months - Non rodent up to 9 months - Dose response evaluation (at least 3 dose levels)	- One species - Duration depends on clinical dosing frequency - Generally, number of clinical doses + 1 additional dose - Dose response evaluation not generally needed
Genotoxicity	- Gene mutation test in bacteria and mammalian cells - Chromosomal damage tests (*in vitro* and *in vivo*)	- Not needed for final vaccine formulation - May be needed for vaccine components (novel adjuvants, additives) - *In vitro* tests for mutations and chromosomal damage should be done prior to first human exposure - Full battery parallel to clinical trial
Reproductive/ developmental studies	- Fertility study (one species) - Embryo-foetal development (two species) - Peri- and post-natal development (one species) - Juvenile toxicity (if needed)	- Fertility study not necessary (histopathology of reproductive organs from general toxicity study is sufficient) - *Prior to exposing pregnant woman or woman of childbearing potential:* - Embryo-foetal development: from implantation until end of pregnancy + additional groups for postnatal evaluation until end of lactation (corresponding to Stage C, D, E of ICH S5A guideline) - Only one species sufficient
Carcinogenicity	- Two-year studies in rat and mice	- Carcinogenicity studies are not required for vaccine antigens or final vaccine product - May be needed for adjuvants/additives, etc.
Safety pharmacology	- *Core battery*: Cardiovascular, central nervous system, and respiratory - *Additiona*l: As needed	- Needed only if data from non-clinical and/or human clinical studies suggest that the vaccine (e.g. one based on specific toxoids) may affect physiological functions - Should be incorporated to general toxicity study
Pharmacokinetics study	- Concentration of active ingredient and/or metabolite in blood and/or other relevant tissues	- Antigen measurement in blood/tissue not needed - Primarily limited to antibody titre (or other markers of immune activation) - Component (e.g.: adjuvant) testing generally not needed - Component testing may be needed for novel adjuvants and to support a different route
Local tolerance	- Site of drug administration should be evaluated in detail - Either as a standalone local tolerance or as part of a general toxicity study	- Similar to non-vaccine pharmaceuticals, more extensive investigations, including evaluation of draining lymph nodes

Reference: [2, 3, 4, 5]

Table 2: Non-clinical safety evaluation of candidate vaccine: Regulatory requirements vis-a-vis non-vaccine pharmaceuticals

Number of species	- Generally, one relevant species
Species selection	- Selected species should be able to elicit an immune response to the antigen - Rodents or rabbit preferred over primates
Route	- Intended clinical route
Dose level	- One full human dose (proposed clinical dose) or at least the dose should exceed human dose on mg/kg basis. Ex: if the proposed dose in human is 0.5 mL, the animal dose should be ideally 0.5 mL
Number of dose groups	- Single dose which gives maximum exposure and max Ab response is sufficient. No need of dose-response data
Dosing interval	- Episodic dosing (usually 2-3 weeks between doses), but should be decided based on antibody response
Dosing frequency	- Similar to proposed clinical dosing frequency + 1 additional dose (n+1 rule)
End points	- Similar to conventional repeated dose toxicity studies (e.g.: OECD 407; FDA red book 2000 guidelines) - Immune end points and local tolerance needs special focus: local inflammatory reactions, and possible effects on the draining lymph nodes, systemic toxicity and on the immune system - Histopathology of immune organs generally front-loaded
Pharmacodynamics	- Quantification of antibody response or markers of immune activation is important to confirm the species selection and validity of the study
Recovery groups	- Yes, generally 2-3 weeks

Table 3: A typical study design for a general toxicity study of a candidate vaccine

Vaccine type	- **Special concerns/requirements**
Nucleic acid- / RNA- / mRNA-based vaccine	- Immunostimulation/inflammatory activation and can have unwanted effects on the host, such as induction of fever or flu-like symptoms and increased expression of autoantigens - Local and systemic inflammation, the biodistribution and persistence of expressed immunogen, stimulation of auto-reactive antibodies and potential toxic effects of any non-native nucleotides and delivery system components - Potential to induce potent type I interferon responses → autoimmunity - Increased endothelial permeability with extracellular naked RNA → oedema - A potential for the extracellular RNA to promote blood coagulation and pathological thrombus formation
Adjuvanted vaccine	- Novel adjuvant: toxicity study with adjuvant alone, or include adjuvant groups as part of toxicity studies with vaccine - Possibility of late granulomas with particles and mineral oils - Hypersensitivity tests may be needed if the adjuvant is immunogenic (e.g. passive cutaneous anaphylaxis, active systemic anaphylaxis assays, IgE measures, and dermal sensitisation potential - Pyrogenicity potential of adjuvant
DNA vaccine	- Assess plasmid biodistribution, persistence (~2-3 months), integration - If integration is expected, the potential for chromosomal instability and tumorigenesis to be addressed - Potential for integration is to be investigated if persistence of plasmid DNA exceeds threshold
Live attenuated vaccines	- Degree of attenuation, stability of attenuated phenotype i.e. reversion to virulence need to be assessed (e.g.: the Cutter incidence) - Potential for genetic exchange with non-vaccine strain - Biodistribution in various tissues
Combination vaccines	- Combined vaccines with known antigen; non-clinical testing may not always be needed; immunogenicity testing may still be necessary - Additional safety studies may be needed if there is concern that combining antigens and/or adjuvants may lead to problems of toxicity (e.g. novel adjuvant)

Reference [6, 7, 8, 9, 10]

Table 4: Special safety concerns associated with different vaccine types

additive-alone group in the toxicity studies that are planned for the vaccine. However, standalone studies investigating genotoxicity, teratogenicity, target organ toxicity, sensitisation, etc. may be warranted depending on the vaccine type and the target population. In such cases, most of the studies recommended in the ICH M3 guideline may be applicable. For adjuvants, in addition to assessing the safety by itself, it is also important to assess whether the combination of antigen and adjuvant exerts a synergistic adverse effect in the animal model, and when species-specific proteins (e.g. cytokines) are used as novel adjuvants, the issue of species-specific response should be considered[8].

The Importance of Lean Study Design

In line with national and international laws, animal usage in research should follow the 3R principles (reduction, replacement, and refinement). When *in vitro* and, to some extent, *in silico* systems can provide the information needed, they should be prioritised over animal studies. When animals are inevitable (as is the case in vaccine toxicity investigations), the following approaches are recommended to be adopted:

Literature review and use of available data: Type and design of the studies should be customised to meet specific scientific objectives in order to generate maximum data with minimum number of animals (use of default study protocols / standard templates are discouraged). A detailed review of all available toxicology / safety data for the vaccine components and the learnings from the similar vaccine type will aid in identifying the potential issues and data gaps with the candidate vaccine. At times, this may help to come up with a study design that involves smaller number of groups or with fewer end points

or shorter duration of administration, yet is able to answer the key safety questions.

Frontloading of toxicity evaluations: The initial non-clinical studies are aimed at determining the activity of the candidate vaccine in relevant animal models. In addition to evaluating immune activation / potency, certain safety end points can also be incorporated without interfering with the primary objective of the study. For example, in-life observation of animals and measurement of body weight, feed consumption, haematology, clinical chemistry, can be easily included as a measure of adverse effect. At sacrifice, critical organs, including immune organs can be collected, and if needed, microscopy can be done to identify any adverse changes even prior to initiating a formal toxicity study.

Integration of multiple end points into toxicology study: In order to save time, money, and animal usage, it is recommended to include multiple end points into a given toxicity study. For example, safety pharmacology end points, evaluation of potential to impair male or female fertility, pharmacodynamic characterisation, etc. can be included as end points in a typical repeated dose toxicity study.

GLP vs Non-GLP

All pivotal toxicology studies should be performed in compliance with principles of Good Laboratory Practices (GLP) (e.g.: 21 CFR Part 58 or OECD principles of GLP, or other appropriate GLP standards). Certain immunological end points, which are part of these pivotal GLP studies, may be unconventional and might have to be evaluated in a lab which is not operating in compliance with formal GLP. Another scenario is that safety information may come from pharmacology studies which are typically performed not in compliance with GLP. In these two scenarios, mere lack of GLP compliance does not disqualify the data, provided the studies were well conducted and documented, and are available for review if requested by the agency.

Can Non-clinical Safety Studies be Bypassed to Fast-track Clinical Development?

In general, the non-clinical studies should support the proposed clinical trial design. For vaccine, the pivotal safety investigations are carried out prior to first-in-human (FIH) trials, especially if the trial designs consist of repeated administration. When there is an urgency to develop a vaccine due to an ongoing medical crisis, as in the case of COVID-19, certain provisions to relax the non-clinical study requirements may be possible. For example, in response to the COVID-19 pandemic, in March 2020 US FDA allowed Moderna Therapeutics to perform FIH testing of its mRNA-based candidate vaccine, mRNA-1273 (within a total of 63 days from sequence selection to first human dosing)[11]. In June 2020, US FDA issued guidelines[12] in which it allowed initiation of human trial without non-clinical toxicity studies in situations where adequate information to characterise product safety may be available from other sources. For example, if the COVID-19 vaccine candidate is made using a platform technology utilised to manufacture a licensed vaccine or other previously studied investigational vaccines and is sufficiently characterised, it may be possible to use toxicology data (e.g. data from repeat dose toxicity studies, biodistribution studies) and clinical data accrued with other products using the same platform to support FIH clinical trials for that COVID-19 vaccine candidate.

In summary, non-clinical programmes should be customised, taking into account various aspects of the investigational vaccine. In general, non-clinical safety studies are abridged when compared to non-vaccine pharmaceuticals; however, novel additives will call for extensive investigations. Among all the effects, potential adverse effects on the immune system and local tolerance are the focus areas of the non-clinical safety programme. The possibility of adopting lean study design should be explored to save time, money, and animal usage. As the issues with each vaccine can vary, early dialogue with the concerned agency will help to avoid the need for additional studies during the later part of clinical development or prior to approval.

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Accelerating Regenerative Medicine Approaches to Type 1 Diabetes Through Direct Cell Reprogramming

Current treatment of type 1 diabetes mellitus (T1DM) depends on the replacement of endogenous insulin by regular subcutaneous injections of exogenous insulin. Unfortunately, exogenous insulin therapy is associated with patient compliance issues and life-threatening hypoglycaemic events. Alternatively, recent convergences of biomaterial and regenerative medicine advances suggest transplantation of stem cell-derived beta cells as an "off-the-shelf" cell therapy treatment approach to T1DM, potentially providing long-term therapeutic benefits to patients, with minimal adverse effects. However, derivation of mature beta cells from stem cells is a lengthy and expensive process requiring multiple cell purification steps. Direct cell reprogramming of source stem cells to target beta cells, guided by analysis of gene regulatory networks using bioinformatic algorithms, offers a potential solution. This article will explore regenerative approaches to resolve T1DM with a focus on how direct cell reprogramming could influence the field.

T1DM is an autoimmune condition that targets insulin-secreting beta cells of the pancreas, resulting in their dysfunction and destruction, and currently affects an estimated 1.4 million adults in the USA[1]. This loss of endogenous insulin underlies the life-threatening state of chronic hyperglycaemia that classically defines diabetes mellitus. The current gold-standard treatment of T1DM is to replace lost endogenous insulin with exogenous insulin administration through regular subcutaneous injections. However, this treatment regime relies on patients regularly monitoring their blood glucose levels and self-administering doses of insulin, which can yield dangerous hypoglycaemic episodes[2]. Cell-based therapies represent an alternative solution, by restoring endogenous insulin production and secretion.

An early iteration of this cell-based approach is cadaveric islet transplantation (Edmonton protocol), which offers proof of principle of the therapeutic effects of restoring endogenous insulin in T1DM through evidence of sustained efficacy in clinical trials[3]. Cadaveric islet transplantation is not currently a viable mainstream T1DM treatment, as healthy cadaveric islet grafts are in short supply, embed variably, and require long-term immunosuppression of recipients. These key limitations of scalability, variable efficacy and immunorejection can be circumvented by deriving islet grafts from expandable sources such as human embryonic stem cell (hESC) and human induced pluripotent stem cell lines, combined with cell encapsulation or *in vivo* beta cell regeneration (Table 1).

Melligen Cells

Human hepatocytes express key molecular elements of the glucose-sensing apparatus present in pancreatic beta cells, including the glucose transporter GLUT2, and the enzyme glucokinase (GCK). By driving ectopic insulin (INS) and GCK expression in a hepatic cell line, HUH7, through transfection, Lawandi *et al.* were able to produce an insulin-secreting cell line, dubbed 'Melligen cells', with physiological glucose-sensing properties. When these hybrid cells were transplanted into mouse models of T1DM, normoglycaemia was restored without inducing bouts of hypoglycaemia[4]. Whilst these preclinical results are promising and support the clinical potential of an expandable line of pseudo-beta cells, there are safety concerns associated with Melligen cells. These cells are derived from a hepatocyte-derived carcinoma, therefore teratoma formation is possible if implanted into humans without encapsulation, and long-term efficacy has yet to be established.

Stem Cell-derived Grafts

Two main strategies exist for deriving functional islet grafts from human pluripotent stem cells (hPSCs). One method involves production of pancreatic endoderm cells (PECs) which are subsequently transplanted into patients. *In vivo*, these PECs differentiate into mature, functional islets. The other strategy involves derivation of functional mature islets from hPSCs *in vitro* prior to transplantation.

Pancreatic Endoderm Cell Grafts

PEC generation from hESCs involves a stepwise differentiation protocol of ~12 days in total, comprised of four short stages (Figure 1A). This protocol mimics the embryonic development of the pancreas *in vivo*, requiring recapitulation of the stem cell niches present at each step of differentiation through media supplementation with specific growth factors and small molecules. Encapsulation of PECs within a biocompatible transplant shields the PECs from immune cell intervention, to allow for subcutaneous transplantation of PECs. In preclinical models, these PECs mature into islets structures with associated endocrine cell types (e.g. beta cells, alpha cells and delta cells), which restore normoglycaemia in diabetic mouse models within 50–70 days, and are maintained long-term (>100 days)[5].

Given that PECs can be generated in a short period from expandable suspension cultures of hESCs, cryopreserved without detrimental effect, and protected from the host immune system, PEC transplantation has great potential as a scalable off-the-shelf cell therapy for T1DM. Despite this, key barriers to PEC use as a mainstream T1DM treatment remain. Firstly, although PECs mature into functional islets *in vivo*, the makeup of these islets is highly variable between individuals, with 50–100% of the grafted cells maturing into endocrine cells[6]. This variability has potential implications for the efficacy of the

Cell therapy	Time to generate graft	Estimated teratoma risk	Estimated production cost	Estimated scalability	Clinical trial status
Melligen cells	Minimal (established cell line)	High (modified cancer cell line) Low (if encapsulated)	Low	Very high	Preclinical
Pancreatic endoderm cells (PECs)	12 days	Moderate Low (if encapsulated)	High	High	Phase 1/2
Stem cell derived islets (SC-islets)	1 month	Moderate Low (if encapsulated)	High	Moderate	Preclinical
In vivo beta cell regeneration	N/A	Unknown (see text)	Low	Very high	N/A

Table 1: Summary of key properties associated with potential T1DM cell therapies.

graft and dose of PECs required to restore normoglycaemia. The non-endocrine contingent could also cause adverse effects, impeding graft viability, and residual non-pancreatic impurities in the PEC graft increase the risk of teratoma formation[8]. Encapsulation can help to minimise these risks by confining the graft to the transplant device and enabling easy removal. Cost is another concern, as although PEC generation is straightforward and quick, the media supplements used to maintain hESCs and generate PECs are extremely expensive[7], and therefore limit the potential of PECs as a scalable diabetes treatment.

Stem Cell-derived Islet Grafts

Generation of terminally differentiated stem cell-derived islets (SC-islets) requires a six-stage protocol of about a month (Figure 1B). The first four stages of the protocol largely overlap with that of PEC generation, with an additional two stages required to produce mature endocrine cells[8,9]. Mature SC-islets can then be encapsulated and administered subcutaneously, enabling off-the-shelf use in a similar manner to PECs.

Although SC-islets require a longer amount of time and greater expense in terms of media supplements to generate than PECs, the extra steps also allow for endocrine and beta cell enrichment of up to 80%, reduce non-endocrine contaminant, and enable SC-islet size determination to avoid large islets, which graft poorly[9,10]. These quality-control steps reduce the variability in SC-islet grafts and create a more defined cell therapy compared with PEC grafts. SC-islet grafts, however, still contain some non-endocrine impurities and other cell types, including up to 5% enterochromaffin cells[9].

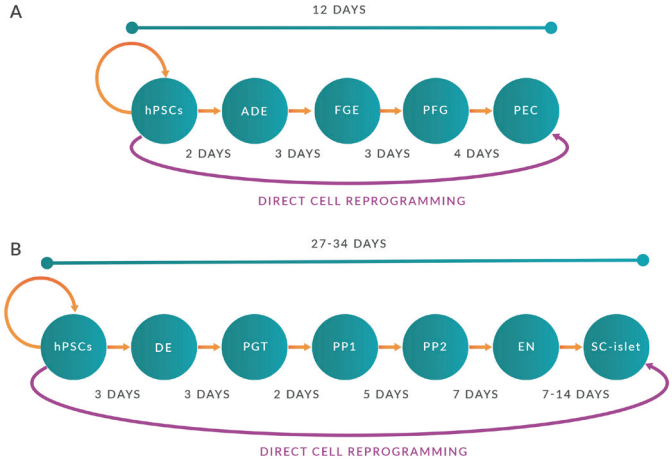

Figure 1: Schematic detailing current protocols for (A) pancreatic endoderm cell (PEC) and (B) stem cell-derived islet (SC-islet) generation from hPSCs. Green lines represent potential single direct cell reprogramming steps. Anterior definitive endoderm (ADE), foregut endoderm (FGE), posterior foregut (PFG), definitive endoderm (DE), primitive gut tube (PGT), pancreatic precursor 1 (PP1), pancreatic precursor (PP2), endocrine progenitors (EN).

An Omics-based Approach to Direct Cell Reprogramming for T1DM Therapy

Predicting Reprogramming Factors

Rather than using trial-and-error approaches to identify key reprogramming factors, the advent of omics data analysis enables the prediction of key reprogramming factors using computer algorithms. These can be used to predict subsets of transcription factors (TFs) required for direct cell reprogramming by identifying genes differentially expressed in source and target (or closely related) cell types. The relative expression levels of target cell-specific TFs can then be individually weighted by their regulatory influence over target cell-specific genes, and ranked accordingly. TFs from these rankings can then be combined to maximize coverage of the target cell transcriptomic network when expressed.[11].

Enhancing *In Vitro* Transplant Production

Currently used protocols for producing transplantable PECs and SC-islets from hPSCs depend on multistage, expensive, and in the case of SC-islets, long differentiation protocols[5,9]. These shortcomings could be mitigated by the development of direct cell reprogramming protocols which accelerate the graft production using a reduced number of steps, or potentially even a single step. Not only would this consolidation of the production protocol enable grafts to be produced at a faster rate, but it would also reduce associated costs by diminishing requirements for expensive media supplements. Furthermore, a direct cell conversion approach may reduce the non-endocrine cell contamination and batch variability currently seen in grafted tissue.

Using an algorithm to establish these novel, TF-driven direct cell-reprogramming protocols for T1DM treatment would require transcriptomic data obtained by RNA-sequencing source and target cell samples. Ideally, this would be carried out using homogenous cell populations to improve the accuracy of the predicted conversion factors. Producing pure beta cell grafts would constitute a more defined cell therapy product than undifferentiated PECs or heterogenous SC-islets, simplifying cell dosing and reducing variability between individual grafts. However, at present, it is unclear whether pure beta cell grafts can function physiologically outside of the islet niche, with clear implications for clinical relevance[12].

***In Vivo* Beta Cell Regeneration**

Direct cell reprogramming strategies could also be employed as an alternative off-the-shelf strategy to treat T1DM. Taking an *in vivo* regenerative approach would avoid the substantial costs associated with generating grafts derived from hPSCs *in vitro*. Additionally, cells converted *in vivo* avoid allogeneic immune rejection, and don't require costly encapsulation like allogeneic *in vitro* grafts. A range of source cell types and tissues, including alpha cells, hepatocytes, gallbladder, and intestinal epithelium, have been successfully converted *in vivo* into insulin-producing (INS+) cells through genetic reprogramming[13]. However, key challenges must be overcome to produce an efficacious, safe and marketable *in vivo* cell therapy for T1DM. These include the selection of an accessible source tissue, achieving targeted transgene delivery within minimal off-target effects, and avoiding autoimmune rejection.

To date, successful attempts at producing INS+ cells by direct cell reprogramming have utilised source cells that are often difficult to access, and are developmentally related to beta cells constitutively expressing key genes associated with beta cell function (e.g. hepatocytes, expressing GLUT2 and GCK)[4,13]. However, most of these INS+ cells do not truly resemble beta cells, and can retain functional expression of source cell genes potentially limiting their therapeutic potential or causing adverse effects. For example, alpha cell-derived INS+ cells can maintain glucagon production, a key alpha cell function absent in canonical beta cells[14]. Taking a big data approach, the potential use of more accessible but uninvestigated source cell types (e.g. adipocytes) for *in vivo* regeneration of canonical beta cells could be examined using algorithms.

Potential transgene delivery systems include integrating lentiviral vectors driving constitutive expression, or an adenovirus or Sendai virus, yielding transient expression[15]. It remains to be explored whether transient conversion factor expression is sufficient to drive beta cell regeneration, or whether constitutive expression is required, and this will determine the delivery system used. Transient expression could also be achieved through small molecule administration to drive direct cell reprogramming, indeed sustained exposure to the neurotransmitter GABA converts alpha cells to beta-like cells *in vivo*[16]. Off-target effects are a major safety concern

with transgene delivery, particularly with integrative vectors, as they could generate tumours. To alleviate these concerns, transient or conditional expression systems could be used to ensure transgenes are expressed only briefly, or specifically in source cells. Furthermore, inducible suicide genes, such as *CASP9*, could be used to selectively kill converted cells if adverse off-target effects are observed post-treatment[17].

Although *in vivo* regeneration of beta cells has the potential to normalise glucose tolerance in T1DM patients, it does not address the underlying autoimmune disease which could target regenerated beta cells and limit their therapeutic capacity. Aside from lifelong co-administration of immunosuppressants, autoimmune rejection could be circumvented by selecting immune-privileged source tissue or through genetic engineering. Candidate immune-privileged source tissues include the gut epithelium. For example, INS+ cells produced by conversion of K-cells (a subtype of gut hormone-secreting cells) were unaffected by autoimmune responses in non-obese diabetic mice[18]. Converted cells could also be rendered hypoimmunogenic by overexpression of immunoregulatory factors such as PD-L1, HLA-G and CD47, and/or targeted knockout of HLA class Ia & II molecules[19,20].

A New Era for Diabetes Treatment?

