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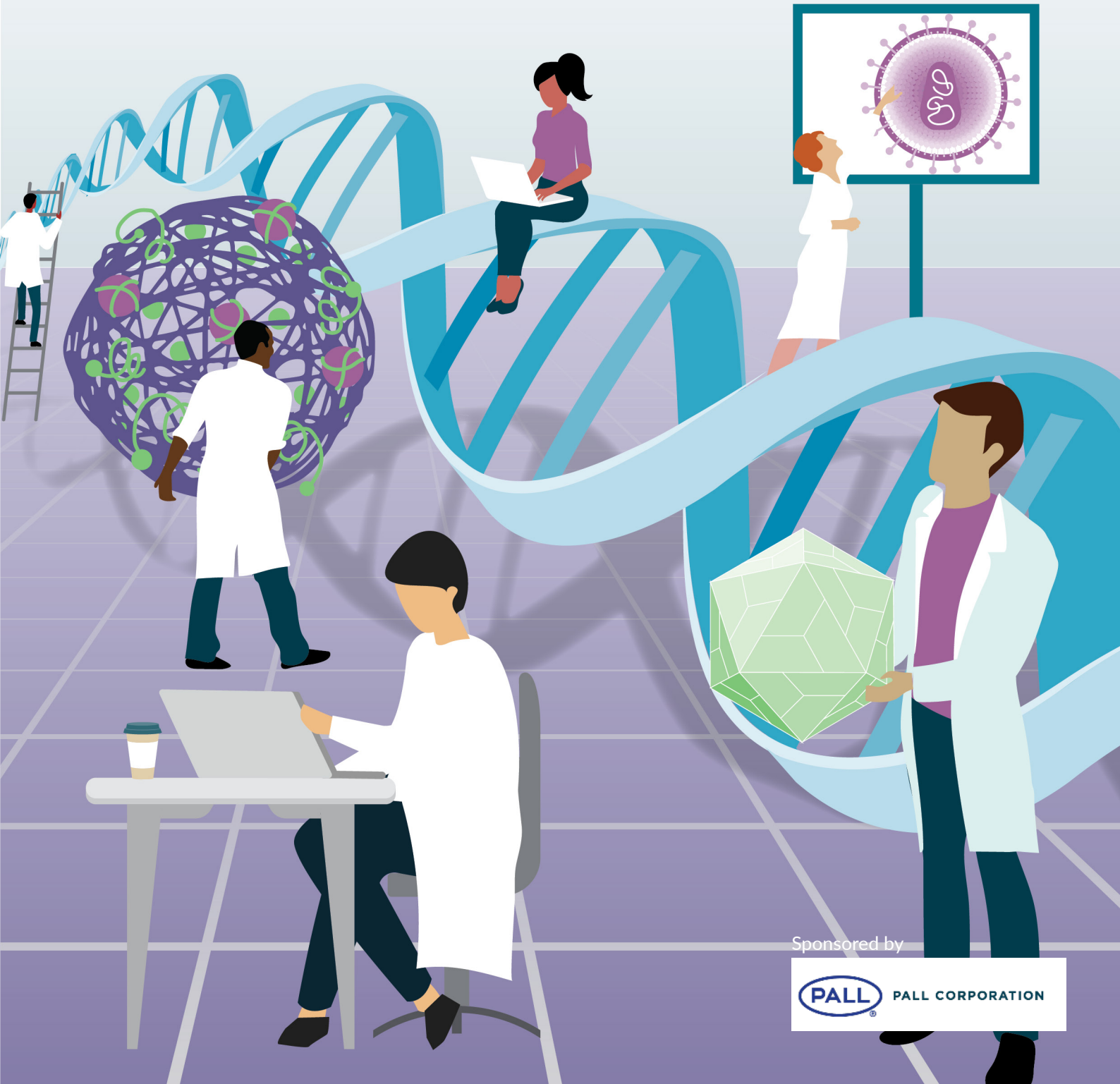
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON: Gene delivery/gene editing platform evolution