Over the last couple of decades, significant advances have been made in deriving off-the-shelf functional grafts from hPSCs to restore T1DM in animal models, and in the development of encapsulation devices for minimally invasive administration. Although some of these devices have entered clinical trials, the complex multi-step differentiation protocols used to produce these functional grafts limit scalability and increase costs, thereby limiting the potential for widespread T1DM treatment. Alternatively, beta cell regeneration could take place *in vivo*, avoiding the need for graft production, encapsulation and implantation, but requiring careful selection of an appropriate conversion factor delivery system and source tissue to avoid dangerous off-target effects. Moreover, regenerated beta cells would need to evade autoimmune rejection, perhaps through genetic engineering. Moving forward, direct cell reprogramming, guided by big data approaches, could facilitate and refine development of scalable and efficacious T1DM cell therapies. By combining improvements such as these with the pioneering scientific efforts detailed in this article, regenerative approaches could bring an end to the almost century-long dominance of exogenous insulin as the gold standard of T1DM treatment.

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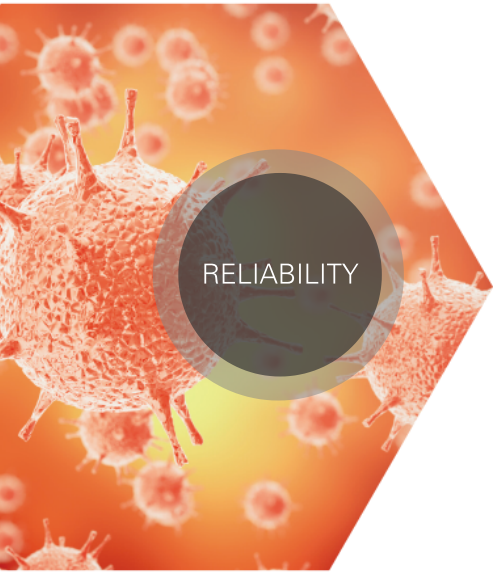

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Analysing Carbohydrates with Ion Chromatography to Develop Better Vaccines

Carbohydrates are key players in the development of successful vaccines against bacteria and viruses. Despite their importance, the most suitable methods to reliably analyse carbohydrates remain largely untapped. Using common analytical approaches, such as liquid chromatography (LC) or gas chromatography (GC), to study smaller carbohydrates requires extensive sample preparation steps with concomitant sources of errors. In vaccine research, where reproducibility and accuracy are of paramount importance, scientists will need to choose the most appropriate technique to examine and characterise carbohydrates that serve as potential vaccine antigens.

Ion chromatography (IC), often overlooked by analytical scientists, provides immense potential in revealing the structure and composition of carbohydrates. It also offers additional information on the resolution of data compared to LC or GC. To keep up with the pressing needs of vaccine requirements during crucial health crises and obtain a thorough understanding of bacterial and viral pathogens, scientists can significantly advance their research by including IC into their workflows.

Here, we discuss how IC can help accelerate vaccine development with robust and sensitive carbohydrate analysis:

Studying Carbohydrates to Develop Better Vaccines

Carbohydrate-based Bacterial Vaccines

Carbohydrates that coat the surfaces of bacterial pathogens serve as potential targets for carbohydrate-based vaccine development. Structurally different from mammalian glycans, these bacterial capsular antigens are isolated from microbial cultures to test for immunogenicity. Bacterial vaccine antigens can sometimes include only the carbohydrate, but are often more effective when conjugated to a carrier protein, forming a glycoconjugate vaccine. When injected, the antigens invoke an immune response, building antibodies against the pathogen. Additionally, adjuvants, such as alum, may also be added to enhance the immune response of patients against a co-administered antigen.

Commercial carbohydrate-based vaccines against *Haemophilus influenzae* type B (Hib) employ the capsular polysaccharide, polyribosyl ribitol-phosphate, made of 5-D-ribitol-(1-1)-β-D-ribose-3-phosphate repeats[1], which was eventually used to develop glycoconjugate vaccines. Multivalent vaccines against *Neisseria meningitidis* infections, targeting multiple serotypes, such as A, C, Y, and W135, also use bacterial capsular polysaccharides. The vaccine against serotype A, for instance, uses a conjugated version of poly-α1,6-N-acetylmannosamine-6-phosphate as its antigen[1].

In the past few decades, well-established vaccines against Hib, *N. meningitidis* as well as *S. pneumonia* have proven to be effective and safe, paving a way for carbohydrate vaccine development against other bacterial and viral pathogens.

Importance of Carbohydrate Analysis in Viral Vaccines

Studying carbohydrates to develop vaccines against viruses involves a more structural and compositional approach. Viruses exploit the host cell machinery to glycosylate their envelope proteins. With mammalian-derived carbohydrates on the viral surface, unlike bacterial vaccines, these sugars can no longer serve as antigens. In viral vaccine development, carbohydrate analysis instead offers insights into the structure, revealing any "exposed" portions of the virus that are potential targets for antibody-based vaccines.

The main target for vaccine development for human immunodeficiency virus (HIV) is a heavily glycosylated viral surface protein: the glycoprotein gp120[2]. Similarly, one of the possible strategies to fight the COVID-19 virus is to destabilise the spike glycoprotein trimerisation[3].

To design promising carbohydrate vaccines with greater efficacy and find unique epitopes against bacterial or viral infections, researchers will need robust, reliable methods to gain maximum knowledge about carbohydrates and identify their structure.

Analysing Carbohydrates in Vaccine Research and Development

Despite being a significant step in vaccine research, carbohydrate analysis hasn't been a major focus in the course curriculum at universities and training institutes. In most technical laboratories, when researchers need to analyse carbohydrates, they often default to using familiar methods such as LC or GC. These analytical techniques, although powerful for several applications, are not always the best choice for carbohydrate analysis.

Carbohydrates are very polar compounds, making it challenging to retain and separate the molecules reliably using high-performance liquid chromatography (HPLC) or GC. Using extreme pH settings to obtain separation can corrode most systems, thereby affecting the results. Additionally, most carbohydrates do not have chromophore groups and, therefore, cannot be detected with adequate sensitivity by an absorbance detector typically employed in LC. Coupling a derivatisation step to allow detection in LC or to make carbohydrates volatile for GC can introduce unwanted errors and additional labour.

Ion chromatography (IC), on the other hand, serves as a suitable technique to analyse the highly polar carbohydrates. At higher pH, carbohydrates are partially ionised and can be separated by anion-exchange mechanisms. This approach, however, cannot be used with LC columns as their stationary phase will dissolve at a high pH with use over time.

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Ion Chromatography:
An Overlooked Method for Carbohydrate Analysis

High-Performance Anion-Exchange (HPAE) chromatography takes advantage of the weakly acidic nature of carbohydrates for highly selective separations at high pH using strong anion-exchange stationary phases. When coupled with pulsed amperometric detection (PAD), it allows direct quantification of non-derivatised carbohydrates at even low-picomole levels with minimal sample preparation and cleanup.

The one feature that differentiates IC from other analytical methods is that it separates and detects carbohydrates without the need for analyte derivatisation. Due to the direct form of analysis, there is no selectivity toward certain carbohydrate structures as may be seen with analytical methods requiring derivatisation, making it simpler to validate the method. These characteristics of IC make it highly suitable for carbohydrate analysis in vaccine research.

Typical steps involved in IC-based carbohydrate analysis during vaccine research:

Structure: IC can be used to study the overall structure of carbohydrates in bacterial or viral strains. During this discovery and characterisation stage, the HPAE-PAD technique helps identify and quantify the types of carbohydrates present, helping researchers determine the range of molecules in the given sample.

Purification and compositional analysis: In developing antibacterial vaccines, the polysaccharides that elicit an immune response are broken down into oligosaccharides. IC is then used to purify and separate the oligosaccharides to perform compositional analysis using HPAE-PAD monosaccharide analysis to identify the carbohydrates present. Isolated bacterial saccharide antigens that trigger an immune response are then chosen as potential candidates.

Stability assays: As the antigenic carbohydrates get conjugated to multiple carrier proteins, IC assays are used to determine the stability of the conjugation in the vaccine vials. These assays ensure that the proteins remain attached during the shelf-life of the vaccine.

QA/QC: Each conjugated carbohydrate often has one unique monosaccharide or repeating polysaccharide unit that can be used to track, monitor, and quantify it during QA and QC assays. For example, in the Hib vaccine, the capsular carbohydrate repeating unit analysed by HPAE-PAD is 5-D-Ribitol-(1-1)-β-D-ribose-3-phosphate[1]. Moreover, IC assays developed during compositional and stability testing can be repurposed for QA/QC testing, saving valuable time required to develop, validate, and optimise methods.

HPAE-PAD was first applied towards vaccine research back in 1992[4] in characterising the carbohydrates for Hib and *S. pneumoniae* vaccines. With the right hydrolysis conditions, the researchers were able to separate sugar alcohols and monosaccharides from each polysaccharide, ultimately using HPAE-PAD to determine the composition, purity and concentration of each polysaccharide. Since then, HPAE-PAD has been applied to several other polysaccharide and glycoconjugate vaccine projects.

Below, we list the versatile capabilities of HPAE-PAD during different stages of vaccine development ranging from initial characterisation to stability testing:

Vaccine	Applications of HPAE-PAD (1)
Hib	- Quantify polysaccharide - Quantify free polysaccharide and conjugated polysaccharide in a conjugate vaccine - Vaccine stability - Assay progress of vaccine preparation - Profile oligosaccharides generated for conjugation - Optimisation of polysaccharide hydrolysis
Meningococcal	- Quantify polysaccharide - Quantify free polysaccharide and conjugated polysaccharide in a conjugate vaccine - Vaccine stability - Profile oligosaccharides generated for conjugation - Optimisation of polysaccharide hydrolysis - Oligosaccharide length for serotypes Y and W135
Pneumococcal	- Quantify polysaccharide - Measure C-polysaccharide contamination - Profile oligosaccharides of serotype 3

Benefits of IC: Sensitive, Rapid and Reliable

HPAE-PAD is a highly selective and specific technique particularly suited for carbohydrate analysis. Pulsed amperometry detects carbohydrates by measuring the electrical current generated by their oxidation at the surface of a gold electrode. It detects only those compounds that contain functional groups oxidisable at that particular voltage. By using appropriate voltage settings, pulsed amperometry enables detection of carbohydrates with higher signal-to-noise ratios, boosting its sensitivity for carbohydrates several orders of magnitude higher than other interfering analytes.

Due to the anion-exchange separation in IC, neutral or cationic components of the sample elute into (or closer to) the void volume of the column. This ensures that, even if these components are oxidisable, they do not usually interfere with the determination of the carbohydrate species of interest.

In addition to these core benefits, IC also brings other advantages to carbohydrate analysis in vaccine development:

Reliability: The selectivity of IC helps determine exactly what carbohydrates are present on the surface of the bacteria or virus. In other analytical methods, the derivatisation step can sometimes result in false positives or false negatives. With no derivatisation steps in IC, these errors are automatically excluded, resulting in highly-reliable data.

Saves time: The direct method of analysis without any steps for derivatisation simplifies sample preparation and saves valuable time for researchers. Moreover, the IC method developed during the research phase to study carbohydrate composition can be reused in QA/QC analysis, relieving scientists from further optimising another protocol. IC workflows are highly compatible with automation to suit future higher throughput needs.

Reproducibility: Minimal manual steps in sample preparation imparts higher reproducibility to the method. Additionally, in the IC setup, parts of the pump head, and the inlet and outlet valves that are in contact with the eluent are made of PEEK (polyether ether ketone) rather than metal. This ensures that even after multiple runs, no metal ions are released into the eluent, maintaining reproducible results. Analytical systems that use metal-based pumps experience merging of analyte peaks

over time as the metal ions strip away from the walls and take up the capacity of the column, interfering with the analytes and their detection.

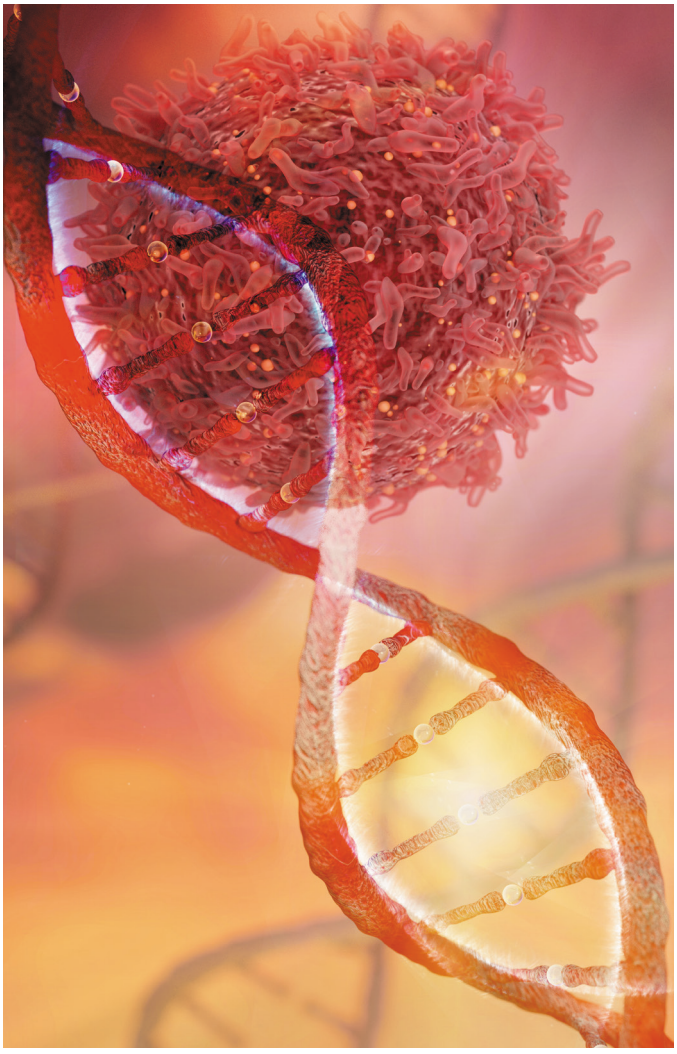
Higher resolution separation: In IC protocols, monosaccharides, phosphorylated sugars and alditols are all identified in the same run without any additional steps or the need to tailor protocols. Using one sample, it's possible to obtain details on all these analytes at once. Conversely, other analytical techniques would require three different derivatisation steps to obtain the same level of information.

Sensitivity: If sample volumes are limited, especially in clinical and early research applications, IC has the sensitivity to determine multiple types of saccharides in a single injection.

Conclusion

The contribution of carbohydrate analysis in vaccine development is undeniable. Cell surface carbohydrates from bacteria, purified and conjugated to carrier proteins, have shown to offer immunogenicity in the case of several infectious diseases. The crux of vaccine development against viruses involves gaining a deeper understanding of its glycoprotein structures and potential binding sites.

As researchers often resort to familiar analytical techniques, such as HPLC and GC, the proven capability of IC to analyse carbohydrates has been neglected. Particularly suited to study highly polar carbohydrates, HPAE-PAD offers a fast-paced, sensitive, and reliable method to identify and quantify sugars in vaccine research, vaccine development, and manufacturing. Within one technique, it offers diverse functionality, such as quantifying carbohydrates, measuring vaccine stability, determining polysaccharide impurities, and beyond.



In addition to characterising carbohydrates on the pathogen surfaces, IC also allows researchers to analyse vaccine formulations, namely determining the purity of the sugars used in formulations as well as validating the quality of raw materials used in production. Employing methods, such as IC, that ensure the quality and consistency of vaccine candidates is especially important during a global health crisis when the research community faces the pressure of rapidly developing an effective vaccine.

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Unlocking the Therapeutic Potential of Antimicrobial Natural Products with Synthetic Biology

Although once a mainstay of drug discovery efforts within the pharmaceutical industry, enthusiasm for the use of natural products as a starting point for the development of new medicines has steadily declined since the early 1990s. As a consequence, many companies have opted to jettison their natural product screening programmes in favour of high-throughput synthesis and combinatorial chemistry, approaches that have ultimately failed to deliver on their early promise. Yet despite their deprioritisation, > 60% of all small molecule drugs in current clinical use can trace their origins back to natural product scaffolds. There is now an increasing realisation that these privileged structures represent the optimal starting point for the development of clinically viable assets. Here, we outline the current state-of-the-art in antimicrobial natural product drug discovery, with a specific focus on how the emerging field of synthetic biology is delivering the tools and technologies required to unlock the therapeutic potential of natural products. We illustrate how these approaches are circumventing many of the problems that have historically plagued conventional screening programmes, enabling the expedient discovery of new molecules with novel functions, and the design and development of therapeutically optimised 'unnatural' natural products.

Introduction

Natural products have been used for therapeutic purposes for millennia. The earliest records date back to Mesopotamia, 2600 B.C., and describe ~ 1000 plant-derived extracts which were used to treat conditions as diverse as parasitic infections, skin disorders and the common cold[1]. The ancient Egyptians and Assyrians chewed on the leaves of willow trees to treat joint pain, and Hippocrates advocated the use of willow leaf extract as an analgesic for childbirth[2]. Efforts to isolate the active ingredients of natural remedies started in earnest in the 1800s, culminating in the discovery of quinine, morphine and salicylic acid; the latter being the active ingredient of willow leaves[3]. Building on these pioneering studies, Bayer successfully developed the antipyretic salicylic acid derivative aspirin, which is still widely used to this day.

Whilst the concept of natural products as a starting point for drug development began to gain traction in the 19th century, it was undoubtedly the discovery of penicillin in the 1920s by Alexander Fleming, and its subsequent manufacture at scale in the 1940s, that ushered in the golden era of natural product drug discovery[4]. The realisation that microorganisms and plants represented a plentiful resource of bioactive molecules with therapeutic potential established a foundation from which the pace of natural product-based drug discovery grew exponentially during the early-to-mid 20th century. This fruitful period served to deliver many of the keystone classes of antibiotics in use today, along with a plethora of allied therapeutic agents. This contrasts starkly with equivalent success rates for antibiotic drug discovery during the past 50 years. Since 1970, only three antibiotics have been developed which are sufficiently chemically differentiated from known molecules to be classified as 'new' assets; the polyketide mupirocin in 1985, the oxazolidinone linezolid in 2000, and the lipopeptide daptomycin in 2003[5].

Interestingly, the degree of representation of natural products and their derivatives amongst successfully realised pharmaceuticals runs counter to the paucity of active research programmes in this area, within the pharmaceutical sector. The late 20th century saw major investments by pharma in high-throughput screening (HTS) platforms, structural biology infrastructure and combinatorial chemistry. The emergence of these methods was coupled with a changing view that natural product-based drug discovery was no longer an economically viable proposition. Screening of natural products was beset by issues of compound rediscovery and the often-intractable issue of developing efficient syntheses for what were often highly structurally complex molecules. Consequently, the time taken to discover, optimise and bring to market a natural product-based drug was deemed to be prohibitively long and expensive, with the focus instead shifting to target-based approaches[6]. This period did, however, see a burst in modifications of natural products, which resulted in second-, third-, fourth- and fifth-generation cephalosporins, for example – but new scaffolds were not being discovered[7].

Regrettably, it is now evident that this decision to transition away from natural products as a starting point for drug discovery has precipitated a decline in the productivity of the pharmaceutical industry, with an emerging view that natural product-based discovery was prematurely jettisoned. This is exemplified in the area of antibiotic discovery, where a failure to deliver new molecules with novel modes of action, in parallel with the emergence of antimicrobial resistance (AMR), is now driving a global healthcare crisis[8]. For compelling commercial and scientific reasons, the reengagement of pharma with natural products is now long overdue. Fortunately, the emerging field of synthetic biology, which seeks to apply the principles and practices of engineering to the design or redesign of biological systems, has in recent years provided researchers with the tools and technologies necessary to circumvent many of the inherent problems associated with the development of medicines from natural molecules. With these game-changing advances, the complexities of natural product hit generation, lead optimisation and scalable manufacture can now be readily addressed, unlocking a myriad of new opportunities. Significantly, these approaches can be readily retrofitted within established drug discovery workflows, minimising disruption and the requirement for infrastructure reconfiguration (Figure 1).

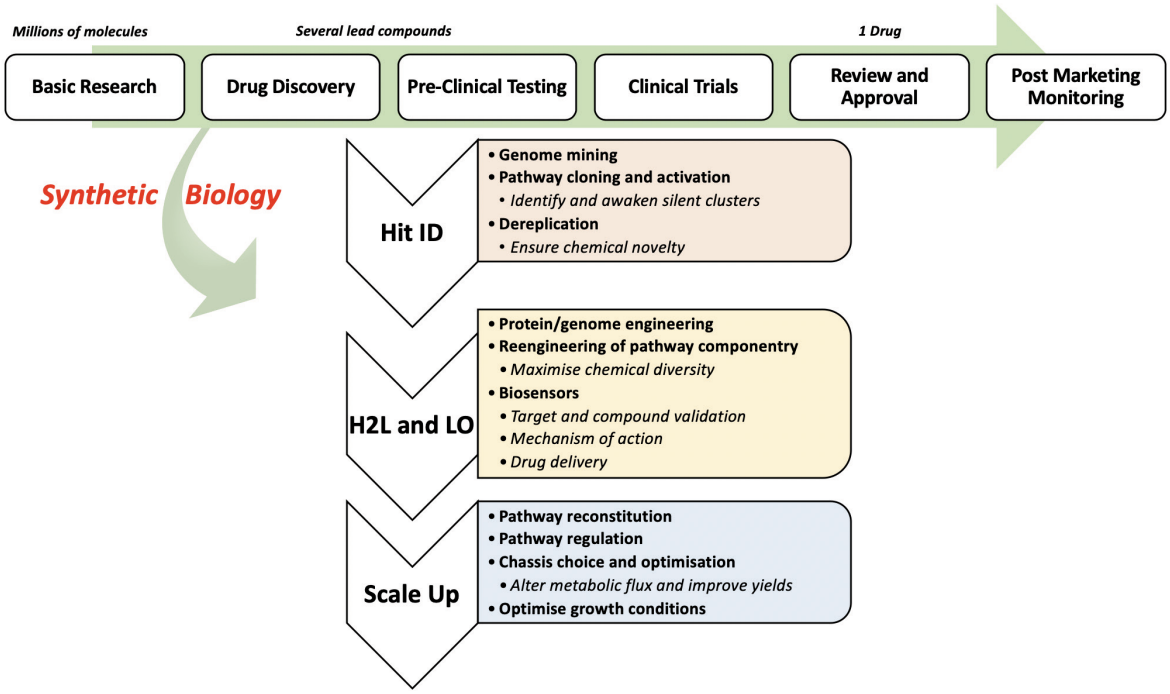

Figure 1. Generalised workflow for natural product drug discovery and development. Aspects of natural product hit identification, hit to lead development, lead optimisation and scale-up which can be expedited using synthetic biology-based methods are identified.

Hit Generation

Classical target-based drug discovery hinges on the identification and validation of a suitable cellular target, which is subsequently subjected to screening, in a high-throughput manner, against proprietary libraries of small molecules. This approach enables the identification of 'hit' compounds, which serve as a starting point for functional enhancement via iterative cycles of medicinal chemistry and binding studies. This approach, by definition, is limited by both library composition and the sensitivity of the assay used and is contingent on an assumption that the observed *in vitro* behaviour can be realised *in vivo*.

In contrast, natural product discovery approaches rely on the identification of bioactive compounds, usually isolated from microbial culture collections or equivalent repositories of plant extracts. Historically, this process has been laborious and expensive, with no guarantee of success. When screening microbial collections, the process is further complicated by the fact that under standard laboratory growth conditions many of the biosynthetic pathways that encode the enzymatic machinery necessary for natural product assembly are inactive, or 'silent', thus significantly reducing the size of the accessible pool of bioactives. Importantly, however, the genes which encode natural product pathways, including those to the four main classes of natural products, polyketides, non-ribosomal peptides, alkaloids and terpenoids[9], are often colocalised into clusters within the producing host's genomic DNA. This subsequently opens up the possibility of 'mining' available genomic sequences for the presence of gene clusters that encode novel biosynthetic pathways, which assemble hitherto unreported chemical scaffolds. The development of next-generation sequencing, and the associated time and cost savings that it brings, has led over the past decade to an explosion in the number and quality of genome sequences available for analysis. This has enabled in silico screening approaches to be developed and applied to the search for novel bioactive compounds using only genomic DNA sequences. This approach circumvents any requirement for wet lab-based screening processes and accounts for all pathways present within a genome, whether expressed under laboratory conditions or not. This method of compound discovery has been greatly aided by the development of reliable genome mining software, e.g. antiSMASH and Pep2Path, which can be deployed to identify all the biosynthetic gene clusters within a target genome and which are also able to make predictions about the likely chemical structure of each pathway product. Consequently, this approach greatly expands the scope of the chemical space available for discovery. The mining of actinobacteria genomes, for example, has revealed that the *Streptomyces coelicolour* (*S. coelicolor*) genome harbours ~10-fold more natural product gene clusters than previously proposed based on the number of isolatable natural products from this bacterium[10].

Once a potential natural product lead compound has been identified *in silico*, it must then be produced within the laboratory in sufficient quantities to enable bioactivity screening to take place. The elaborate chemical scaffolds of natural products frequently present a significant challenge for synthesis. Thus, compound generation is often best achieved via pathway expression in either the native host, where feasible, or more commonly via expression in a heterologous host. Genomic information and prediction of the structure may also be used to adjust growth conditions to access the molecule of interest. Natural products impose a high metabolic cost upon the producing organism, which generally results in low expression levels. Strains of the bacterium *Escherichia coli* (*E. coli*) and the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) have been selectively engineered to overcome this hurdle. The use of these chassis microorganisms, in tandem with innovative gene cloning methods, e.g. transformation associated recombination (TAR) cloning, allows for large stretches of DNA to be easily manipulated and transferred to heterologous hosts. This technique can be used in the cloning of entire clusters from bespoke native producers into well characterised, metabolically optimised surrogates for expression.

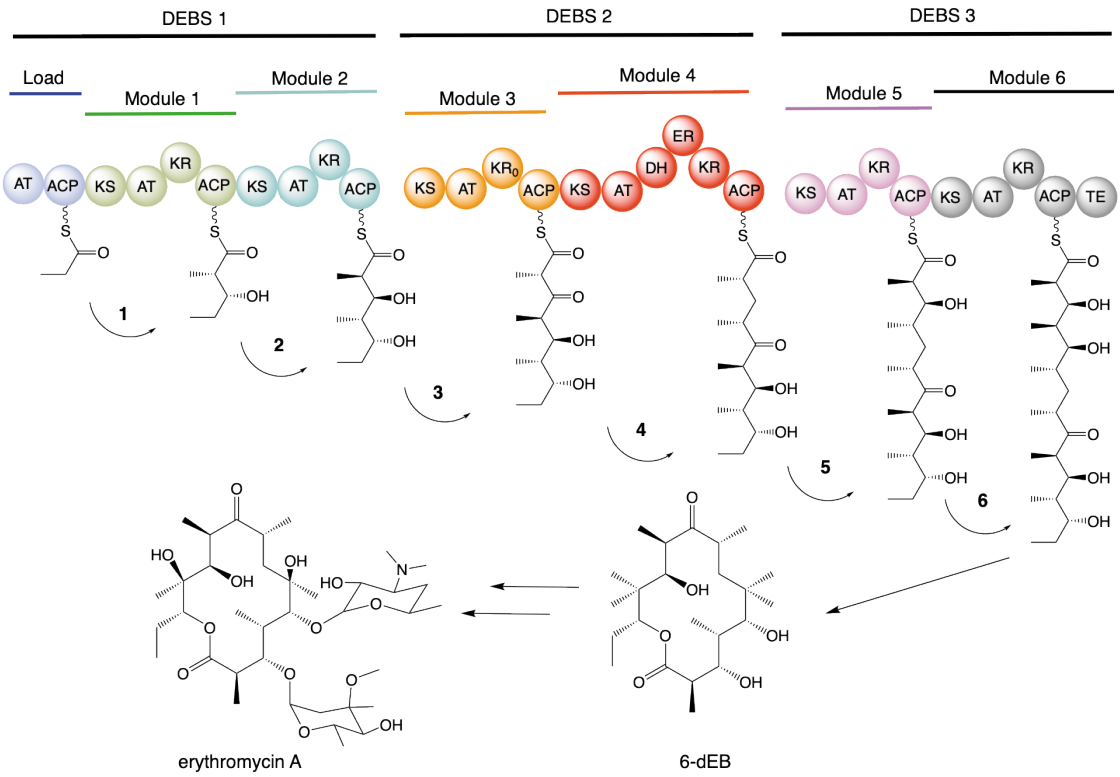

Figure 2. Biosynthesis of 6-deoxyerythronolide B synthase (DEBS) and the biosynthetic route to erythromycin A. Individual synthase domains are organised into discreate modules that catalyse single chain extension events, exemplifying the modular, assembly line-like route to polyketide natural products. Numbered arrows indicate the direction and order of product chain extension and transfer.

Together, the approaches outlined above can be applied to mitigate many of the major bottlenecks in early-stage natural product drug discovery. However, the identification of a hit, and its subsequent isolation and characterisation, is of little value if the compound under investigation has minimal bioactivity, or is a previously reported molecule, or close relative thereof. This issue of chemotype replication in natural product drug discovery is generally considered the primary reason as to why pharma has shifted its focus away from natural products, as dereplication is non-trivial, laborious, and resource-intensive. The development of the antibiotic resistance platform (ARP) by Wright and colleagues has, however, provided a fit-for-purpose tool, which can be used for quick, low-cost antibiotic dereplication, as well as for the discovery of antibiotic adjuvants (inhibitors of resistance). The ARP currently comprises 15 antibiotic resistance genes that have been transformed into *E. coli*. Natural product extracts, or secondary metabolite-producing microorganisms, can be tested against these resistant strains either by agar-overlay or using an agar-plug method. *E. coli* colonies will survive if they house the corresponding resistance gene for the antibiotic produced, allowing rapid, robust identification of the molecules present and prioritisation of antibacterial assets. With respect to adjuvant discovery (antibiotic adjuvants are non-antibiotic compounds that improve antibiotic activity), this system has been used to identify several molecules that enhance the activity of aminoglycosides against the resistance determinant nucleotidyltransferase ANT(2")-la, thus resensitising strains to aminoglycoside antibiotics. This offers a starting point for the rational design of inhibitors to improve the efficacy and longevity of this class of natural product drugs[11]. The ARP platform, along with equivalent dereplication approaches, are of the utmost importance for directing effective antibiotic natural product drug discovery programmes.

Hit to Lead Development and Lead Optimisation

Following hit identification, hit to lead (H2L) development and lead optimisation must take place. These processes are necessary to establish functionally optimised candidate scaffolds that are best suited for clinical use, e.g., increased affinity for their cellular target and reduced off-target effects. Conventional drug discovery approaches employ iterative cycles of medical chemistry coupled to compound testing to achieve this desired outcome. This is time-consuming, expensive and poorly suited to automation. In contrast, a natural product-focused synthetic biology approach employs genetic manipulations of the biosynthetic pathway to a given target compound, such that the resulting engineered pathway assembles functionally optimised unnatural-natural products. This requires an intricate knowledge of the biosynthetic process to the parent compound. The application of this approach is best illustrated using the example of the broad-spectrum polyketide antibiotic erythromycin, a clinically used compound first isolated from the soil bacterium *Saccharopolyspora erythraea*[12]. Similarly to other polyketide natural products, erythromycin is biosynthesised in a stepwise manner, via a series of sequential condensation reactions catalysed by an assembly line-like mega-enzyme complex, termed a type 1 polyketide synthase (PKS; Figure 2)[13,14]. The colinear gene-protein-bioactive compound relationship common to these systems makes them an ideal target for synthetic biology-based combinatorial approaches, as exemplified by the work of Jiang and colleagues in 2013. In this study, the deoxysugar pathways employed for polyketide tailoring were transferred to an *E. coli* host engineered to express the erythromycin PKS. The resulting *E. coli* strain was shown capable of biosynthesising a suite of novel erythromycin analogues with desirable characteristics[15].

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Another elegant example can be seen in studies of daptomycin, a lipopeptide produced by a non-ribosomal peptide synthase (NRPS), which employs an analogous assembly line-like biosynthetic process as that employed by PKSs. The primary differentiator is the use of amino acids as substrates by NRPSs, as opposed to carboxylic acids in PKSs. In this example, a heterologous condensation-adenylation di-domain was fused to the biosynthetic enzyme DptD, with the resulting engineered pathway consequently incorporating an asparagine at the C13 position of the product chain. Interestingly, this optimised lead molecule showed increased antimicrobial efficacy vs. *Staphylococcus aureus in vivo*[16].

Combinatorial biosynthesis can also be employed to generate biosynthetic chimeras, which incorporate enzymatic machinery from different natural product biosynthetic pathways, often originating from different microorganisms. Saponins are large, highly decorated polycyclic structures that comprise one or more glyconemoietes combined with a triterpoene or steroid derivative. They are produced by multiple plant species and exhibit a variety of biological activities. The sapogenin backbone is formed via multiple cytochrome P450 mediated oxidation-reduction reactions. Synthetic biology-based methods have been successfully used to incorporate a non-cognate cytochrome P450 from *Bupleurum* (CYP716Y1) into the sapogenin pathway of an unrelated plant species. Expression of this chimeric pathway in yeast results in the production of a novel non-natural saponin, which is of major industrial value[17].

A similar approach has been applied to enable the site-specific halogenation of natural products. Although more common in biosynthetic processes than previously thought, halogenation reactions are nonetheless considered highly desirable modifications, with the isolation of halogenated natural products from plants and microbes widely considered to be a non-trivial task. Runguphan and colleagues expressed the chlorination biosynthetic machinery from a soil bacterium in the Madagascar periwinkle, which subsequently produced chlorinated alkaloids[18]. This proved the viability of a synthetic biology approach for natural product optimisation in plants, which are generally viewed as less tractable targets for combinatorial biosynthesis.

Whilst the above examples predominantly involve the substitution or augmentation of natural product pathways to alter product chemistry, one may also exploit the inherent promiscuity of a pathway by, for example, the feeding of non-cognate precursor substrates. This approach has been successfully applied to the Rhizoxin PKS from the *Rhizopus* symbiont *Burkholderia rhizoxinica*, which biosynthesises a potent phytotoxin antibiotic. Here, a range of unnatural precursors were synthesised and their biotransformation by a reconstituted Rhizoxin PKS module monitored *in vitro*. Interestingly, the resulting products were shown to resemble the clinically relevant antibiotic cycloheximide[19]. Not only did this study demonstrate the potential of feeding natural precursors to produce new molecules, but it sheds light on the biochemistry underpinning the production of cycloheximide.

Scale-up

The capacity to produce clinical leads at scale is one of the most important elements of any drug development process. Even the most efficacious compounds will not transition to clinical use if they cannot be produced in sufficient quantities. Scale-up therefore represents one of the most significant challenges in natural product drug development. Given the chemical complexities of natural products, they more often than not must be produced via fermentation of a suitable production host. This imparts a significant metabolic burden on the chosen host, which is often intolerable for the natural producer[20]. However, emerging advances in chassis optimisation via genome engineering, along with improvements in cell culturing methods, are now being applied to overcome this challenge.

The primary consideration when developing a natural product fermentation process is the choice of production host. Biosynthetic pathway expression in a heterologous host is often tractable, but it is highly dependent on the compatibility of the pathway gene and consequent polypeptide sequence with the chosen chassis, e.g. codon usage, availability of precursor substrates and chaperones. For these reasons, the optimisation of natural host microorganisms has become an area of major interest.

Ribosome engineering is a well-established method for host optimisation. This technique was originally applied to a strain of *Streptomyces*, a bacterial genus known to harbour numerous silent gene clusters. A mutation in the ribosomal S12 protein resulted in the production of the blue pigment antibiotic actinorhodin. It was subsequently demonstrated that the mutations introduced into the ribosome coding sequence promotes the binding of bacterial alarmone guanosine 5'-diphosphate 3'-diphospahte (pp-Gpp), produced on the ribosome, to RNA polymerase, thus increasing its affinity for promoter regions involved in secondary metabolite production[21]. A vast array of bioactive secondary metabolites have subsequently been produced at scale using this method, including daptomycin, erythromycin and vancomycin. Ribosome engineering has also been used in the discovery of new natural products with antibacterial properties[22].

Another method for increasing natural product titres in host strains is that of metabolic engineering. This approach involves making defined changes to the sequence of a producer's genome, in an effort to direct metabolic flux towards the desired product. Metabolic engineering is a particularly attractive method for yield enhancement in actinobacteria, which are amongst the most prodigious producers of microbial natural products. For example, incorporating metabolite-responsive promoters into the genome of *S. coelicolor* resulted in a 9.1-fold increase in the production of the antibiotic oxytetracycline[23]. Other examples of metabolic engineering efforts in actinobacteria include riboswitches, natural product-specific biosensors for dynamic product regulation, and multiplex site-specific genome engineering (MSGE). This latter approach enables target clusters to be amplified in the natural host and has been used successfully in actinobacteria to overproduce the antibiotic goadsporin 2.3-fold[23].

With respect to heterologous hosts, a number of different cell chassis have been explored. Commonly used examples include *E. coli* and *S. cerevisiae*, which both benefit from fast doubling times, having well characterised genomes and proteomes, and the availability of robust molecular genetic tools which enable

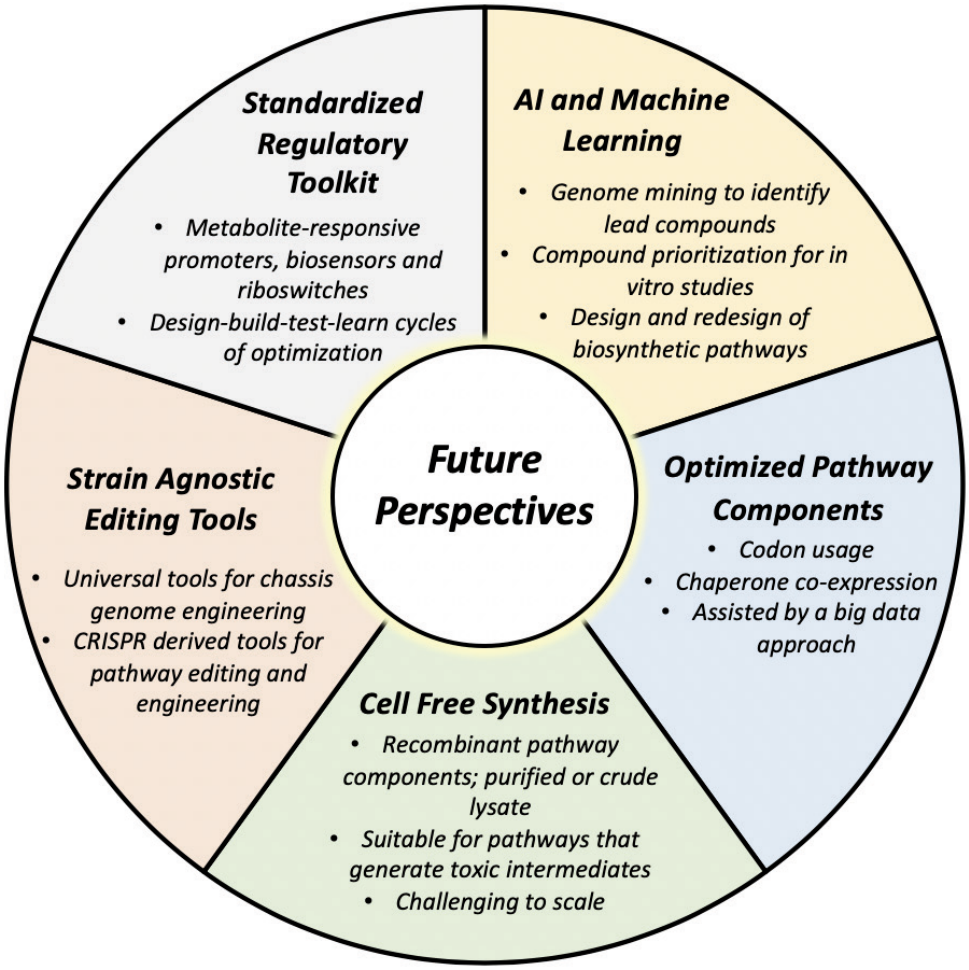

Figure 3. Emerging opportunities for the use of synthetic biology in natural product drug discovery and development. Methods are categorised based on application area and alignment with standard drug discovery workflows.

their manipulation. Interestingly, *E. coli* and *S. cerevisiae* can be deployed in a combinatorial co-culture approach, which has been successfully used to produce oxygenated taxenes at scale. In this example, an *E. coli* host synthesising taxadiene was grown in a co-culture with *S. cerevisiae* expressing enzymes required to perform site-specific oxygenation reactions. This elegantly demonstrates the benefit of distributing metabolic pathways among a microbial consortium[24]. In terms of antibiotic natural product production Streptomyces strains are still considered the gold standard. For example, the repertoire of post-translational modification systems in Streptomyces is more extensive and sophisticated than that of *E. coli*, enabling a ready supply of precursor molecules and cofactors required for polyketide, non-ribosomal peptide and terpene biosynthesis[25].

In addition to chassis choice, one must also consider provision of the requisite enzymatic machinery required to assemble the target product. Databases such as NP.searcher[26] can be used for the prediction of gene clusters during the genome mining phase of development, and ATLAS[27] and RetroPath2.0[28] can be used to design synthetic pathways based on known biochemical reactions. There are also a wealth of transporter databases available that can be used to find a suitable candidate to enable product efflux[25]. A key emerging enabler of these methods is artificial intelligence, which may also be used to predict alternative pathways to target compounds that may be more tractable for scale-up[29]. Protein engineering and directed evolution approaches also offer mechanisms for the enhancement of product titres, e.g. by increasing enzyme specificity for a target substrate, reducing off-target reactions, or for the development of non-natural biosynthetic pathways[30]. Once an optimised biosynthetic route has been formulated and an appropriate chassis selected, pathway reconstitution in the host must be undertaken. Modern DNA assembly methods, either *in vitro*, e.g. Golden Gate assembly, or *in vivo*, e.g. TAR cloning, in combination with CRISPR/Cas9 based methods are now enabling DNA constructs of > 1.5 MB to be routinely successfully reconstituted[31]. In tandem, the development of dCas9 (deactivated Cas9) and CRISPRi (interference CRISPR) may be used to achieve regulatory control over reconstituted pathways in a manner that is inherently more tuneable than was previously possible[25].

Remaining Challenges and Future Prospects

The past decade has seen major advances in our fundamental understanding of natural product biosynthesis. These insights, coupled with the tools and technologies of synthetic biology, are now driving a resurgence of interest in the use of natural products as a starting point for drug discovery efforts. Figure 3 highlights the major areas where emerging synthetic biology tools could impact drug discovery processes. Interestingly, pharma's deprioritisation of natural scaffolds means that they are now poorly positioned to retransition into this area, with the most innovative work in this field now being undertaken

in academia, or by emerging biotech small to medum-sized enterprises (SMEs).

With respect to compound discovery, genome mining is now enabling the identification of new biosynthetic pathways and the prediction of their corresponding natural products at a rate once considered improbable. The issue is no longer one of target identification, but rather one of target prioritisation. Here, artificial intelligence appears set to make major contributions, enabling autonomous screening of genome databases and the application of predictive tools that can rank candidate pathways and associated metabolites based on chemical novelty and drug-like properties. Similarly, our capacity to selectively manipulate biosynthetic pathways, enabling access to bespoke non-natural natural products, is advancing rapidly. The promise of combinatorial biosynthesis is being realised, with effective tools for pathway redesign and optimisation now readily accessible.

Despite these advances, issues still exist. The development of fit-for-purpose chassis organisms remains a major obstacle to success, with future efforts undoubtedly focusing on the establishment of general-purpose heterologous hosts which can be employed for compound manufacture agnostic of pathway identity and/or native producer. Ultimately, this may necessitate the development of cell-free manufacturing processes, but such systems are still very much in the development phase, particularly for compound manufacture at scale[32]. Improved genetic manipulation tools are also a priority, specifically those which can be applied in a strain independent fashion. Undoubtedly, these will leverage recent game-changing progress in the development of the CRISPR-transposon system[33].

Without question, the next decade will witness the re-emergence of natural products as a favoured starting point for drug discovery. This will be most keenly felt in the area of antibiotic development, where the move away from natural products in the 1990s has resulted in a catastrophic decline in the rate of asset discovery and development. Future natural product drug discovery workflows will be less dependent on physical infrastructure and access to extensive compound and strain collections and will instead be founded on *in silico*-led distributed development programmes, which are inherently nimbler and can be pursued with significantly lower operating costs. When it comes to natural products drug discovery, the model very much is back to the future.

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Conflicts of Interest
The authors declare no conflicts of interest.