CELL & GENE THERAPY INSIGHTS

Volume 8, Issue 4

CONTENTS

Spotlight

Gene delivery/gene editing platform evolution

Latest articles

Vector Channel

Vector characterization

CELL & GENE THERAPY INSIGHTS

Volume 8, Issue 4

Report

Business Insights

CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

FOREWORD

Gene delivery & gene editing platform evolution

DR RODINO-KLAPAC is a gene therapy pioneer who has dedicated her professional life to advancing medicines designed to treat genetically based diseases. With professional experience across industry and academia, she is renowned for her contributions to molecular genetics and gene therapy that have advanced the field. She is author to a vast body of published, peer-reviewed work, the recipient of multiple awards, a National Institutes of Health (NIH) Fellow appointee, and current Board member of the Association for Regenerative Medicine, as well as a member of the American Society for Gene and Cell Therapy, the American Academy of Neurology, and the American Association for the Advancement of Science. She is the former head of the Laboratory for Gene Therapy Research at Nationwide Children's Hospital, established the Gene Therapy Center of Excellence within Sarepta and leads the Company's Gene Editing Innovation Center, directing a team of researchers to discover and develop novel gene replacement and gene editing therapies. She co-founded and served as chief scientific officer of Myonexus Therapeutics, a gene therapy company focused on limb-girdle muscular dystrophies acquired by Sarepta in 2019. She currently serves as Sarepta's Executive Vice President, Chief Scientific Officer. Her work has led to 11 investigational new drug applications and she is the co-inventor of SRP-9001, an investigational micro-dystrophin gene therapy, and the inventor of five investigational gene therapies for limb-girdle muscular dystrophy. She is the inventor of over 50 US and over 70 international published patent applications. She earned her PhD in molecular genetics form the Ohio State University and graduated summa cum laude from Kings College in Wilkes-Barre, Pennsylvania, with a Bachelor of Science degree in Biology.

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Welcome to the May 2022 *Cell & Gene Therapy Insights* Spotlight! This edition discusses recent R&D progress and current challenges in the ongoing development of the technologies

that will drive the continued growth and expansion of the gene therapy field. We take a look across the ever-increasing range of viral and non-viral delivery and gene editing platforms, and ask how these tools are looking to the future to adapt and drive the improvements in safety, efficacy, and efficiency that would be required to produce a step change for the biotechnology industrial sector.

Adeno-associated virus (AAV) continues to drive *in vivo* gene therapy as the dominant delivery platform of choice, and the field is flourishing with efforts to engineer around limitations such as pre-existing neutralizing antibodies as well as understanding safety related to systemic dosing. AAV thought-leaders Katherine A. High, Roland Herzog, and Genine Winslow lend their perspectives.

One of the most significant breakthroughs in gene editing of 2021 was the positive clinical data readout from Intellia Therapeutics and Regeneron for their CRISPR/Cas9 genome editing candidate, NTLA-2001, in patients with transthyretin (ATTR) amyloidosis. Intellia's CSO, Laura Sepp-Lorenzino, shares her reflections on that groundbreaking development, and discusses current and future trends and priorities in gene editing platform evolution and clinical application.

Non-viral gene delivery continues to grow as a technology area, and to progress into the clinical development setting. Metin Kurtoglu examines an RNA-based cell transfection alternative to the traditional lentiviral vector transduction approach for the engineered T cell immunotherapy field, while Umar Iqbal and Jagdeep K. Sandhu explore the utility of lipid nanoparticles (LNPs) as a targeted delivery vehicle for therapeutic mRNA. Like LNPs, exosomes continue to grab plenty of attention as both an emerging delivery platform of potential significance and a promising therapeutic modality in its own right. Antonin de Fougerolles *et al.* review recent advances in the field.

The potential for gene therapy and gene editing to make meaningful differences for patients remains astounding. Important advancements have been made with regards to manufacturing and clinical development based on experience and careful scientific investigation in the past 5 years alone. As we look to a future that may include other modalities such as non-viral delivery, the field with benefit from the lessons learned from AAV-based gene therapy.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Encouraging signs for a new generation of AAV-driven gene therapy

David McCall, Commissioning Editor of *Cell & Gene Therapy Insights*, **speaks to Dr Katherine A High**, President, Therapeutics, AskBio.

Dr Katherine High trained in internal medicine, hematology, and molecular genetics, and began her faculty career at UNC-Chapel Hill. After moving to the University of Pennsylvania, she conducted pioneering bench-to-bedside studies of gene therapy for hemophilia. These led to a series of basic and clinical investigations that characterized the human immune response to AAV gene delivery vectors. Her work evolved to encompass clinical translation of genetic therapies for multiple inherited disorders. As the inaugural director of a Center at The Children's Hospital of Philadelphia, where she was also an Investigator of the Howard Hughes Medical Institute (HHMI), Dr High assembled a multidisciplinary team of scientists and physicians to discover and develop new gene therapies for genetic diseases. In 2013, her Center at CHOP spun out as Spark Therapeutics, where she led the team that achieved the first FDA approval of a gene therapy for a genetic disease. After Spark was acquired by Roche in 2019, Dr High did a (virtual) sabbatical as a Visiting Professor at Rockefeller University, and in 2021 joined AskBio as President, Therapeutics. Dr High is an elected member of the National Academy of Medicine (US), the American Academy of Arts and Sciences, the Royal College of Physicians (London), and the National Academy of Sciences (US).

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Q What drew you to your current role at AskBio, and what have been your key activities in your first year at the company?

KH: I left Spark Therapeutics in February of 2020 with the intention to begin my sabbatical at Rockefeller University the following month – and I think we all know what happened then! During the pandemic, Rockefeller effectively closed unless you were working on COVID-19 and so my sabbatical was a virtual one. I continued to talk to the folks at AskBio over this period.