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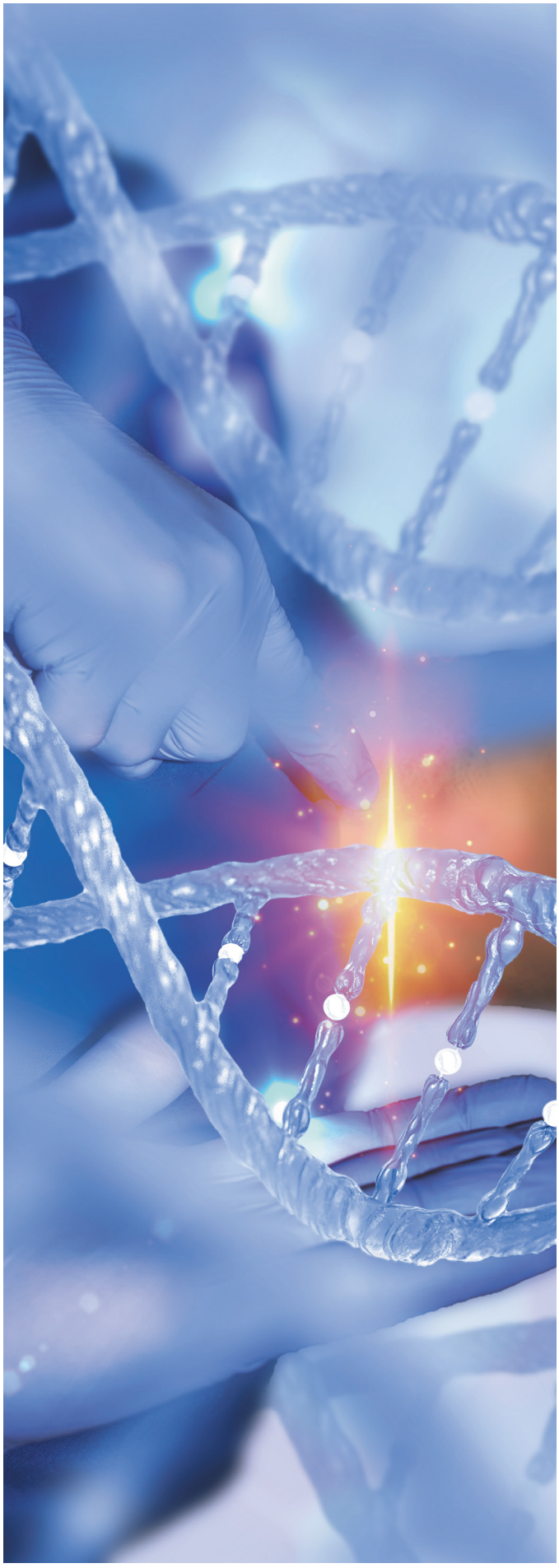

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Faster Drug Discovery with Picodroplet Technologies

Automated platforms based on picodroplet microfluidic technologies are one of the most promising tools for improving drug discovery efficiency. By encapsulating single cells in miniaturised, aqueous picolitre compartments, called picodroplets, these technologies provide a high-throughput and sensitive method to identify high-affinity, high-potency drug candidates with better biotherapeutic profiles and faster developability timelines. Automated, picodroplet-based workflows can help to streamline the drug discovery process, increase throughput, and reduce time and operational costs.

Challenges in Drug Discovery

As the demand for new therapeutics surges, the acceleration and optimisation of drug discovery processes have never been more crucial. However, the discovery process remains complex, time-consuming, and inefficient, increasing timelines and development costs.

This efficiency problem is attributed to various causes, one of which is the resource- and labour-intensive nature of screening large cell populations for rare antigen-specific, antibody-secreting cells during drug discovery. Traditional hybridoma-based strategies involve laborious screening efforts that create major bottlenecks in finding lead candidates for progression to antibody optimisation and clinical candidate selection (Figure 1).

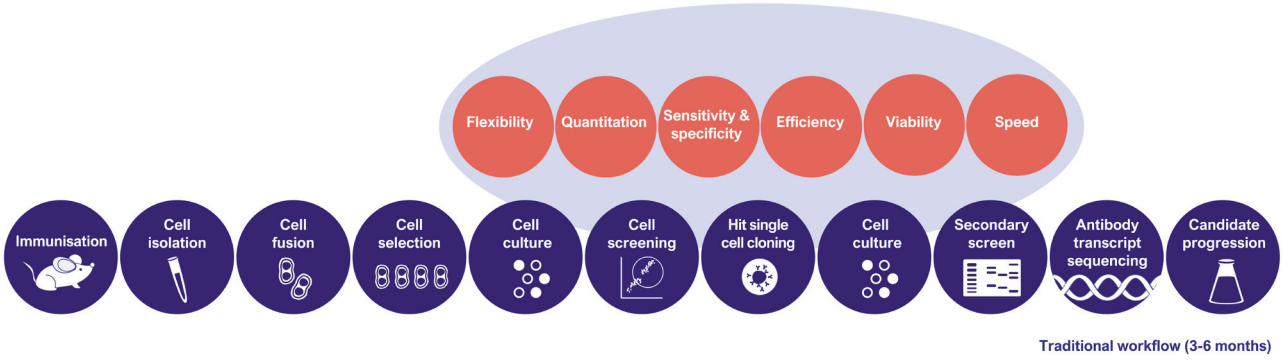

Figure 1. Traditional workflow in hybridoma screening

Advances in automated, high-throughput screening technologies that enable the screening of millions of antibodies to identify new drug candidates can partially overcome the problem. One typical method involves screening the purified B cells directly using flow cytometry, bypassing traditional hybridoma fusion and phage display approaches.

Flow cytometry has the advantage of being very high throughput, and antibodies secreted by B cells can potentially be screened using cold capture, a technique used to prevent the full secretion of antibodies by trapping them at the cell surface. However, this technique produces a representation rather than a direct measurement of the antibody secretion profile by a single cell. There are several other limitations to this screening method, including altered cell function and reduced cell viability. Alternative screening methods include ELISA and Elispot; however, these techniques often need to be executed manually. Consequently, it becomes too costly and time-consuming to analyse large populations[1].

After multiple rounds of screening and selection, the positive cells must then be sub-cloned into monoclonal populations (lead panels) by employing semi-automated methods like cell-in-well imagers and cell sorting; this multi-step approach adds even more complexity and hands on-time, slowing down the discovery process even more[2].

Integrated Drug Discovery Platforms

To remove common bottlenecks and find rare variants faster, biopharmaceutical companies are now looking to picodroplet microfluidics. Picodroplet microfluidic technologies conduct complex multi-step assays with high reliability, cost-efficiency, and throughput in a picolitre-sized aqueous droplet (picodroplet) format. Using this approach, individual cells, or multiple cells in pools, are encapsulated in the picodroplets for high-throughput screening (Figure 2). Picodroplets act as a bioreactor to compartmentalise cells and facilitate growth, eventually trapping secreted molecules such as antibodies, making them easily accessible for characterisation[3].

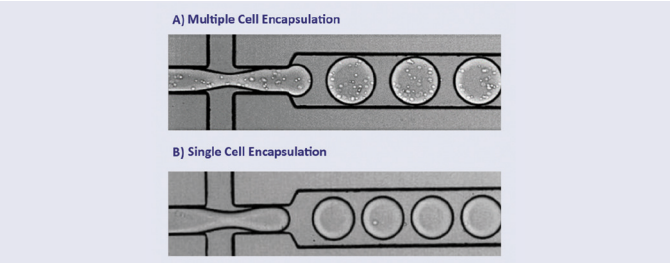

Figure 2. These images show the encapsulation of multiple cells or single cells per picodroplet. A) A large population of cells (>1 million) diluted to a concentration of $1x10^8$ cells/mL in medium resulting in multiple cells per picodroplet. B) Cells diluted to a concentration of $1x10^6$ cells/mL to obtain a population of picodroplets containing single cells.

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Emerging fully integrated picodroplet systems offer a unique opportunity to improve the antibody drug process and increase the number of targets that generate biologics. These technologies not only simplify the screening of one to tens of millions of encapsulated cells and their products, but combine the subsequent selective sorting, cell isolation, imaging, and single-cell dispensing stages into an automated platform.

Compared to conventional systems, automated picodroplet systems offer significant advantages in high-throughput single-cell screening, rapid-yet-gentle cell processing, and high-sensitivity quantitative assays. These capabilities facilitate high-throughput research to interrogate larger repertoires and find more functional properties in just days[4]. For example, using a fully integrated platform, researchers can analyse up to 40 million cells (B cells) in two days with each picodroplet containing ~30 cells in the first of a two-run protocol). These systems can also be used to analyse up to 200,000 single cells (B cells or hybridomas) for antigen-specific antibody-secreting cells, isolate high-potency candidates of interest, and directly dispense single cells into individual wells of a microtitre plate, in a single day.

As a result of these advancements, researchers can now 'mine' for the rarest cells that naturally occur in a heterogeneous population to isolate the most valuable antibodies with the greatest antigen-binding affinity and specificity. A process that, when following a traditional, multi-step discovery workflow, can take several weeks (Figure 3).

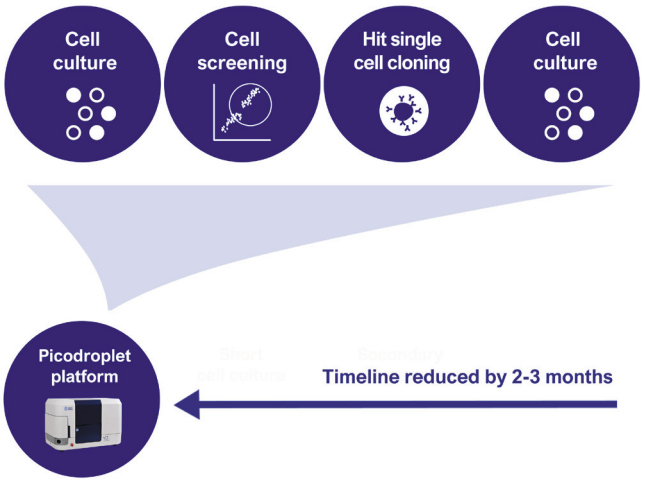

Figure 3. High-throughput screening workflow

Importantly, these platforms maintain the cells in a highly viable state throughout the discovery process, as picodroplets provide a uniquely protective environment to support cell integrity during incubation, shielding cells against shear stress as they flow through the microfluidic channels.

Additionally, the miniaturised format requires much smaller sample volumes, allowing the concentration of the molecules secreted by the cell to accumulate quickly. This provides a more sensitive and accurate measurement of antibody secretion levels to help find rare antibodies with desirable characteristics at a dramatically reduced cost per test.

An Automated, Picodroplet-based Workflow

Fully integrated picodroplet systems consist of five stages; cell isolation, assay, sorting, imaging and dispensing. Streamlined workflows enable researchers to get a complete run-through in a day, starting from the cell sample and ending up with picodroplets dispensed into the wells of a microtitre plate, significantly reducing timelines for the discovery and development of antibody-based therapeutics (Figure 4)[5].

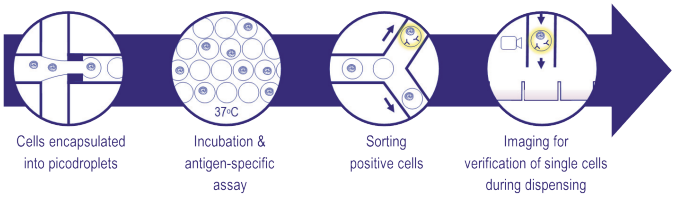

Figure 4. Integration of antigen-specific screening, sorting, imaging and dispensing using a fully automated microfluidic process.

By streamlining the whole discovery workflow into one, easy-to-use instrument, biopharmaceutical companies can remove much of the complexity of the process and critically, switching to a picodroplet-based technology requires very little additional resources, training time, and maintenance.

The stages of an automated, picodroplet-based workflow typically include:

1) Cells encapsulated into picodroplets: The target cell population is prepared in a preferred culture medium and supplemented with an appropriate animal-origin-free antibody-based detection reagent for the selected secretion assay. The cell suspension is then gently processed through microfluidic channels and mixed with an oil containing a biocompatible surfactant, which ensures stable picodroplet formation and encapsulates a single cell (or pools of cells) in each picodroplet.
2) Incubation and secreted protein assay: Approximately two million picodroplets are collected and incubated *in situ* at 37°C to activate cell metabolism and allow the assay signal to develop. Assays may include antigen-specific assays for hybridoma screening or B-cell mining, but this assay format can be adapted and tailored to many different antigen targets.
3) Sorting positive cells: The picodroplets are sorted by fluorescence detection and gating, with positive 'hits' being actively channelled for collection. The population of cells selected for collection can be defined and adjusted according to each specific experiment.
4) Visual verification and dispensing: After completion of the sorting phase, positive picodroplets are selected, imaged, and dispensed to individual wells of a 96- or 384-well microplate prefilled with preferred culture medium.

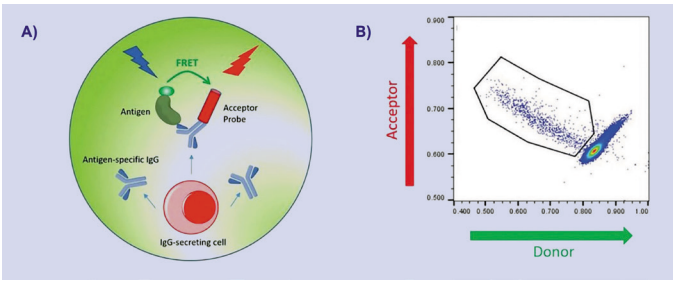

Figure 5. A picodroplet-based antigen-specific assay. (A) Antigen-specific IgG secreted from the encapsulated cell is recognised by the donor conjugated antigen and by the acceptor-conjugated IgG-specific probe. (B) Scatterplot of FRET signal generated from hybridomas screened for secretion of anti-human TNF α IgG.

An example of an antibody discovery experiment which has used the described workflow is included in Figure 5. Josephides *et al.*[3] used an automated, picodroplet-based workflow for the high-throughput screening and selection of antigen-specific clones generated from a mouse immunised with human tumour necrosis factor-α (TNFα). A population of hybridoma cells was analysed with validated detection probes to find TNFα-specific, IgG producing clones (Figure 5A). A subpopulation of cells with a high acceptor-to-donor fluorescence ratio, indicating secretion of human TNFα-specific IgG, was then gated for collection and further analysis, while the remaining picodroplets were diverted to waste (Figure 5B).

The Bottom Line

Researchers can now automate the antibody drug discovery workflow to perform studies with higher sensitivity and speed than conventional systems. High-throughput capabilities enable the screening of hundreds of thousands of individual cells or up to 40 million cells (in pools) to rapidly identify antibody-secreting cells and isolate rare cells secreting antigen-specific antibodies. This enables the discovery of optimal drug candidates from an entire cell library, while ensuring good viability of the cells throughout the process. Overall, picodroplet microfluidic technology presents a compelling opportunity to streamline labour-intensive and inefficient drug discovery, leading to lower operational costs and reduced time to market. In doing so, automated, picodroplet-based technologies address the major challenges faced in the discovery workflow, which are:

- Flexibility: offers adaptable assay design for specific needs
- Measurement: provides quantitative assays of antibody secretion
- Sensitivity and specificity: detects antibodies of interest
- Efficiency: screens the entire cell population at high throughput
- Viability: maintains high levels of cell viability
- Speed: reduces total drug discovery workflow timelines

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Ensuring Reproducibility in Biomedical Research – The Role of Data, Metadata, and Emerging Best Practices

> **"Science is one of the very few human activities – perhaps the only one – in which errors are systematically criticised and fairly often, in time, corrected."**
>
> **Karl Popper**

Facing the Reproducibility Crisis

Science is built upon continuous cycles of generating hypotheses, testing them, and refining the models based on these findings. Over many years of research, scientists collaboratively build new models to explain the world around us. To advance our understanding, and move ever deeper, it is of critical importance to base new theories, models, and explanations on solid data. These foundations are tested by other scientists independently, thereby verifying each others' results. This process of repeatedly reproducing new findings is at the core of science. Yet, a growing body of evidence suggests that we cannot take the reproducibility of published scientific findings for granted any longer.[1] In a survey published by *Nature* in 2016, among the 1576 researchers surveyed, 52% agreed that there is a significant crisis of reproducibility in science.[2]

This problem is particularly troubling in the life sciences, given that basic biomedical research is the cornerstone of drug discovery, and thus has direct implications for human health. Hence, the reproducibility requirements in this space should be among the strictest. However, studies conducted by leading meta-research experts, biotech companies and pharmaceutical corporations found the prevalence of irreproducible pre-clinical research to exceed 50% – by some estimates even 80%[3,4] – causing waste in excess of $28 billion[5] every year. If the 'self-correcting' nature of science is to be preserved, stakeholders in the scientific community must take urgent and decisive action to tackle the reproducibility crisis and ensure we safeguard our ability to make real scientific progress.

When looking at the underlying reasons for lack of reproducibility, many factors have been discussed. Most experts agree that shortcomings in reporting, differences in assay execution, as well as issues with the identity and quality of research reagents are central.[6,7,8] In this context, it is important to stress that not all results of irreproducible research are necessarily false. There are cases where the information needed to replicate a study is either absent or inscrutable. When the biotechnology company Amgen tried to replicate fifty-three papers deemed as 'landmark' studies, they successfully replicated only six of them. Interestingly, in these six studies, the authors had paid close attention to controls, reagents, and provided a detailed description of the complete data set.[9] However, even when details of experimental execution are provided, the results data often remain elusive due to lack of access, correct formatting, clear terminology, or contextual information. Presently, there are no universally accepted guidelines that govern the formatting, storing, and contextualisation of research data. However, without reliable and transparent data reporting, in scientific reproducibility will be hard to achieve.

Turning a Crisis into an Opportunity

Yet, concerns regarding reproducibility can also be seen as an opportunity to make science itself better. Consistent with their self-correcting norm, scientists are actively addressing the alarming rise in irreproducible findings, and stakeholders in the scientific community are taking concrete actions to remedy this situation. It is clear that implementing more careful stewardship of data is one important step towards alleviating the reproducibility crisis. Scientific societies and publishers are therefore pushing to increase experimental rigour and reporting transparency. For instance, the Federation of American Societies for Experimental Biology (FASEB) issued a set of recommendations to enhance the reproducibility and transparency of research.[10] The Center for Open Science brought together publishers, funders, and societies to create the TOP guidelines providing a set of transparency standards for journals.[11] The UK Reproducibility Network functions as a grassroots initiative of scientists on the ground, and a coordinator for institutional actors that commit to best practices for reproducible research.[12]

These, among other efforts, highlight the willingness of the community to address the widespread lack of reproducibility in biomedical research. Emphasis on rigorous reporting and full transparency is a step in the right direction, yet enforcing them will place additional pressure on individual scientists. Many approaches have been implemented to improve the handling of scientific data. For instance, *Nature* has initiated an 18-point checklist for authors "to ensure that all technical and statistical information that is crucial to an experiment's reproducibility or that might introduce bias is published". A particularly ambitious approach towards improving reproducibility by proper data stewardship is embodied in the FAIR Guiding Principles for scientific data management.[13] Based on four foundational principles – findability, accessibility, interoperability, and reusability – FAIR provides a set of concise and measurable principles to enable the reuse of research data. Furthermore, FAIR principles place specific emphasis on enhancing the ability of machines to automatically find and use the data. FAIR principles will not only help researchers get the most value from their data, but have become mandatory requirements by major funding bodies such as the European Commission. In this context, it becomes clear that the process of FAIRification must start from the moment data is being generated, and cannot only be an afterthought once data has been already collected.

Beyond Data: Metadata

We argue that calling for appropriate data management is just the first step. The notion of what constitutes 'raw data' varies from researcher to researcher and this ambiguity can lead to complications. Indeed, researchers tend to share with

their peers only the data and procedures they deem critical to reproduce their results. However, most experiments involve a plethora of seemingly minor steps which are often omitted from the records but have, nonetheless, a measurable impact on the results. This can be a problem even within a single research group. In the aforementioned *Nature* survey, more than half of respondents had trouble reproducing their own results, presumably due to the lack of details in their internal records.

The importance of recording the 'data beyond data' – metadata – was one of the key takeaways of a three-year controlled trial for reproducibility undertaken by DARPA, the US Defense Advanced Research Projects Agency. Their Biological Technologies Office ran a pilot where two teams were funded for each project: one for research and one for independent validation and verification (IV&V).[14] With this effort, DARPA wished to encourage the adoption of some of the best practices from the engineering community into the life sciences. Many of the reported difficulties and delays in this pilot could be attributed to a lack of reporting of environmental data, reagents, and execution-related details.[15] For instance, it took the participating teams more than a year to discover that a mismatch in the results occured from mistakenly assuming that commonly used reagents from different vendors could be used interchangeably. In another example, investigators noted that failing to account for the flow rates used when washing cells from culture dishes could lead to discrepancies as well. These observations highlight the importance of keeping complete records not only of 'data' in the conventional sense, but also of the tools, conditions, and workflows that led to that data in the first place. Duly recording and sharing these metadata is essential in our efforts to enhance the reproducibility of biomedical research. This is especially important in the era of artificial intelligence, where algorithms play an increasingly important role in all aspects of research and data handling.

Programmes such as DARPA's IV&V are compelling but can most likely only be implemented by institutions with the flexibility and vast resources of DARPA. Tackling the reproducibility crisis requires approaches that avoid creating extra challenges on already overburdened scientists or demand vast amounts of resources. Along with the efforts to improve the quality of research, new tools to lower the barriers for the adoption of reproducibility practices must be developed.

Applying Engineering Practices to the Wet Lab

DARPA's pilot on reproducibility aimed to introduce IV&V practices commonly used in electronics and software design to the wet lab, but implementing these practices in the biomedical setting is far from trivial. The variability of biological systems and experimental protocols makes IV&V efforts more challenging than in software and electronics where the building blocks and processes are standardised and, to a great extent, predictable. Thus, the widespread adoption of IV&V programmes in biology will require a significant reduction of 'experimental entropy'. And while biological processes are expected to remain less predictable than their electronic counterparts, fully automated workflows are the most consistent way to control for external variables. Enhanced control in execution and (meta)data recording brings wet lab work closer to engineering methods and opens the door to the introduction of the best practices of that discipline. This is often referred to as Biology 2.0.[16,17]

Figure 1 shows the robotic laboratory operated by Arctoris Ltd in Oxford, UK.

Recent advances in laboratory automation can make this possible, and thereby help foster reproducibility in the life sciences. Automated workflows promote the adoption of unambiguous protocols that can be widely distributed among stakeholders for later replication. Furthermore, automated experimental pipelines can track all the parameters necessary to fully describe an experiment and create detailed records to understand experimental variations. In light of the high costs of automated equipment, it is unlikely that individual research groups or biotech companies will have the budget to develop, build, and maintain their own automated facilities. Access to shared automated laboratories can alleviate this concern and is being compared with the introduction of Amazon Web Services and other cloud providers in the software space, enabling a reduction in capital expenditure, and greater flexibility in resource use. The emergence of these third-party facilities – such as cloud labs and biofoundries – is bringing the possibility of using state-of-the-art automation closer to an ever-growing number of scientists.[18] Some of the most prominent players in this space include US cloud labs Strateos and Emerald Cloud Lab, Oxford-based Arctoris, and US synthetic biology foundries Ginkgo Bioworks and Zymergen.

The Role of Automation

Increasing the share of research workflows conducted in an automated fashion eases the adoption of proper metadata capture and record-keeping. Automated workflows allow for full audit trails that capture details on the experimental conditions such as temperature, reaction times, and humidity, which influence the experimental results. Automated platforms can use these audit trails to enable researchers to repeat experiments under precisely the same conditions. Furthermore, robots do not fare well with ambiguity. Writing experimental protocols for machines compels researchers to be precise and avoid overreliance on tacit knowledge. Current protocols often include vague instructions such as "incubate overnight" or "shake gently". According to Google Scholar, "shake gently" has already been used 3400 times in publications in the first half of 2020 alone.[19] Moving from vague descriptions to unambiguous, encoded instructions together with precise execution routines is an important component of moving towards full experimental reproducibility.

Moreover, automation can help ease access to laboratory infrastructure and accelerate the research process. Robotic laboratories accessible online allow for global access to state-of-the-art equipment and resources. Automation can also increase experimental throughput, running 24/7 without human errors or variability while facilitating the data and metadata collection before, during, and at the end of an experiment. This complete set of experimental data and connected metadata can truly explain the results and provide an easily accessible roadmap enabling full validation. Taken together, these unique features have the potential to increase the chances of success for many research projects. Several efforts to trigger the widespread adoption of automation in biology are currently being pursued by companies and research centres across the world.[19,20]

The adoption of automated laboratories requires a shift in how scientists think of their profession, moving towards a far greater emphasis on experimental planning, and choice of parameters, instead of experimental execution. There are attempts to describe protocols in an algorithmic rather than a free-flowing text fashion, with programming languages such as BioCoder,[22] Antha,[23] and Autoprotocol[24] designed for standardising and automating biology protocols emerging in recent years. This opens up the interesting prospect of applying an even broader range of software engineering practices such as containerisation and continuous integration to biological experiments. In principle, fully automated laboratories can save digital 'snapshots' of the lab bench's internal state, including all the relevant metadata at an exact point in time. These 'containers' can then be stored and easily shared. This would allow the original authors, colleagues, and other scientists to reproduce what was done with minute detail. Software developers need to *continuously integrate* modifications in the code and test for malfunctions before deploying each release. Similarly, automated workflows also allow researchers to implement analogous policies regarding changes in the experimental protocol. Tracking all these metadata opens up the possibility of truly understanding and replicating experiments under identical conditions – both across time and geographies – and to explore the role played by each parameter individually.

Challenges Ahead

There is a clear and urgent need to rethink the way biomedical research happens and to put our path to new knowledge on more solid foundations. Addressing this challenge will require a multipronged strategy. The incentives within the scientific system will have to shift and start rewarding reproducibility

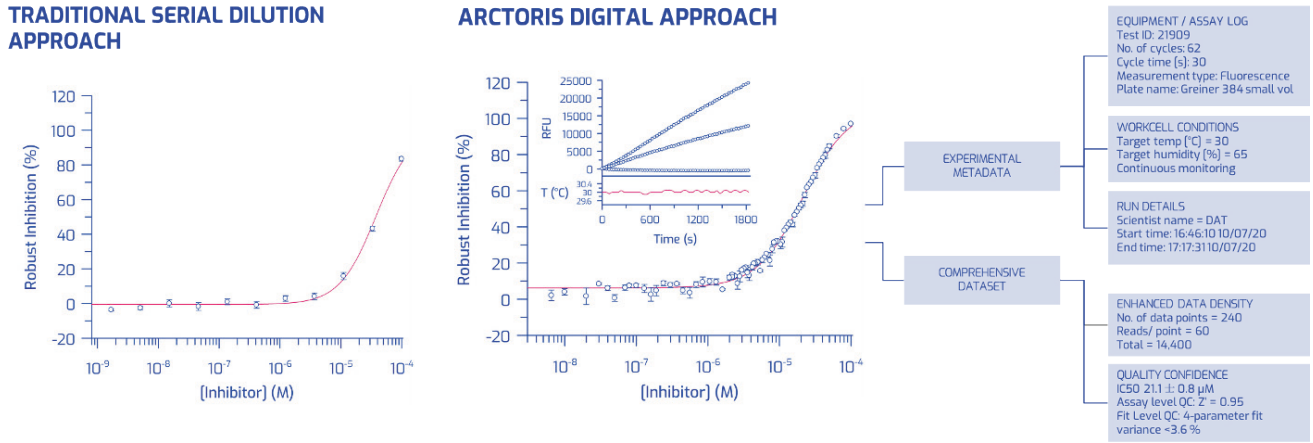

Figure 2 contrasts a traditional biochemical IC50 assay without contextual information with an IC50 assay generated in a fully automated setting, showing higher data density as well as full metadata capture.

and not just novelty. Generating reproducible work and embarking on efforts to replicate other groups' research should be recognised as a task as important as starting a new research project and be rewarded accordingly. This will involve significant modifications in the way projects are evaluated for funding and the criteria committees consider for promotions. Furthermore, funding agencies must learn to set aside budgets for reproducibility work. Even science journalists will need to do their share and disseminate scientific advancement with greater appreciation for the diligent work that goes into keeping the foundations of scientific advancements strong. Achieving this will require a significant effort from all the stakeholders involved.

While automation is poised to ease the adoption of practices that foster reproducibility, there are still important challenges ahead. There is a need to adopt standards for experiment and data description and contextualisation. Efforts in this space include, for example, work on data standards and data models under the umbrella of the Pistoia Alliance, and on increased compatibility between different vendors' equipment by the Standardisation in Lab Automation group SiLA. However, there often are competing standards, including, for example, several dozen ontologies for experimental parameters currently in use. To make sure the solutions currently being developed and trialled fulfil the needs of researchers in academia and industry, it is paramount that scientists and automation experts work closely together to enable the widespread adoption of research practices that are built on reproducible, transparent, audited protocols with full data and metadata capture, ideally entirely conducted in an automated fashion.

Over the next 10 years, adoption of these best practices will lead to a pronounced shift in the individual scientists' tasks and responsibilities. As automation takes over the execution and record-keeping of assays and experiments, researchers will have the opportunity to redefine their roles and place more emphasis on experiment and project planning and data analysis and interpretation – genuine scientific tasks that require the creativity and ingenuity only humans possess.

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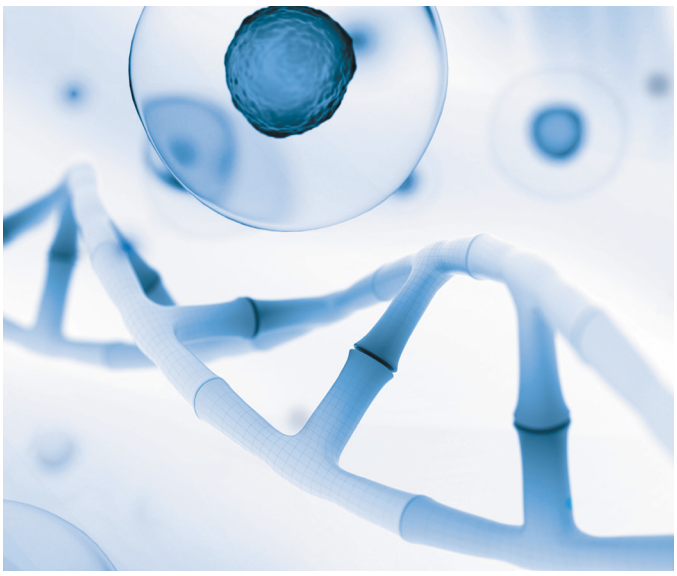
Re-programming CHO by Gene Editing, the New Frontier in Bioprocessing

For many years, Chinese hamster ovary (CHO) cells have been the cornerstone of the success of biopharmaceutical proteins. Their flexibility and adaptability to bioreactor culture conditions, compared to other mammalian systems, make them the system of choice for one of the most successful protein therapeutic classes, monoclonal antibodies. However, very little has changed since the approval of the first commercial product ever manufactured in mammalian cells in 1987. The advent of new gene editing technologies is revolutionising how the industry relates to CHO cell hosts. Gene editing, particularly CRISPR-related platforms and functional genomic screening are opening the door to new tailor-made cell hosts able to deliver desired product characteristics and show optimal culture performance in bioreactors. Long gone are the days where product and processes were subservient to the whims and behaviour of expressing cell lines.



Why the Cells from a Humble Rodent Became the Industry Standard

Since the approval of the first recombinant biotherapeutic, insulin, in 1982, the pharmaceutical industry has experienced an explosion in the development and commercialisation of protein therapeutics. The development and improvement of industrial manufacturing platforms has been a key enabler for this. Today, monoclonal antibodies and architectures derived from them constitute more than half of the protein therapeutics on the market and are, by far, the largest group of biopharmaceuticals currently in clinical development. At the heart of this success are the CHO cells that, since their establishment as a cell line, have become 'the' de-facto manufacturing platform for the large majority of protein-based therapeutics on the market and in development[1].

Chinese hamsters had been used as a laboratory model since the 1910s, but it was in 1957 when Theodore Puck managed to establish stable cultures of what would later turn out to be the "mother of all CHO cells", the strain CHO-K1 from which all existing bioprocessing CHO cell lines in use today derive[2]. In those early days, CHO cells already showed several interesting properties that made them attractive as a cellular model:

- They remain in uninterrupted culture for many generations without immortalisation or transformation.
- They have short doubling time (16–22 h).
- They are genetically simpler than other mammalian cells (Table 1).

Cell	Organism	Number Chromosomes	DNA size (Mb)	Doubling Time
HEK293	Human (kidney)	64	~9000?	34h
CHO-K1	Chinese Hamster (ovary)	21	2450	16–22h
Saccharomyces cerevisiae	Yeast	16	12.2	1.2–2h
Pichia pastoris	Yeast	4	9.4	2–5h
Bacillus subtilis	Bacteria	1	4.2	27 min
Escherichia coli	Bacteria	1	4	20 min

Table 1. Common cells used in the production of biotherapeutics

These features have led to CHO cells becoming an ideal model for research and biotechnology applications. On one hand, they present significant advantages for bioprocessing by allowing bioreactor cycle times to be substantially shorter. Also, their genetic simplicity makes them a favourable target for gene editing today.

Why Has CHO Not Been Displaced by Other Expression Platforms?

Many other platforms, particularly microbial organisms, are considerably easier to maintain in culture and are often able to produce large amounts of recombinant proteins but have not managed to displace the predominance of CHO. This is due to a combination of several factors:

- **Post-translational modifications (PTMs).** Microbial systems (even yeasts) are not very effective at replicating desired PTMs in proteins, particularly complex glycosylation patterns. By contrast, CHO in most cases (chiefly monoclonal antibodies) manages to do a reasonably good job.
- **Manufacturing costs are still a minor fraction of the price of a drug.** Typical manufacturing costs of monoclonal antibodies expressed in CHO cells can be as little as 1–5% of the final drug price[3].
- **Microbial or plant-based platforms are not as 'cheap' or 'fast' as one might expect.** Although microbial and plant-based systems can occasionally bring advantages in terms of bioprocessing costs and timelines, they have not yet managed to dramatically outperform CHO in terms of cost or overall development timelines.
- **Regulatory** (traceability and safety-related) and **infrastructure hurdles** for developing new therapeutic products can dissuade from switching platforms. Also, once a company has invested in developing the manufacturing structure for a given product class, they will not move away if it does not bring substantial benefits (reducing time, risks, and costs, or improving process performance).
- **CHO is still the industry 'standard'.** CHO is the leading platform for producing biotherapeutics in large quantities and at acceptable costs. One can transfer a CHO-based

process anywhere in the world and almost all existing CDMOs have experience and infrastructure to use CHO cells successfully.

Are New Gene Editing Platforms Marking the Onset of a New Age for CHO?

Very little changed with CHO during its first 50 or so years as a cell line. Initial efforts concentrated in moving away from adherent cultures requiring complex roller-bottles or multi-stack infrastructure that were poorly scalable. Probably, the most important event in CHO's history as a protein expression host was its adaptation to suspension culture. This transition allowed significant improvements in cell culture process control and consequently substantial increases in productivity and product quality. Further to this, the discovery of metabolic inhibitors; methotrexate (MTX) and methionine sulfoximine (MSX) for two particular enzymes; di-hydro folate reductase (DHFR) and glutamine synthetase (GS) respectively, provided selection markers to facilitate the selection of cells expressing a given gene of interest. Aside from this, the only substantial manipulation worthy of mention was the generation of the DHFR (double negative) mutant in the 1980s, giving rise to the CHO DG44 strain. Since then, nothing much happened to the CHO hosts cells used in bioproduction.

The arrival of the new millennium with the publication of the first CHO genome and emerging genetic technologies, reignited more systematic gene modification efforts[4,5]. Early projects included the generation of GS knockouts in CHO-K1 derivatives to improve selection pressure, whilst eliminating the need for MSX (potentially neurotoxic), and the generation of mutants with reduced fucosylation to enhance the effector-function activity of monoclonal antibodies *in vivo*[6,7].

New gene editing platforms have been appearing ever since[4,8]:

- Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), were the first genomic-editing technologies to be used in CHO. These platforms were successfully used to generate selection marker knockouts as well as other variants. However, these platforms present some limitations. They require considerable expertise in the design of the DNA-recognition domains and are not as 'high-throughput' and flexible when compared to more modern platforms, like CRISPR-based methods. This makes them unsuitable for high-throughput screening approaches and restricts their use to pre-validated targets. Still, they remain powerful, and efficient approaches for gene editing remain actively in use in the industry.
- Systems based in recombinant adeno-associated virus (rAAV) were next used for gene editing in CHO cells. Recombinant AAVs do not integrate in the host genome and are unable to generate replication-competent viruses in CHO. The platform constitutes a reliable method for gene editing, and the design of the edits is simple and does not require sophisticated knowledge or technologies. Also, the intellectual property situation around the use of rAAV is straightforward. However, the technique has a relatively low efficiency, and is slow in comparison to CRISPR-based methods.
- CRISPR-Cas9 is one of the latest platforms to arrive and from which many different variants have emerged. The CRISPR-Cas9 system does not require sophisticated design constraints, making it easier to implement, but also faster in performing edits. The modularity of the system enables multiplexing of gene edits. Also, high-throughput screening with large guide RNA libraries can be used to interrogate the impact of different genes in cellular function. CRISPR is extremely efficient compared to other gene editing platforms and this, combined with its speed, makes it the method of choice for many researchers. However, the IP behind CRISPR technologies is complicated, which could discourage the industry from fully exploring its commercial applications in the short term.

Gene-edited CHO Cell Lines for Biotherapeutic Production

The generally risk-averse bioproduction industry has been relatively slow in embracing new technologies to modify the genome of expression cell hosts. This may have been compounded by the complexity of technology access (including IP landscape), restrictive commercial licensing terms, perceived technical difficulty or even doubts about its benefits altogether. However, the increasing complexity of biotherapeutic molecular architectures (i.e. multi-specific scaffolds and complex fusion molecules), combined with an increased urgency for taking products to the clinic and streamlining development are forcing the industry to seek alternative technologies and processes.

In recent years gene editing technologies have been employed to solve various problems:

- **Incorporating desirable PTMs in the product.** CHO cells are known for not being able to produce the PTMs required for some therapeutic molecules. This has been addressed recently by several groups by incorporating enzymatic activities that were missing from CHO; for example, sialidases that could extend the product half-life.

- **Pharmacology and efficacy of product though glyco-engineering.** The glycoform attached to the Fc fragment of antibodies is known to play an important role in the pharmacology and effector-function activity of monoclonal antibodies and Fc fusion proteins. Equally, as mentioned above, the presence of specific human-like sialylation patterns can increase the half-life of protein therapeutics[9].

- **Safety of product.** The safety of biopharmaceuticals can be affected by the presence of potential pathogens in the preparation (virus) or the immunogenicity of the product. Strategies to address these risks include reducing viral permissivity of CHO cells and/or eliminating host cell proteins (HCPs) that could increase the risk of immune responses in patients[10,11].

- **Simpler, cheaper processes.** Downstream processing (purification) of protein biotherapeutics is a key bottleneck in bioproduction and probably the single most expensive unit operation, largely due to the costs of resins and time required to perform. Reduction or, whenever possible, elimination of difficult-to-remove contaminants could potentially have a significant impact in the economics of bioproduction but also in the quality and safety of the therapeutic product itself[11,12].

- **Process robustness and productivity.** CHO cultures require large amounts of energy to grow and express products. In bioprocessing, CHO cells are coached to produce as much protein as possible whilst reducing the expenditure of the cells in 'unnecessary' activities (like synthesising DNA, or non-desired host cell proteins, including proteases). Several lines of research are approaching this in different ways: from manipulating the metabolic circuitry in CHO cells, to eliminating HCPs, to promoting anti-apoptotic behaviour[12,13].

- **Consistency and speed in cell line generation.** The introduction of landing pads in CHO to direct the integration of the desired transgene into a specific location in the genome has been proposed as advantageous in increasing consistency of expression across different cell lines and also potentially accelerating the generation of expressing cell lines[14]. Such landing pads can also be used in combination with mammalian display technologies[15].

- **Streamline antibody discovery and development.** Mammalian display technologies allow the incorporation of additional selection criteria beyond simple ligand binding affinity. This 'cell-based developability' at such an early stage of development facilitates the identification and design of good binders that also are able to fold, assemble and express more favourably, reducing manufacturing and product stability risks that might derail product development later on, often at a very high cost[16].

The Future of Gene Editing in Bioproduction

The current revolution in gene editing is shifting the bioprocessing landscape and opening possibilities to manipulation[4]. Early gene editing platforms (ZFNs, TALENs, rAAVs, etc.) require a good understanding of the desired edit to be performed and the expected phenotypic result, which can often be a question of trial-and-error. In contrast, the simplicity of design afforded by CRISPR-derived platforms has enabled the generation of large screening libraries that make the complexity of whole mammalian genomes a manageable problem. This is where CRISPR screening, combined with comprehensive computational models that integrate different cellular pathways, can become a powerful tool in the identification of novel targets suitable for gene editing.

Genome-wide functional genomics CRISPR screening (Figure 1) is becoming a powerful tool in the identification of genome-phenotype functional relationships, primarily due to its simple design and 'programmability' compared to other platforms[17]. The availability of multiple CRISPR variants allows very sophisticated analysis combining knockout generation with gene modulation via CRISPRi (interference) or CRISPRa (activation) approaches, which can be particularly useful for genes that are either essential or can play different roles depending on relative abundance.

Multiple gene edits can be obtained simultaneously in a single cell, allowing targeting of complex interactions to achieve significant phenotypic effects as a result. This has recently been demonstrated by the simultaneous knocking out of a variety of HCP genes that synergistically contributed to a reduction in general HCP load and favouring the gene of interest productivity in CHO[12].

As mentioned above, CRISPR-based mammalian display has been proposed as an alternative to landing-pad recombinase-mediated display systems. This approach can potentially increase the size of libraries available for screening but also help merge the interface between antibody discovery and engineering with bioprocess development and manufacturing[16]. On the other hand, base editing and prime editing technologies are opening the door to simpler gene editing to fine-tune the activity of specific effectors relevant to a given product or process. Finally, the emergence of new CRISPR systems is opening alternative commercialisation routes to gene editing that might be currently blocked due to complex intellectual property landscapes. In addition, new smaller CRISPR systems might create opportunities to integrate nucleases in more sophisticated multi-functional architectures[18].

What is Next for CHO?

The industry is just peering out into a brand-new landscape where drug developers will have access to novel, even tailor-

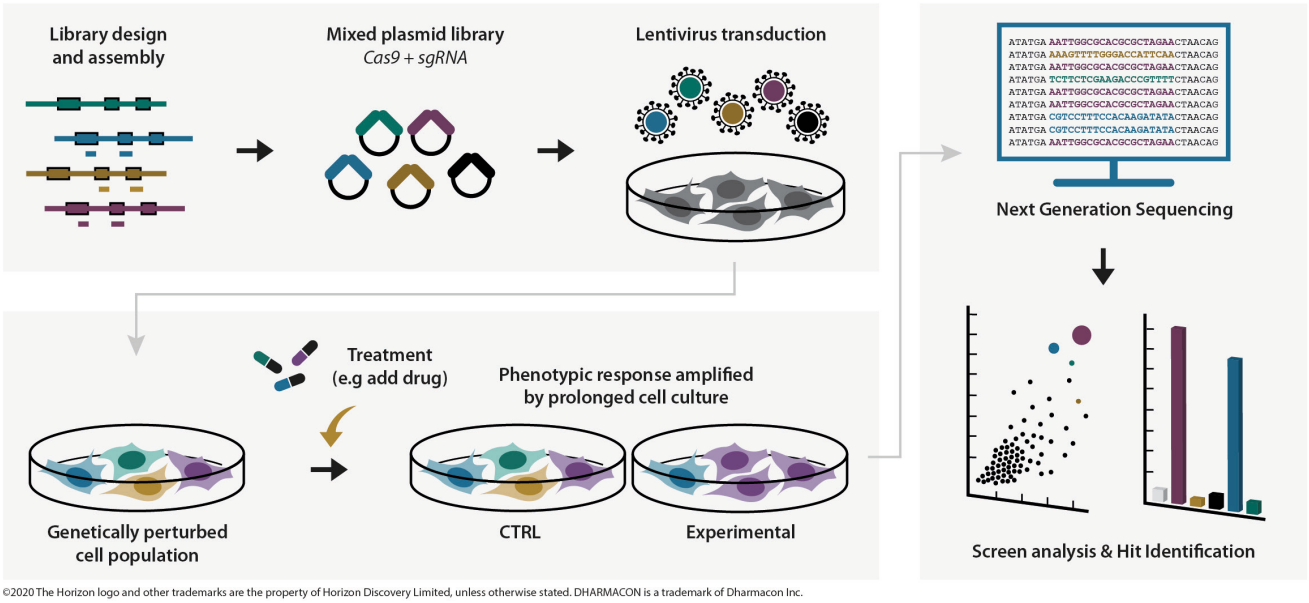


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Figure 1: An example of a genome-wide functional genomic CRISPR screening workflow

made, expression platforms to accommodate their needs and enable them to produce protein biotherapeutics in entirely new ways. One cannot help but be surprised at how the industry has historically adapted its processes to the whims and biological designs of CHO cells, with substantial investments in infrastructure and technology over the years. For example, the market for media, cell culture and bioreactor technology is estimated to be in excess of $1 billion per year, whereas the market for downstream processing, including resins, filters and purification technologies is about ten times as large. However, the investment made by the industry in 'taming' CHO cells by re-programming their genomes, pales in comparison. Now the door is open to adapting the host design to 'ideal' or 'optimal' bioprocessing conditions and development needs. These may vary broadly depending on requirements introduced by disease condition, or specific commercialisation strategies, which could impact the required production scale, development timeline, on-demand manufacturing, or non-standard chemical composition. These new paradigms will shape future manufacturing practices and will have at their core more diverse, robust, and flexible cell hosts, which are still likely to include those derived from CHO cells.

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Addressing the Challenges of Selective and Sensitive Bioanalytical Assay Development

It's a remarkable thought: antibodies have evolved over millions of years to become a critical component of immunity, but in a few short decades we have dramatically expanded their potential for solving other problems. Our general understanding of an antibody is a unique immunoglobulin, shaped by the immune system in response to whatever infection we may have contracted. However, recombinant antibody technologies allow us now to consider antibodies as precise, replicable, manufactured components that can facilitate biological research and cure diseases.

Modern biological research is made possible by using antibodies as molecular tools, utilising their ability to bind specifically to a target, but more recently antibodies are being used as therapeutics; today, over 70 monoclonal antibody drugs are used to treat autoimmune, cardiovascular, infectious diseases and cancer[1]. Such therapeutic antibodies can provide the benefits of impressive specificity, high affinity, a long *in vivo* half-life, and strong biological potency.

In this article, we will discuss the critical reagents required in the bioanalytical assays necessary for development of monoclonal antibody drugs, and will explain how antibody phage display technology can address the challenges of creating selective and sensitive ligand binding assays (LBAs) for analysing this type of drug.

Development of Antibody-based Biologics and Biosimilars

Therapeutic monoclonal antibodies belong to a class of drugs known as large molecule biologics. There are several types of biologic drug on the market, and many more are in clinical trials. Most are full length monoclonal antibodies, but other modalities are making their way through the development process – these include antibody Fab fragments, single domain antibodies, antibody drug conjugates (ADCs), bispecific antibodies, fusion proteins, and CAR-T cells. In 2017, seven of the 10 top-selling drugs worldwide were monoclonal antibodies, and in 2018, eight of the top 10 best-selling drugs were large molecule biologics[2,3]. An expanding market demands the best tools for the bioanalysis of these drugs.

Discovery and development of any drug is an enormous investment, typically taking many years and costing millions of dollars. If it reaches clinical trials, a pharmaceutical company must then demonstrate that their drug shows positive safety and efficacy and is appropriate for clinical use.

As the therapeutic antibody market rapidly expands, revolutionising the treatment of many chronic and complex diseases, many original antibody drugs are coming off-patent and so a lucrative biosimilars market is growing. A biosimilar is a biological product (for example another monoclonal antibody drug) that is highly similar to an originator drug (known in this context as the reference product). Development costs of biosimilars are much lower than originator drugs, with a timeline of roughly seven to eight years[4]. These faster, cheaper pipelines mean that biosimilars can be sold at a lower price than originator drugs, benefiting patients and healthcare providers. Accordingly, the biosimilar monoclonal antibody market has a compound annual growth rate (CAGR) of 30%, predicted to grow by 8.65 billion USD during 2020–2024[5].

For a biosimilar antibody drug to gain market approval, the manufacturer must demonstrate that there are no clinically meaningful differences in the safety, purity, and potency of the biosimilar compared with the original reference product. When seeking regulatory approval, for example from the FDA or EMA, biosimilar manufacturers must submit robust analytical, non-clinical, and clinical data that will be reviewed to understand the level of similarity of the proposed biosimilar to the reference product. The types of bioanalytical data submitted include structural and functional characterisations, clinical pharmacokinetic (PK) and pharmacodynamics (PD) studies, clinical immunogenicity testing, and clinical safety and efficacy.

Challenges for the Bioanalysis of Monoclonal Antibody Drugs

To ensure that biopharmaceutical and biotechnology companies submit the robust data required for regulatory approval of both original biologics and biosimilars, it is imperative they use trustworthy tools. Poor quality critical reagents can delay early drug discovery as well as the subsequent drug development process and can lead to incorrect conclusions, creating significant financial losses.

Ligand binding assays (LBA) are used to assess the immunogenicity of biotherapeutic molecules, and to determine drug dose concentrations from pharmacokinetic analyses[6]. They are essential to demonstrate that a biosimilar is functionally equivalent to an originator drug. The Global Bioanalysis Consortium (GBC) classifies bioanalytical antibody tools as critical reagents affecting the accuracy and performance of LBAs.

Each drug type creates specific challenges for bioanalytical assays, with no single analysis method suiting all biologics. Biopharma companies and their contract research organisation (CRO) partners need the expertise to develop robust and transferable methods using the most appropriate testing platforms and reagents, and large, accessible repertoires of bioanalytical antibodies enable bioanalytical scientists to achieve these goals.

When it comes to determining drug effects in humans, designing appropriate assays for the different modalities of biologics has its challenges. In the development of an LBA, a bioanalyst must be certain that they are measuring exactly what they want to measure, with no interference from serum matrix proteins, soluble targets, receptors or other unrelated factors. Measuring drug levels in patient sera during a pharmacokinetic

(PK) study can present challenges for selectivity. Antibody drugs are engineered to be as human as possible in sequence and structure in order not to cause an immune response in the patient, which could lead to loss of efficacy and unwanted or dangerous side-effects. A PK assay must therefore be able to differentiate between the drug and the excess of immunoglobulins present in the patient sample, where the concentration of an antibody drug can be a million times lower than serum antibody concentration, hiding the drug from detection. To mitigate this, PK bridging ELISAs require well characterised capture and detection antibodies that are highly specific to the drug.

Despite efforts to design antibody therapeutics to be as human as possible, they are at risk of being recognised as foreign by the immune system, which produces anti-drug antibodies (ADAs) in response. ADAs can interfere with the drug's action and have the potential to cause serious adverse events. A vital part of the bioanalysts risk assessment is to evaluate the potential immunogenicity of the drug by detecting and characterising the ADAs elicited *in vivo*. Ligand binding and cell-based assays are typically used to determine the presence and levels of ADAs, their neutralising activity and isotype, and a surrogate positive control or calibrator is an important component of such assays.

Anti-idiotypic Antibodies are Critical for Assay Development

An antibody binds its target antigen at its unique binding site called the paratope. The whole of the variable part of the antibody, including the paratope is called the idiotype, and the additional unique regions are called idiotopes (Figure 1). In the special case where the target antigen of the antibody is another monoclonal antibody such as a monoclonal antibody drug, and it binds to the idiotope, it is called an anti-idiotypic antibody.

Anti-idiotypic antibodies that bind specifically to one monoclonal antibody drug are critical reagents for bioanalytical method development. They can be used for capture and detection in PK assays to measure free or total drug levels in preclinical or clinical samples, and as a control or calibrator in ADA assays.

The availability of these critical reagents with a choice of binding properties (Figure 2) enables the design of assays for different bioanalysis requirements:

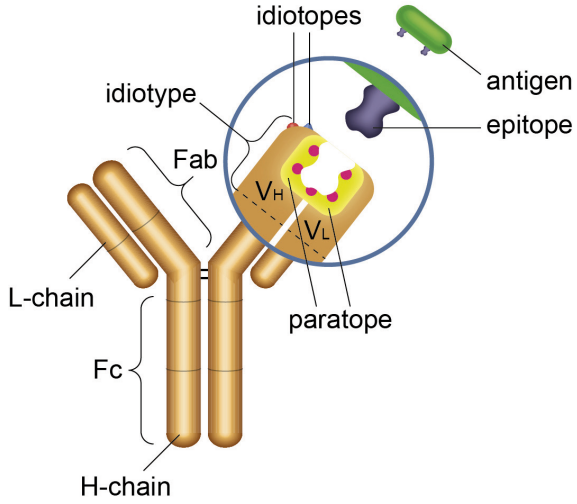
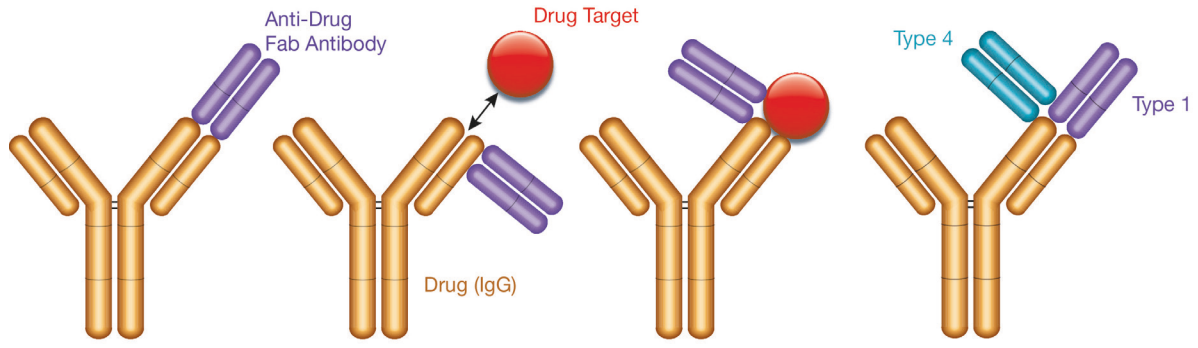


Figure 1. The antibody idiotope is the unique set of antigenic determinants of the variable region of an antibody.

- Inhibitory (type 1) anti-idiotypic antibodies are used as capture and detection reagents in PK assays to detect free drug, and as a surrogate positive control or calibrator in an ADA assay
- Non-inhibitory (type 2) anti-idiotypic antibodies are used as capture and detection reagents in PK assays to measure total levels of the drug; they bind to the drug at the idiotope, but outside the paratope, so can detect the drug when free, partially bound or fully bound to its target
- Drug-target complex-specific binders (type 3) only recognise the drug when bound to its target, enabling setup of a PK antigen capture ELISA that detects bound drug exclusively, and providing an alternative option when a bridging ELISA is not feasible, e.g. when detecting a monovalent Fab or single domain antibody
- Drug type 1 complex-specific binders (type 4) recognise the drug in complex with an inhibitory anti-idiotypic antibody and may be useful for a PK antigen capture assay when the drug-target is not easily available or is very costly, and when generating type 3 antibodies against the drug-target complex is not possible



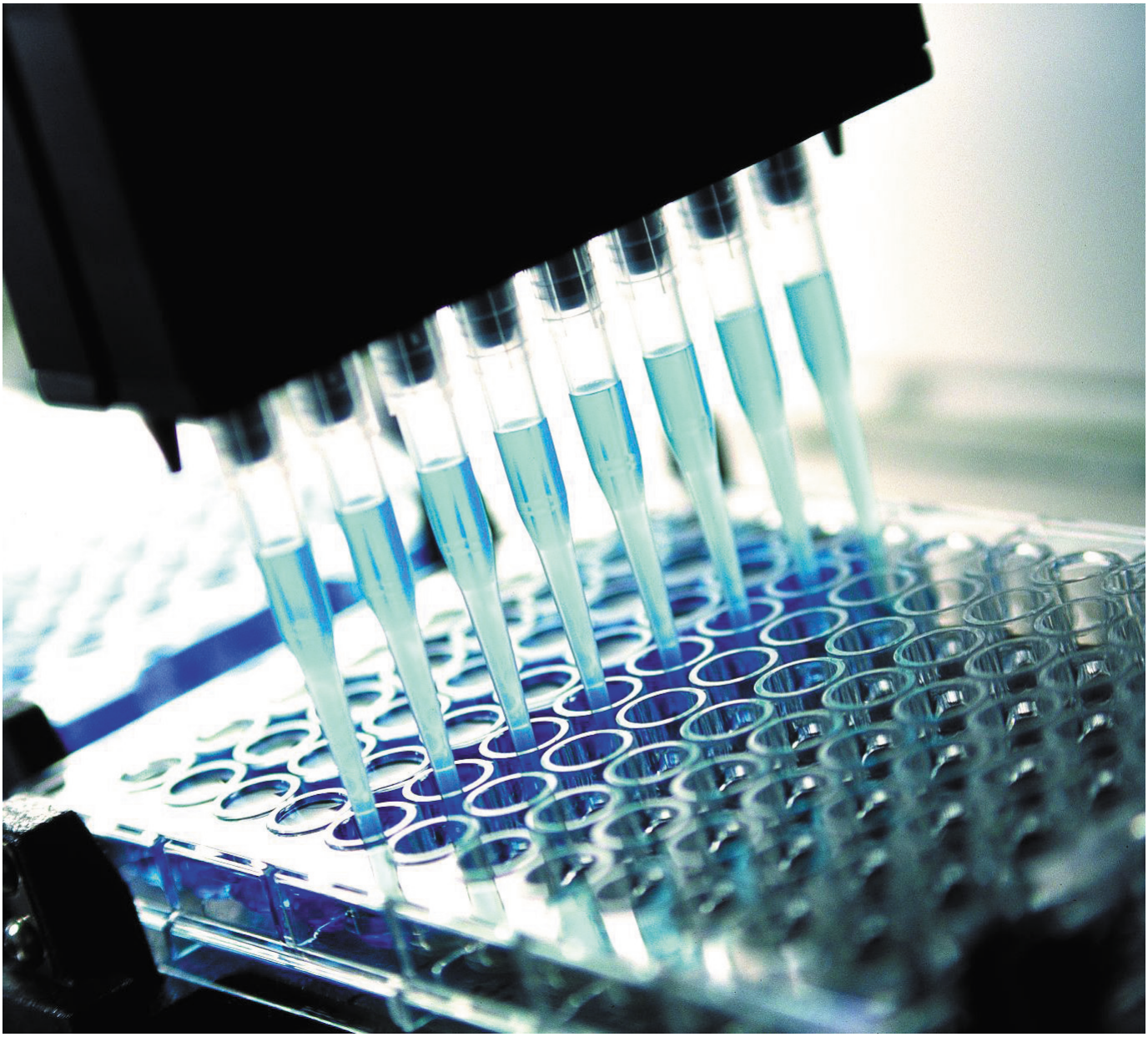
Type 1
- Paratope-specific
- Inhibitory
- Neutralising
- Detects free drug

Type 2
- Not paratope-specific
- Non-inhibitory

Type 3
- Antibody-antigen complex-specific
- Non-inhibitory
- Exclusively detects bound drug

Type 4
- Non-inhibitory
- Exclusively detects Type 1 antibody drug complex

Figure 2. Anti-idiotypic antibody binding modes and properties



Any antibody used for bioanalytical assays must be sensitive enough to detect the drug at very low levels, and specific enough to only detect the target and nothing else[7]. *In vitro* antibody phage display technology can provide the antibody tools that bioanalysts need to ensure that their assays meet these strict requirements and overcome the limitations of antibody generation by traditional methods of animal immunisation.

Antibody Phage Display Libraries Provide High Quality Bioanalytical Reagents

The specialised antibodies with the different binding modes and properties described above can be generated with high specificity, high affinity, and consistency using *in vitro* technologies. The use of synthetic, naïve antibody phage display libraries avoids reliance on the immune response of an animal to generate antibodies, and results in the production of recombinant antibodies with a defined sequence, which can be well characterised and reproduced indefinitely via production in *E. coli* or a mammalian cell line. These libraries cover an enormous immune repertoire and antibodies can be generated targeting almost any conceivable antigen. Applying *in vitro* guided selection methods enables generation of the type 1 and type 2 anti-idiotypic antibodies described for PK and ADA assays, and the specialised type 3 and 4 drug-target or drug antibody complex binders. Furthermore, strategies can be devised to generate antibodies targeting specific epitopes of just a few amino acids, such as the unique linker region in a fusion protein drug, or the linker and toxin of an ADC molecule. These highly targeted antibodies are virtually impossible to produce using traditional animal immunisation methods.

An example of a synthetic naïve phage display library is HuCAL®, Human Combinatorial Antibody Libraries[8]; the latest version, HuCAL PLATINUM®, contains 45 billion functional human antibody genes in Fab format and has been used in combination with CysDisplay® (a modified phage display method) to generate thousands of highly customised antibodies, including antibodies specific to the drug-target complex for five marketed biotherapeutics: ranibizumab, adalimumab, golimumab, trastuzumab, and omalizumab[9].

When generating anti-idiotypic antibodies using HuCAL technology, antibody selection is carried out for the monoclonal

antibody drug in the presence of antibodies matched for isotype sub-class, which serve as blockers. This avoids enrichment of specificities that bind other regions of the antibody drug, and helps ensure idiotype specificity. Selection is also performed in the presence of human serum to avoid matrix effects in the final assay that could be caused by cellular components interfering with the ability of the antibody to bind to its target.

These non-animal-derived recombinant antibodies are sequenced as part of the selection process and are therefore precisely defined, which allows them to be reproduced indefinitely. Production of recombinant antibodies can be controlled to ensure high levels of consistency between batches, contributing to reproducibility of experimental results. Recombinant antibodies can be engineered in different formats with ease, including monovalent and bivalent Fab antibodies with a variety of purification and detection tags, and full length antibodies of different isotypes. Anti-idiotypic antibodies selected from HuCAL PLATINUM are fully human in sequence, and in full length immunoglobulin format can act as a surrogate positive control in an anti-drug antibody assay, closely mimicking the antibodies produced by the patient in response to the drug treatment. The advantage of long-term, consistent supply of a recombinant avoids the inherent problem of batch variation suffered by a control derived from animal serum, or worse, the exhaustion of supply during the study, which would require development of new reagents, qualification and revalidation of assays. The advantages of recombinant antibodies allow the bioanalytical scientists to expand their options for assay design and choose the best reagents for use on the different technology platforms available to them.

Beyond the Assay – Reducing the Use of Animals in Science

In a document published in May 2020, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) recommended that animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications[10]. The argument for reducing animal use is not just an ethical one, and the EURL ECVAM report explains that non-animal-derived antibodies offer significant scientific and economic benefits over animal-derived antibodies. This recommendation reflects substantial trust in *in vitro* technologies as the future for novel antibody production. Just as the scientific community was urged to move away from ascites-based antibody generation several decades ago[11], now it is called to switch from animal immunisation approaches altogether in favour of *in vitro* antibody generation technologies.

With growing numbers of original biologic drugs in development and an expanding biosimilar market, the need for easy access to robust and reproducible critical antibody reagents will increase. This demand, coupled with the increasing advocacy to move away from animal-derived antibodies, will lead to improvements and new innovations from companies invested in non-animal-derived antibody generation technologies, which in turn will contribute to the success of bioanalytical scientists and the shared goal of advancing human healthcare.

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The Power of Recombinant Human Albumin
New Advances in Cell Therapy Optimisation, Cryopreservation and Formulation for Novel Therapies

The Case for Recombinant Human Albumin

The pharmaceutical and biotech industry is changing. We have already seen a shift from small molecule-based drugs to more complex biologics; but now a range of more advanced therapies are entering the scene, such as cell therapies or virus-based drugs in oncolytic vaccines for use in gene therapies. These more complex and specialised therapies are offering incredible new therapeutic potential, but also come with their own unique set of challenges – challenges that across these innovative types of therapies can be addressed by a known and established excipient: albumin. But not all albumins are created equal, and choice of albumin source can make a big difference in outcome. As in any drug product, purity and batch-to-batch consistency are paramount regulatory considerations for a very good reason; they impact performance, efficacy, and safety. When it comes to albumin, oxidative modification or the presence of impurities in formulations can alter its binding capability and negate its stabilising effect.

Innovative recombinant human albumin (rAlb) products overcome this obstacle. Certain pioneering rAlbs are proven effective and versatile stabilisers that can protect protein, peptide, vaccine and cell therapy products from aggregation, surface adsorption, oxidation, and precipitation. Unlike plasma-derived human serum albumin (HSA), rAlb offers a reliable albumin source with excellent batch-to-batch consistency and security of supply. Highly pure rAlbs do not support post-translational modifications (PTMs), offer unsurpassed cGMP quality and meet USP-NF standards.

Cell therapies have many applications across a broad range of conditions; however, the use of stem cells and other cell types can be problematic due to issues around their preservation or cultivation. Manufacturers generally shun the use of animal- or human-derived components in cell culture media due to challenges with variability, risk of infections, morphology alterations and differentiation. rAlb presents numerous benefits for both upstream and downstream cell suspensions. Offering a safer solution for optimised cell performance, it can be easily implemented in both formulation studies and later large-scale production. Through acting as a nutrient carrier to cells, rAlb ensures optimal growth, brings stabilisation of cell membranes and co-formulated proteins, and empowers manufacturers to achieve improvements in the viability of cells. Not only does this advance the performance of novel products, but it can be fundamental in streamlining the regulatory pathway for the approval of new stem cell therapies.

Other advanced biopharmaceuticals include virus-based treatments, such as viral vectors, cancer vaccines and oncolytic virotherapy. Such innovative, virus-based treatments hold life-changing potential, but embracing this opportunity is not without its challenges. Manufacturing large volumes of viral vectors calls for efficient, safe production that ensures the stability of the final product. Virus diversity, the very thing that provides clinical benefits, also means that it can be difficult to find a uniform solution. rAlb, through the general stabilising properties of albumin together with the recombinant benefits, can provide the answers to many of these challenges, allowing developers and patients to embrace the opportunities of the next generation of advanced biopharmaceuticals.

Addressing Industry Needs

Plasma-derived human serum albumin (HSA) is a highly useful component in the stabilisation of cell culture and preservation media. Present at approximately 40g/L within blood, the protein acts as a buffer, or reservoir, for smaller entities including metals, hormones, fatty acids and toxins, shuttling these around the various tissues and body compartments, from areas of high concentration to areas of low concentration. Albumin also constitutes about 75% of the colloidal oncotic (colloidal osmotic) pressure of blood and the single free cysteine of albumin makes up most of the reducing equivalents.

While many of albumin's properties make it an extremely valuable component in the development of stem cell therapies, the source of albumin can make a big difference. Blood-borne contaminants (mycoplasma, viruses and prions) theoretically present in HSA, in addition to potential concerns about the reliability of supply and the performance variability of undefined serum, create concerns. This necessitates more well-defined, well-characterised and controlled forms of albumin, such as rAlb. Unlike its blood-derived counterparts, rAlb offers an animal and human component-free material produced at GMP level and of high consistency and purity. It permits complex culture media to be chemically defined and controlled, while affording higher cell growth reproducibility. The method is also supported by regulatory authorities as comprehensive information is available for all constituents of the product.

With stem cell therapies becoming viable treatment options for numerous life-threatening conditions, interest has turned to different technologies that can help optimise their development and application. Albumin, a long-established ingredient of cell culture media, has proven its ability to facilitate growth of many cell types, and has seen its properties expanded for use in the cryopreservation and formulation of stem cell therapies due to a move towards more well-defined, high quality and fully recombinant cGMP recombinant albumin sources. This protein is bringing new advancements in the cryopreservation and formulation of stem cell therapies. When used in cryopreservation, yeast-derived rAlb has been demonstrated to be functionally superior to alternative approaches and this offers tremendous benefits for pioneering new treatments.

Albumin in Cell Therapy

Advanced therapies, such as cell therapy, typically have more

complex value chains along with an inherent variability that often complicates standardisation of production needed to scale up and reach patients worldwide. Throughout development from upstream culture to cell transformation, preservation and final administration, consistent performance is a difficult task to achieve. Aggregation, shear, surface interaction growth rate and reproducibility can all also cause serious issues. The protection of cell identity and the confidence of cell survival during transformation can lead to further problems, while quality control of final products, storage and transportation, apoptosis, cell death, loss of identity and safety all pose significant risks. This has generated the demand for improved knowledge and command of cell viability and variability throughout the value chain.

rAlb can shield cells against chemical and physical stress during processing, particularly when the cells are subjected to this in a higher degree (for instance the more stress, the higher the benefit of recombinant albumin). It can also inhibit aggregation of cells in formulation and stabilise cells during freeze and thaw, while maintaining the safety and efficacy of products during storage. rAlb does not contain any blood-derived impurities which could otherwise activate unwanted cell pathways. These properties can bring considerable enhancements to cell therapy applications and ensure that optimal results are realised from products.

Cryopreservation Benefits

The capacity to preserve stems cells is extremely significant to their use in stem cell therapies and facilitates the completion of quality and safety testing before use, as well as transportation of the cells between sites of collection and processing facilities. Cryopreservation removes the need for a continuous process, generating increased flexibility and logistical advantages. Developing a cryopreservation protocol for a particular cell type requires a specification of pre-freeze processing, introduction of a cryopreservation solution, a freezing protocol, defined storage conditions, thawing conditions, and post-thaw assessment.

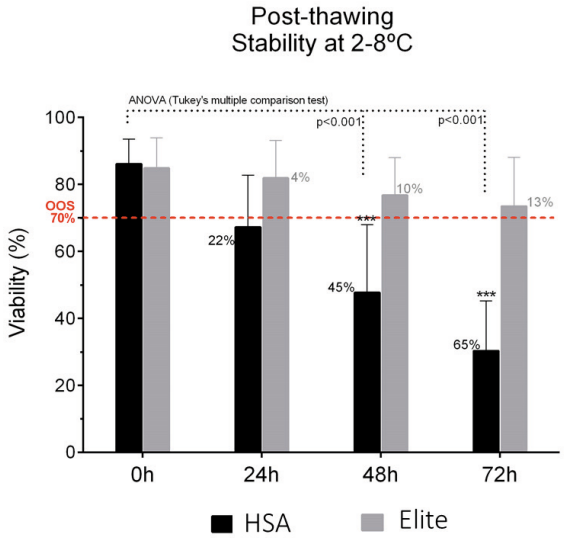


Figure 1: Increasing Product Shelf-life, The graph shows the feasibility of hMSC post-thaw, contrasting Recombumin® Elite rAlb to HSA. When relying on HSA, the viability drops significantly below the release criteria (out of specification – OOS) of 70% viability, whereas Recombumin® Elite maintained viability of the cells after thawing significantly better compared to HSA. The product still met the acceptance criteria at 72 hours while products conditioned with HSA were out of specification at 24 hours. This study was carried out in collaboration with University of Barcelona, Xcelia and Banc de Sang I Teixits, Barcelona.

Cryopreservation is commonly utilised to produce sizeable stocks, or cell banks, that can be stably stored for short and long periods, ready to be recovered and used as required. Demand is mounting for this step to be devoid of serum or any animal-derived components. As a cryoprotective agent, yeast-derived rAlb can bring several unique improvements to the process, helping stem cells to tolerate media change and transition. Its buffering capacity and ability to stabilise entities in solution can help to optimise processes for manufacturers. The purity and source of yeast-derived rAlb also plays a significant role as it can prevent cells from progressing to late state apoptosis compared to plasma-derived albumin, exemplified with mesenchymal stem cells (data not shown). This warrants a considerably greater viability post-thaw of stem cells that have been cryopreserved, as shown in Figure 1, and guarantees an extended shelf-life than was formerly attainable using traditional approaches.[1]

In the case study above, rAlb maintained viability of the cells after thawing significantly better compared to HSA, significantly increasing product shelf-life.

Another study, in collaboration with the international biomedical research institute Centre for Genomic Regulation in Barcelona, sought to investigate cell health post-cryopreservation of induced pluripotent stem cells (iPSCs), including viability, potency and how cells perform in terms of colony formation.[2] Data, as shown in Figures 2 and 3, demonstrates that the inclusion of rAlb in the cryopreservation media improved iPSC colony formation post-cryopreservation compared to standard solutions, with no change to pluripotency of the cells.

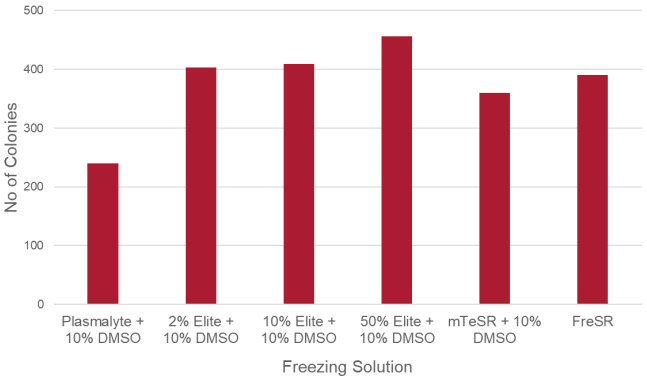


Figure 2: Improving iPSC Colony Counts Post-cryopreservation

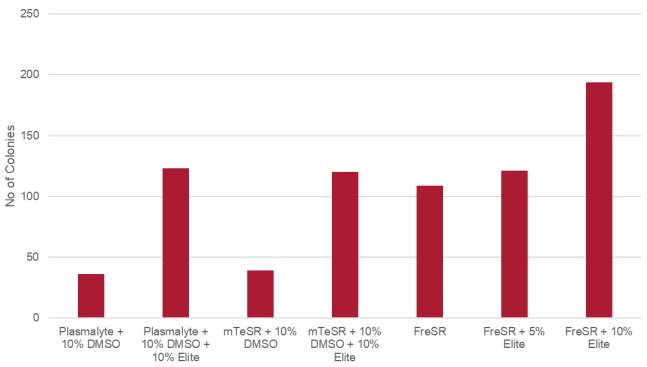


Figure 3: Improving iPSC Colony Counts Post-cryopreservation

Formulation Benefits

To create a new, functional therapy that also presents high levels of reproducibility, the correct formulation is imperative for stem

cell products. Formulation usually takes place directly after stem cell generation or thawing, contingent on the requirements of the application. Several assays must be performed following formulation and prior to a product being administered to patients, therefore the longer that stem cells can be maintained in a stable state, the greater the applicability and flexibility of the therapy. An approximate rule of thumb is that one-day stability permits for on-site administration, two-day stability permits nationwide distribution, and three-day stability allows for worldwide use.

Preparation of stem cells in a controlled medium attains an easier release of the therapy, which means that analysis can concentrate on the therapy itself instead of the possible effects and impurities from the medium. The controlled nature of the formulation can also reduce variation in the background of biological assays. Nevertheless, due to carry-over between process steps, it is not enough only to employ controlled media in the generation of the final formulation. If a controlled final formulation is desired, this must be designed into the process sufficiently upstream to ensure enough dilutions and exchanges have taken place to mitigate any risks from uncontrolled substances. An alternative is to design the process to include the controlled media as early in the process as possible, to thereby eliminate the requirement to wash out the uncontrolled substances and to aid regulatory acceptance.

Albumin in Virus-based Treatments

In recent years, virus utilisation has extended from infectious diseases into areas such as gene editing and cancer prevention. In the early days, researchers using viruses to deliver genes of relevance experienced setbacks, but progress has been fast. Data has accumulated, and lessons have been learned. New modalities have enriched safety and efficacy, and more and more viral vector-based therapies are now approaching the market, presenting a new set of production, manufacturing and stability challenges.

As already discussed within the cell therapy section, albumin, with its validated multi-functional stabilisation properties, can help overcome many of these challenges and ensure patients receive optimal treatment with maximum effect. rAlb readily adsorbs to both hydrophobic and hydrophilic surfaces in a single monolayer, preventing the non-specific adsorption of biopharmaceuticals during manufacture, formulation and storage. For example, just 1–2mg of rAlb is needed to coat 1m^2 of surface. This allows developers to avoid losses during downstream processing and increase overall yield, while also avoiding losses on container surfaces and providing safer dose control. Further, it helps to prevent surface-induced denaturation, cutting the risk of reductions in efficacy and increases in immunogenicity.

Albumin binds to a wide range of compounds, using both ionic and hydrophobic interactions, and also disperses uniformly throughout a solution. This effective solubilisation confers an insulating quality on viral vectors and vaccines, protecting them during manufacturing and handling and, ultimately, maintaining potency and efficacy. It means the excipient can be used to prevent aggregation or changes in higher order structure, which can impact the quality, stability, safety, and efficacy of products. It also stops aggregation formation during downstream processing and final formulation, and protects fragile particles from shear stress, adding to the purity of the final product. This characteristic also helps to stabilise vaccines during freeze and thaw, increasing long-term stabilisation and improving cold chain processes.

Albumin has one natural free thiol group, C34, which has been evolutionarily optimised to react with aggressive oxidising species. This enables albumin to protect biopharmaceuticals by scavenging against oxidation modification when in formulation.

Case Examples of Albumin Optimising Virus-based Therapies

Novel rAlbs that have been commercially validated in marketed and late-stage clinical drug and vaccine candidates and accepted by leading regulatory agencies present a natural choice for the safety-conscious manufacturers of innovative viral vector-based vaccines.

An example of this includes the case of Amgen's HSV-based vaccine, which is very sensitive to temperature changes. In its original marketed formulation, it cannot be exposed to transient ambient temperatures for more than 60 seconds, which of course leads to significant operational and logistical issues. However, in a patent application[3] from Amgen, addition of recombinant albumin was shown to significantly increase the liquid stability of the virus at 2–8°C.

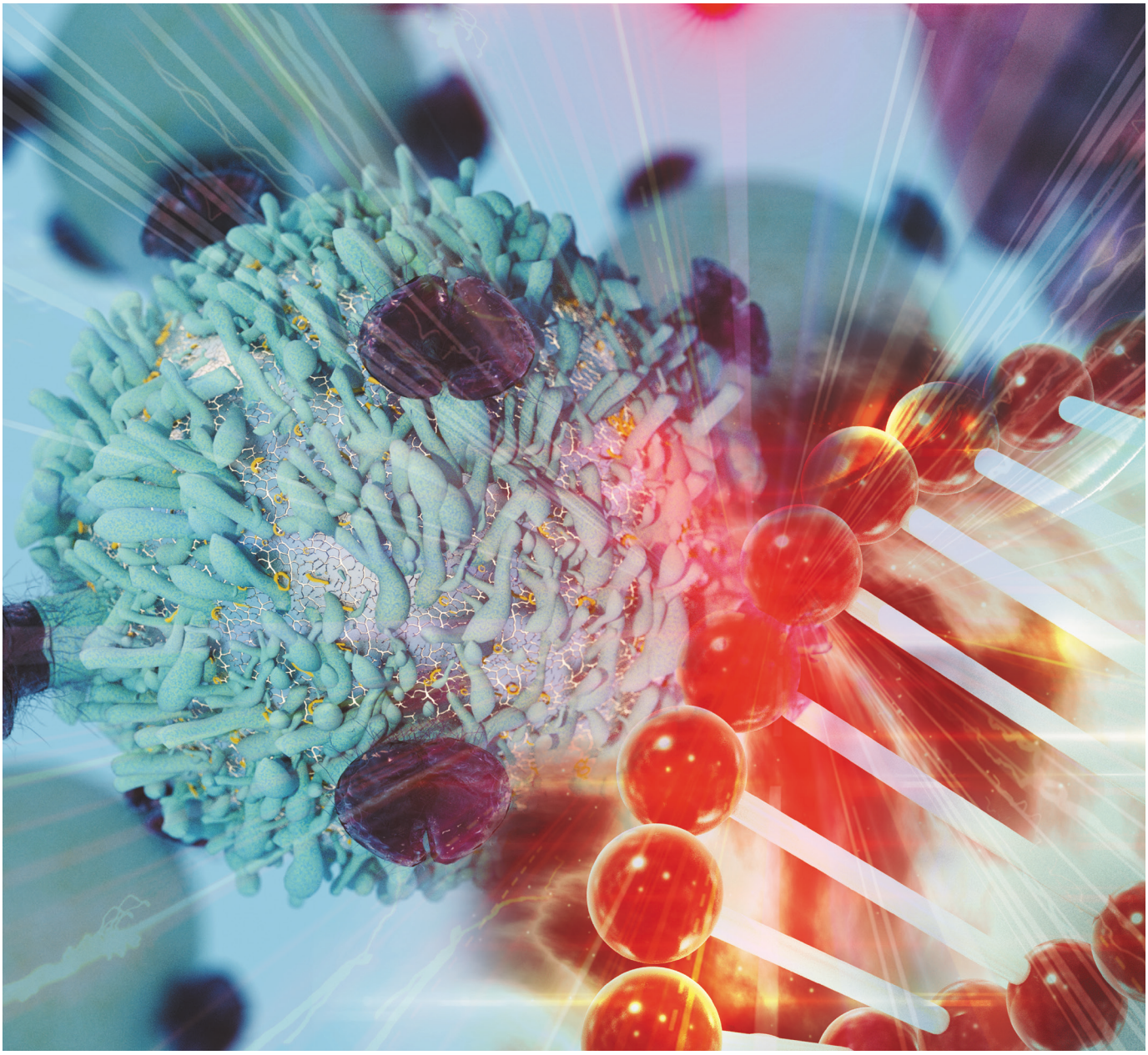
An additional example of the benefits of albumin in the viral vector application is a study by the University of North Carolina[4] that demonstrated incorporating human serum albumin improved AAV vector transduction –7-fold, resulting in a concomitant increase in expressed and active protein.Cell therapies are modified to alter their function, to activate because of immune cells, or to express certain proteins or antigens on their surface. When such modifications are conducted using viral vectored gene therapies to deliver recombinant DNA, chimeric antigen receptors, or CRISPR-Cas9 modifications, rAlb may improve the efficiency of such activities.

The consequences of the above study are that albumin may act as a "chaperone" assisting in the interaction between the AAV vectors and the cells. AAV enters cells via the endosome, which is also the pathway by which albumin is recycled. Whether such vectors are used to modify cell therapies, or to serve as standalone therapeutics, the addition of albumin can dramatically improve the performance of the modified cells.

Conclusions

Stem cell-based therapies represent some of the most cutting edge and sophisticated therapeutic developments currently in the biopharmaceutical industry, conferring substantial benefits for patients in addressing a broad spectrum of conditions. With a wide variety of stem cell types offering enormous therapeutic potential in the treatment of diseases, including multiple sclerosis, cardiovascular disease, liver disease and many forms of cancer, recent advancements have triggered an increase in their use, meaning that the concept is no longer such a foreign one. Likewise, the ability to unlock the life-saving potential of the next generation of virus-based drug treatments is in sight.

The field of advanced biopharmaceuticals faces several hurdles, however the use of rAlb can address many of



these. rAlb's natural stabilisation properties enable the formulation of challenging drug, vaccine and cell therapies, and its multitude of beneficial characteristics play vital roles at various stages of the development pathway.

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Patenting Antibodies at the European Patent Office

With antibodies accounting for seven out of the top ten global drugs[1], it is of critical importance that those companies that invest huge sums of money into R&D in this technology space are able to protect their investment from unlawful competition. Whilst the patent system provides a pretty good framework for achieving this, the approaches taken by patent offices in different parts of the world can vary widely, potentially impacting one's ability to secure optimal patent protection (in terms of territorial scope and/or patent claim scope). Unsurprisingly, therefore, the risk of failing to achieve commercially viable patent protection in a key jurisdiction (plus loss of an important revenue stream) provides a strong incentive to operate at the highest possible patentability threshold. Here, we will focus on the European Patent Office (EPO), and on the key issues you will likely need to address in order to obtain patent protection in Europe for a new antibody therapeutic.

Obtaining Patent Protection for Antibody Subject-matter at the EPO

For antibodies directed at a new target, such as a new antigen structure that has not been targeted before, it may be possible to obtain broad patent protection at the EPO for antibodies that bind specifically to that target. However, these broad patents are becoming less common because it is now unusual to discover such a new target. Most antibody patents, therefore, relate to antibodies that bind to a known target, and where other antibodies binding to the same target have been described.

The EPO allows the patenting of new antibodies, but only when said antibodies also demonstrate an unexpected technical effect (i.e. an inventive step) when compared to antibodies that were known before. Unlike some patent offices around the world, which may concede that a new antibody is inventive because of a unique structure or sequence it possesses when compared with previously known antibodies, a difference in structure or sequence alone is not enough to establish inventive step at the EPO. Moreover, this remains the case irrespective of whether said unique structure or sequence maps to the framework regions or to the complementary determining regions (CDRs) of the antibody. Thus, a new antibody against a known target will only be considered inventive by the EPO if it shows an unexpected property, or if it was unexpected that such an antibody could be produced at all.

A key component of the EPO's reasoning is that many techniques in the field of antibody production are routine, and that antibodies against a given target can be produced in large numbers without any inventive input being needed. For example, the EPO considers it routine to immunise animals with an antigen, to obtain a large number of different antibodies against that antigen that are produced by the animals, and to screen the resulting antibodies to confirm binding to the antigen of interest. Because the generation of antibodies in such immunisation methods is essentially random, the EPO assumes that essentially all antibodies against that antigen could be found eventually by just routine trial and error experiments, given a sufficient amount of time and resources. As a starting point, therefore, the EPO will assume that any antibody that has been produced against a known target could have been found in a routine way, and so is not inventive. Thus, the burden lies with the applicant of a patent application to convince the EPO otherwise.

The EPO also considers that other techniques in the antibody field, such as humanisation and affinity maturation, are now routine, and again that trial and error would eventually produce any effective humanised or affinity-matured variants of a starting antibody. This suggests that over time, it may become increasingly difficult to persuade the EPO that antibody claims are inventive, as more techniques for antibody production, optimisation and selection become routine in the field.

Thus, for new antibodies that bind to a known target (particularly if other antibodies against that target are known), the applicant must demonstrate there is something that makes said antibodies surprisingly better than other antibodies that were known to bind to the same antigen (else surprisingly better than would have been expected based on what was known about the target antigen and corresponding antibodies).

In theory, any kind of advantage can be relied upon. For example, this might relate to the way that the antibody binds to its target, such as improved specificity, cross-reactivity, or affinity; it might relate to improved properties of the antibody *in vivo* or *in vitro*, such as improved pharmacokinetic properties, low immunogenicity, or improved biological activity; or it might be based on other properties of the antibody which do not relate directly to its binding properties, such as improved storage stability, improved formulation properties or improved expression levels.

In practice, any advantage relied on must be surprising in its own context. For example, for a humanised antibody, the EPO might reasonably expect that it will have reduced immunogenicity compared to an antibody that is not humanised, so such a technical effect alone is unlikely to be enough to confer an inventive step. However, if a humanised antibody were to retain a high affinity for its target antigen, this might reasonably be considered surprising and thus supportive of an inventive step.

Conversely, if an asserted technical effect is found to be unsurprising, then it is unlikely the EPO will allow any patent

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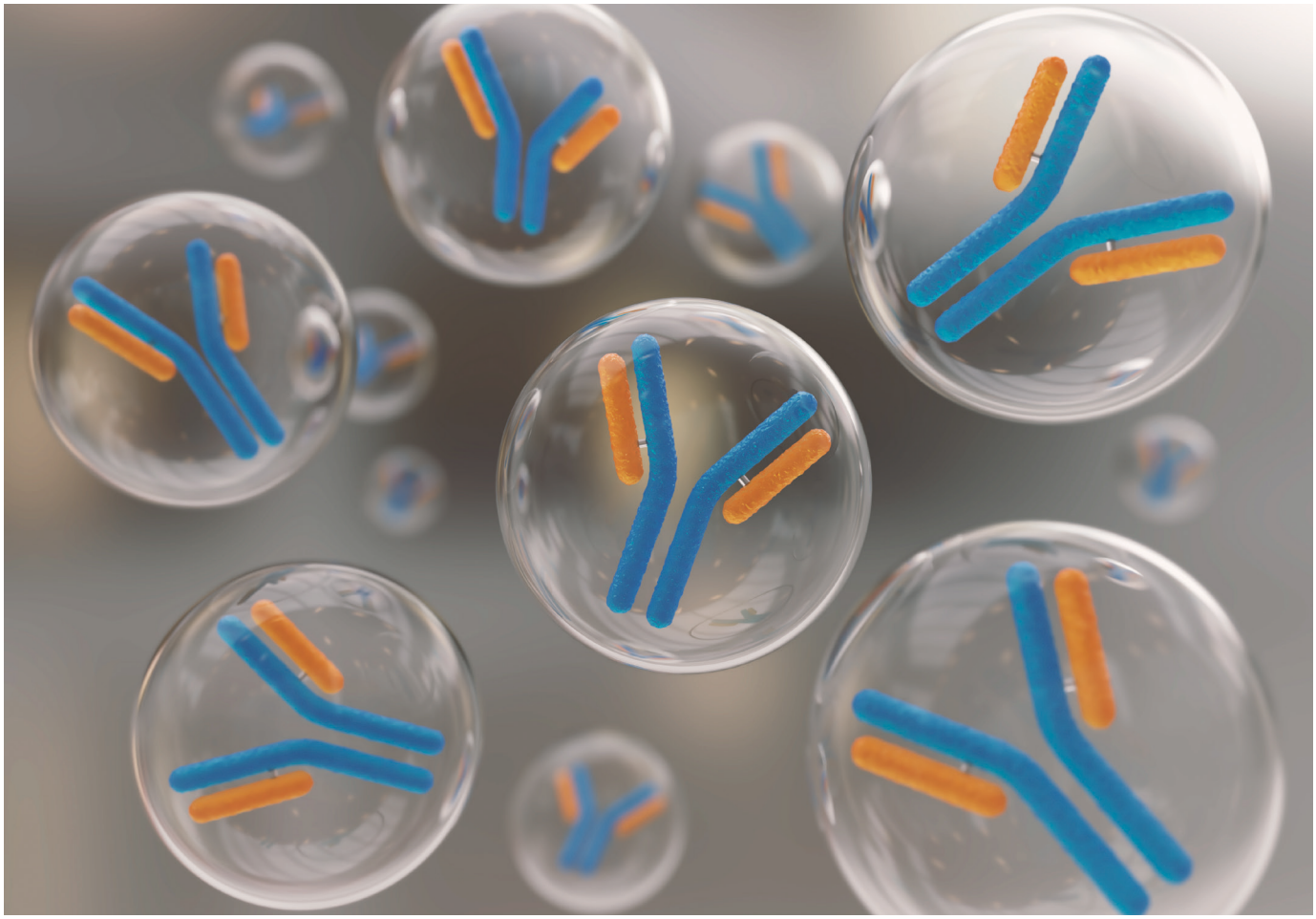

claim to the antibody, irrespective of whether the antibody in question is defined by reference to all six CDR sequences, both variable domain sequences, or even the full amino acid sequence of the antibody molecule. In such scenarios, even a very narrow "picture" claim is unlikely to be awarded.

Having established the presence of an unexpected technical effect (and thus inventive step), the next question to be considered by the EPO is that of claim scope. Namely, how broad can I claim variants of the antibody?

This assessment flows from analysis of the scientific principles that underpin the asserted unexpected technical effect, and attempts to ensure that the scope of patent claim awarded is commensurate with the level of scientific contribution the invention provides above and beyond the prior art. To put this another way, the EPO's general approach is the unexpected technical effect relied on to support inventive step must be demonstrated across the full scope of the awarded patent claims. In more detail, it must be at least technically credible that all the antibodies across the scope of your defined claims demonstrate the same unexpected technical effect / advantage. The EPO's analysis will, therefore, involve considering the properties or features of your antibody that are responsible for that advantage.

For example, you may be able to establish that your antibody is inventive because it has particular binding selectivity for one antigen and not to another. If your inventive step is based on the binding specificity or selectivity of your antibody, then the EPO is likely to take the view that the advantage might reasonably be shared by other antibodies that have the same set of six CDRs, or both variable domain sequences of your antibody. For this type of advantage, the EPO will, therefore, usually insist that you limit your patent claims to antibodies that include all six CDR sequences, or both variable domain sequences, of your antibody. It can be possible to obtain broader protection than this, but to do so, it is likely that you will need evidence that the same advantage is also found for other antibodies that do not have these particular sets of sequences. For example, to obtain a patent that does not require all six CDR sequences to be present, you might need data showing that antibodies with fewer than six CDRs, or antibodies having particular variations in the CDR sequences, will retain the same inventive advantage. The scope of patent claims that you will obtain will depend upon the related antibody sequences that you can persuade the EPO will retain the inventive advantage.

Another common advantage that is used to establish an inventive step at the EPO is improved affinity. The EPO considers that the choice of framework regions, as well as the CDR sequences themselves, may considerably influence antibody affinity. This means that if inventive step is based solely on an antibody having improved affinity for a target, then the EPO is likely to require the framework regions and the CDR sequences to be defined in the patent claim. In practice, this means that you will be asked to limit your

patent claims to antibodies having the same heavy and light chain variable region sequences as your antibody. Again, if you wish to obtain broader patent scope, then it is likely that the EPO will require supporting evidence that the improved affinity would be retained with other framework regions.

The same principles will be applied by the EPO to any advantage that you are relying upon to obtain an inventive step. If the advantage is linked to a particular structure or feature of your antibody, then the EPO is likely to require that structure or feature to be defined in the claims. For example, if your advantage relates to improved effector functions, then the EPO may require particular Fc domain sequences to be recited in the claims. If the advantage relates to a physical property of the antibody, such as its stability or production yield, then the EPO may consider that to be a property of the molecule as a whole, and so require the full antibody sequences to be defined in the claims.

If you need to rely on a technical advantage over other antibodies, then how can you establish that such an advantage exists, and when do you need to provide that information? The EPO considers that it must be derivable from your original patent application that the invention had been made before that application was filed. This does not mean that your patent application needs to provide absolute proof of the advantage. Indeed, it may not be possible to include the ideal comparisons in your application to prove that an advantage exists. For example, the EPO requires that an inventive step is established when compared to what it considers to be the "closest prior art". In this field, that is likely to be an earlier antibody that binds to the same antigen and that has similar properties. However, you may not know at the time of the patent application being filed what other antibodies may exist to the same target, and you may not be able to determine which antibody the EPO will later consider to be the "closest". Even if you are aware of earlier publications describing other antibodies, those antibodies may not be publicly available, and so it may not be possible to carry out any direct comparison in order to confirm that an advantage exists.

What the EPO will look for in the patent application is enough information to make it technically plausible that the advantage would be achieved. Your patent application might include data demonstrating particular effects or measuring particular parameters for your antibody, and might, therefore, provide data that could be used for a subsequent comparison with other antibodies, or it might include technical reasons why an effect or advantage can be plausibly derived from the available data.

If you can meet this threshold and persuade the EPO that your advantage was technically plausible from the information in your original patent application, then you may be permitted to rely on additional evidence, not included in the original patent application, to confirm the existence of the advantage. For example, you may be able to submit *in vivo* data confirming effects that were shown *in vitro*, or you may be able to submit comparative data confirming that your antibody does show an improvement when compared to particular antibodies that the EPO has selected as the "closest".

In conclusion, the EPO takes a technical and scientific approach when considering inventions in the antibody field. Every case will be judged on its own facts, but in general, the EPO will start from a number of preconceptions about what could have been done in a routine way, and the burden is likely on you to counter those preconceptions in order to persuade the EPO that your antibody is inventive.

An antibody that is new, and that is effective at binding its desired target, is unlikely to be considered inventive by the EPO unless it also exhibits some kind of unexpected technical effect (e.g. an advantage) when compared to other antibodies against the same target. This is worth considering when you draft a new patent application in this field. Ideally, your patent application will include some data supporting the superior properties of your antibody, or it will at least include a technical rationale to make it credible that your antibody has such an advantage. You should also consider the scope of claim that the EPO is likely to allow based on the advantages that you can establish. If you want to obtain broader claims than are likely to be allowed by default, then you may need to obtain more data before filing the application, to show that your advantage can be obtained with a broader range of antibodies than might otherwise be expected. Most importantly, when preparing a patent specification, you should think ahead to present a tiered range of (plausible) technical effects that may be relied on to provide potential fallback positions during prosecution and beyond.

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Martin MacLean

Martin has over 20 years of IP experience with particular expertise in patent portfolio management for corporates and government, and has conducted over 100 European Patent Office (EPO) hearings. He has a strong biotech background and specialises in technologies such as protein therapeutics, antibodies, vaccines, expression systems, diagnostic assays, and 'green' agrochemicals. Martin is top ranked in Chambers UK, noted for being "a real guru in the healthcare and pharmaceutical field" in The Legal 500, and has been ranked in IAM Strategy 300 and IAM Patent 1000 for a number of years.

Hazel Ford

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Building a Patient-centric CGT Supply Chain

As more cell and gene therapies (CGT) move toward commercial production, biopharmaceutical innovators developing these potentially life-saving treatments will need reliable, fast and efficient technologies for seamless collection, processing and delivery. With the timeframe between harvest and reimplantation so critical, the industry needs to develop a "vein-to-vein" manufacturing and delivery system that keeps the patient at the centre. Industrialising the vein-to-vein supply chain is discussed to advance commercial production of these revolutionary therapies –including autologous therapies, matched-allogeneic therapies and personalised cancer vaccines. This article will also examine supply chain best practices to ensure effective manufacturing and delivery of CGTs to the patients who needs them the most.

Autologous cell therapies are today one of the most advanced types of cell and gene therapies in the race to demonstrate efficacy and commercialise. While autologous immunotherapies hold a very promising future, based on current commercially approved drugs such as Yescarta® and Kymriah®, and latest clinical trial results, developers of these therapies face significant supply chain challenges.

Autologous therapies, which are cell therapies manufactured from a patient's own cells, are manufactured by a complex supply chain, and final products must meet specific release requirements. Due to the criticality of the starting material, logistics around cell sourcing, patient scheduling for collection/infusion, transportation, and manufacturing needs to be coordinated, ensuring the highest standards, regulatory compliance, and safety throughout the process. Many current solutions for this vein-to-vein supply chain logistics are antiquated in nature, relying on phone and email interactions between all key stakeholders (Figure 1). As demand increases, it is clear that process industrialisation is warranted.

Challenges

Chain of Custody (COC) and Chain of Identity (COI) of the Patient Material

Autologous and/or patient-specific therapies pose challenges concerning turnaround times, as well as the chain of identity and custody to ensure that the final drug product is returned to the right patient.

Chain of identity (COI) and c hain of custody (COC) represent some of the greatest challenges for cell and gene therapies and are new requirements for many biopharma companies. They can also bring significant risks.

COI refers to the identity of the patient, starting material, and final product. COI is defined as the permanent and transparent association of a donor or patient's unique identifiers to their tissue and/or cells from order through collection, manufacturing, administration, and post-treatment monitoring.

COC refers to tracking the stakeholders, facilities, and locations involved in the handling of the starting material and final product. COC is defined as the permanent data capture from the start of tissue and/or cell collection through product administration of information related to staff that handled the product, actions performed by those staff, and the location/ date/time of those actions.

COI and COC are essential for compliance. Every patient product must be handled with well-defined and consistent procedures. Patient confidentiality and safety cannot be jeopardised. Each handoff (couriers, third-party manufacturers, hospitals) must be tracked and controlled to prevent errors and comply with regulatory requirements. Regulators recognise that manual processes cause data entry and mishandling errors, and encourage automation to reduce risk.[1]

Therefore, it is of primary importance to ensure that the traceability of the patient material throughout the entire process, starting with the shipment from the investigator sites,

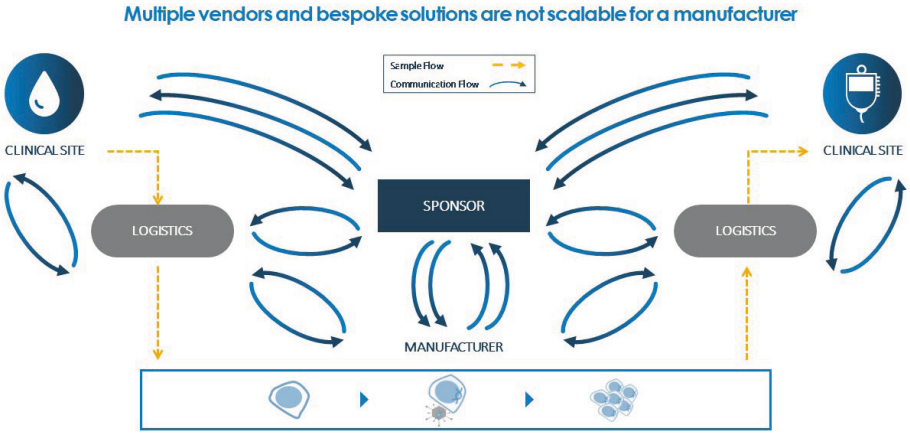

Figure 1: Complex interactions between supply chain stake holders

hospitals, or apheresis centres, to the manufacturing site and back to the patient is captured.

It is imperative to establish processes and technology that will guarantee that neither the samples nor the advance therapy medicinal product (ATMP) are at risk of cross-contamination at any point in time and can manage and optimise a high level of manufacturing demand.

Each sample and therapy is unique in that it requires traceability down to the tube or vial level. Thus, all players within the supply chain such as hospitals, clinics, manufacturing sites, and supply chain organisations must all communicate and coordinate at all times along the various points within the product lifecycle without impacting or increasing the turnaround time.

Challenges for Timing and Scheduling of Tissue Collection and Treatment

Challenges of tissue collection begin at the time of harvest. The starting cellular material taken from the patient is often stored at ambient temperature, which puts a strict time limit of 24–48 hours for clinicians and couriers to transport the cellular material from the patient to the manufacturing facility.[2]

Another challenge is the scheduling of manufacturing around the sample collection from patients. There needs to be increased flexibility around timing, taking into account changes or delays of delivery to the manufacturing site of the patient material. Constant communication is needed between the manufacturer, the clinic and/or patient on any shipment date changes of the final product.

Therefore, it is critical to provide real-time visibility to all supply chain partners. If key stakeholders are not apprised of product status or delays and delivery issues, trust diminishes, and patients are at risk. Manual supply chain management processes are error-prone and cannot scale safely.

Today more than 85% of the autologous cell therapies are developed by small to medium-sized biopharma companies.[3] One of the main pain points of small to medium-sized biopharmaceutical companies is their lack of a strong IT support organisation, which ensures that they are GMP compliant and build the IT infrastructures and digital interfaces required for them to meet their patient/customer needs.

Enabling an effective supply chain of identity and custody guarantees that each produced treatment is returned to the correct patient within the required time. This is not only critical for patients but also for developers.

Transportation and Logistics

As it relates to logistics management within the advanced therapy space, including cell and gene therapies, the current infrastructure utilised for the distribution of small molecule and/or biologics has proven to be insufficient. The fragility and urgency required in managing these advanced therapy products is not what past supply chains were built for. The industry is seeing accelerating clinical progression for personalised therapies for diseases from haemophilia to cancer in as little as four years. In January 2019, former FDA Commissioner Scott Gottlieb stated, "We anticipate that by 2020 we will be receiving more than 200 INDs per year, building upon our total of more than 800 active cell-based or directly administered gene therapy INDs currently on file with the FDA. And by 2025, we predict that the FDA will be approving 10 to 20 cell and gene therapy products a year based on an assessment of the current pipeline and the clinical success rates of these products."[4] This timeline compression has placed enormous pressure on existing supply chains, which were never set up to effectively manage risk for a drug product that is modified for a single individual – and in most cases does not have back-up doses if something goes wrong during transport or storage.

One common observation is that current cold chain container qualification and management processes are insufficient in effectively managing risk during personalised medicine distribution. This is due to the fact that these therapies require utilisation of equipment that must maintain exacting conditions, where a single temperature excursion may damage or destroy an unreplaceable sample utilised in the manufacture of a patient-specific therapy. Basic requirements for the traceability of equipment utilised in the manufacture and storage of drug substance and drug product must be implemented and utilised in the drug distribution space. In addition, integration of multiple informatics and supply chain IT systems is an absolute requirement in order to maintain efficiencies, optimise facility and equipment utilisation, as well as effectively manage risk.

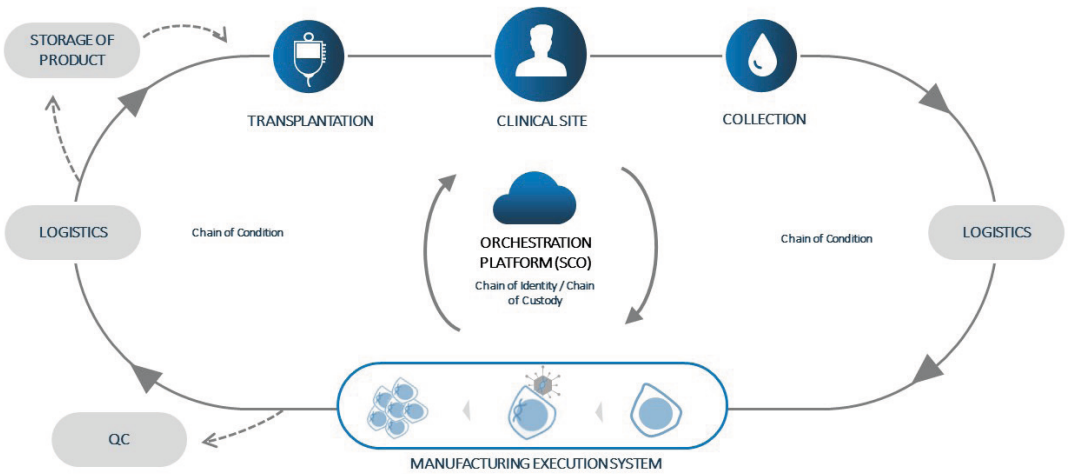

Figure 2: Partnership solution to link workflows of key stakeholders

Manufacturing Shop-floor Traceability
Aside from vein-to-vein traceability of the patient material, manufacturing execution systems (MESs) or "shop-floor" systems are also crucial to ensure that the COC as well as the COI are both documented and are guaranteed during the manufacturing process. Shop-floor systems are instrumental in providing visibility of the manufacturing activities in the overall supply chain of cell and gene therapies, therefore, gaining efficiencies in the area of batch record issuance/review and in the reduction of deviations. As the number of commercialised autologous products increase, shop-floor systems will need to be implemented by manufacturers and these systems will need to be seamlessly integrated with the other vein-to-vein supplier systems.

Potential Solution
As clinical timelines shorten, and the supply chain complexity increases, there is a growing need to provide a standardised and industrialised network of solutions. By developing a network of partners, an ecosystem can be created that is intended to seamlessly connect, allowing for information and material to flow through the key stakeholders without interruptions or errors (Figure 2). A standard offer for vein-to-vein supply chain logistics may increase speed to the clinic, elevate compliance, and provide a path for a commercialised therapy. Central to this offer would include a) an integrated SCO platform, b) a cold chain logistics solution, and c) an MES platform during manufacturing.

a. Supply Chain Orchestration (SCO) Backbone
To solve the challenge of implementing and managing a safe and secure supply chain, leaders in personalised medicine are implementing a new technology "backbone" – specialised software to assure that critical supply chain components such as COI and COC are properly maintained. These personalised therapy management (PTM) solutions help ensure safety, compliance and efficiencies for manufacturers.

One key feature of PTM software is the capability to control and manage COI and COC. Developing a proper COI involves capturing all of the critical unique identifiers associated with a patient, and ensuring they are properly linked. Additionally, this COI needs to be visible to all of those responsible for handling or manufacturing the drug product or treating the patient. COC functionality ensures handoffs of critical patient materials (both collected cells and finished product) are tracked (ideally via barcode scan) and signatures controlled to prevent the wrong materials going to the wrong location or patient. These events are summarised in a COC report for each patient, which can be provided to regulators to demonstrate process control.

Another critical software capability is a "control tower" view to provide clear, end-to-end visibility of each patient's starting material as it moves through the supply chain. This view can be tailored to different supply chain participants (CROs, case managers, healthcare providers, patients), providing only the detail necessary for the relevant role to protect patient privacy and data security. Along with visibility, these systems provide alerts when processes aren't moving as expected, as well as a control centre to manage changes, including rescheduling of key supply chain events.

Finally, this digital system provides reporting and analytics capabilities to drive continuous improvement of the supply chain, with the goal of boosting efficiencies, reducing end-to-end delivery times, and increasing success rates of patient treatments.

b. Transportation and Logistics
As mentioned previously, establishing traceability, or "Chain of Compliance®" (Figure 3) processes will effectively manage and meet International Organisation for Standardisation (ISO) 21973 recommendations for the distribution of advanced therapies. Chain of Compliance® is establishing full traceability of the equipment and processes used in managing the environmental control of the commodity. This includes transportation container performance and requalification history, commodity history, courier handling and performance history, calibration history and correlation competencies that can link in field events to equipment performance.

The reason that ISO released updated standards for advanced therapy distribution in guidance 21973 is that it provides recommendations around establishing the ability to collect, interpret, and leverage comprehensive data enabling a significantly more intelligent supply chain. Rather than reactively trying to determine what has gone wrong after multiple failures, it becomes possible to take a proactive approach. Moreover, effective implementation provides historical traceability of logistics processes, equipment, and third-party support entities enabling one to critically assess its complete supply chain and minimise failures and risk. What's more, having complete data gives you the ability to learn and improve over time to significantly reduce the risk of product failure. Robust data reveals patterns that you can leverage to minimise risk, year after year.

Five Essentials for Effective Chain of Compliance® Implementation
1. Start to continuously track individual equipment performance.
2. Track and archive what is being put into your equipment and who is moving it.
3. Ensure you have comprehensive (re)qualification and maintenance records for your equipment.
4. Integrate all four chains – custody, condition, identity, and compliance – into a single data stream for cross-referencing and accountability.
5. Insist on world-class Informatics, and real-time integrity measurements.

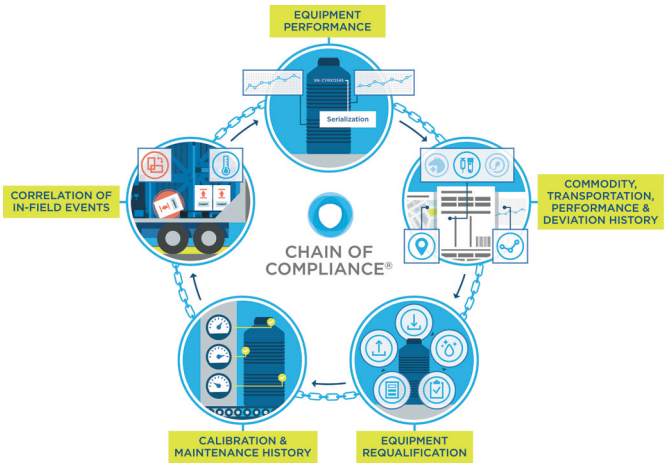

Figure 3: Complexity of Chain of Compliance®

c. Manufacturing Execution System Solution

The system that is central in the digitalisation of the shop-floor activities is the MES. The purpose of the MES system is to document manufacturing activities at the time they occur, by using digital capabilities to ensure the manufacturing process complies to pre-approved recipes. For example, an MES system will be able to determine if an incubator is suitable to be used for a patient based on the allocation, maintenance, and cleaning status.

Digitalisation of the COC at the manufacturing site is important to enforce the COI and mitigate the risk of cross-contaminations. It is a fundamental element to consider for commercial and clinical manufacturing, thus allowing manufacturers to parallelise the open phase of cellular processing. The complex process of implementing the MES systems is in part due to sites' requirements to change the way they work; however, the initial digitalisation of the COC is an imperative step within the MES journey. Upon the digitalisation of the COC, the expansion of the MES scope to digitalise the batch record (electronic batch records, EBR), with integration into planning systems (enterprise resource planning, ERP), shop-floor instruments, and other lab/quality systems shall be planned.

What's Next

In the past year, due to the global pandemic, the cell and gene therapy industry has experienced an unprecedented strain on the supply chain supporting clinical trials. Increased complexities of coordinating patient treatments, transportation limitations, and availability of critical raw materials has highlighted the need to strengthen the supply chain ecosystem for autologous cell therapies. In addition, it's clear that the value in the supply chain development goes beyond traditional CAR-T like autologous cell therapies. These types of solutions would be critical for other applications including *in vivo* viral vector gene therapies and well as allogeneic cell therapies. Additional ecosystem solutions that product developers need to think about include clinical site management, case management, robust procurement of critical raw materials, testing services, bio-services, and distribution.

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Marc Puich is Vice President of Business Development at Vineti, where he focuses on leading the global sales activities for the Vineti Personalized Therapy Management (PTM) platform. Prior to joining Vineti, Marc served as Vice President at Werum IT Solutions, the leading provider of Manufacturing Execution Systems for the Pharmaceutical Industry. There, he was directly involved with sales and implementation of Werum's PAS-X MES, as well as supporting organizations as they continued to drive benefits post go-live. Marc came to Werum with ten years of consulting experience as a partner focused on the pharmaceutical and biotech sectors at Tefen USA. He holds a Bachelor's degree in Physics from the University of California at Berkeley, and a Master's degree in Industrial Engineering and Engineering Management from Stanford University.

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Mark Sawicki is chief commercial officer of Cryoport and brings over 20 years of business development and sales management experience, having consistently delivered on corporate revenue and market share goals in the pharmaceutical and biotechnology industries. He holds a bachelor's degree in biochemistry from the State University of New York at Buffalo and a PhD in biochemistry from the State University of New York at Buffalo, School of Medicine and Biomedical Sciences. He also received graduate training at the Hauptman Woodard Medical Research Institute. Sawicki has authored more than a dozen scientific publications in drug discovery with a focus on oncology and immunology.

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