I'll tell you the things I really liked about AskBio. Number one, I think that Jude Samulski's long study of adeno-associated virus (AAV) had led him to make some innovations in manufacturing that I believe are going to result in a superior product – for example, getting away from plasmid DNA as a starting material (moving to wholly synthetic doggybone DNA, thus avoiding plasmid-related impurities in the product) and working on improved yields. There was also a big investment made by the company in the manufacturing facility in San Sebastian, Spain.

I liked the programs they have in hand. I liked the fact they are attempting to move gene therapy beyond single-gene disorders to complex acquired disorders.

I think in retrospect that the CEO, Sheila Mikhail, was prescient in making the decision to agree to the acquisition by Bayer, because it's certainly a very difficult time for publicly traded gene therapy companies to get traction right now – it's a positive to be part of a larger organization instead of having to try to raise money in the capital markets at the moment.

I knew beforehand that I liked Sheila a great deal and I thought it would be fun to work with a woman CEO, and it has been. It's been great to work with the colleagues at Bayer, too, and there are plenty of people at AskBio who know a lot about drug development. And I don't think there is anyone in the world who knows more about AAV virology than Jude Samulski.

Q You mentioned the move into larger patient population indications, and AskBio has both Parkinson's disease and congestive heart failure in its R&D pipeline. Tell us more about the current promise and challenges you see at the cutting edge of gene therapy application in these disease areas and healthcare settings

KH: Firstly, I should mention that there will be presentations about both of those programs at ASGCT, so I can't really talk about them in detail. But let me just say that the data looks encouraging.

What will be different about these indications as opposed to single-gene disorders is that when you have a well-chosen single-gene disorder, a well-designed vector, and a well-executed trial, if some of the patients respond then generally, most patients will probably respond. That has to do with the homogeneity of the population you are addressing and the predictable effects of the transgene product. In other words, if you look at the AAV gene therapies

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that have already been approved, they have been approved on just a few dozen patients. That's because if you have a single-gene disorder, and you put that gene back into the right target tissue, you are going to have profound effects on the clinical phenotype. However, I think the complexity of these acquired disorders is that they are almost certainly multi-factorial.

It is early days for gene therapy in these larger indications, but I suspect that when all is said and done, you will see some patients responding and others not responding, and that won't be because the transgene product doesn't work. It will be because the patient populations are more heterogenous, and some of the patients get to the same final common disease pathway via a different route. I think that in some ways, these complex acquired disorders may eventually turn out to be more like oncology, where we generally have to use three or four drugs in order to get high response rates.

These are just my own thoughts – the field doesn't have enough experience yet with trying to apply a single-gene solution to complex acquired disorders for anyone to be able to predict the future with confidence. But I'm very glad we are starting on this journey – I think it's a strategy that holds a lot of promise.

Q The FDA's acceptance of the multi-luminance mobility test (MLMT) as an approvable endpoint for voretigene neparvovec is celebrated as a landmark moment for the gene therapy field, opening up new pathways and giving confidence to AAV-based gene therapy developers. How do you reflect today on the development of MLMT and its lessons for the field moving forward?

KH: During the development of voretigene neparvovec, both the US FDA and the European regulators were very insistent that we needed a primary endpoint that would measure how a *person* functions in a visually dependent activity of daily living. They did not want visual acuity, they did not want visual fields, they did not want light sensitivity (as primary endpoint) – they wanted something like the mobility test. However, all the existing mobility tests failed to take into account the level of environmental illumination, which is the primary defect in RPE65 deficiency – a lack of sensitivity to light. That is why we had to design and then validate a novel test.

We developed that test in dialogue with the FDA, and they made a number of meaningful suggestions. One of them was that we should videotape all performances of the test, then send the videos in shuffled order to a group of independent graders along with

"... the existing mobility tests failed to take into account the level of environmental illumination, which is the primary defect in RPE65 deficiency – a lack of sensitivity to light. That is why we had to design and then validate a novel test."

a very detailed grading rubric, including how much to take off the score for colliding with obstacles, how much to take off for the test subject needing to be redirected because they couldn't see the arrows, how much to take off for taking more than three minutes to complete the test, and so on. In addition, we ran a separate study to establish the performance characteristics of the test in both sighted people and people with inherited retinal dystrophy. For that study, we also collected data on all test subjects' visual acuity and visual fields. Consequently, we were able to establish the relationship between the performance on the test with those well-accepted measures of visual function.

We made 12 different designs of the course itself that all had the same number of turns, the same number of arrows, and the same number of obstacles. And again, we kept statistics on each one of the 12 tests separately to determine whether they really did meet the criteria of equal levels of difficulty, in terms of the scores obtained. The 12 different courses were presented to patients in random order, and that was really to attempt to reduce the learning effect. Of course, there are visually impaired people who learn to get around by memorizing details of paths, so that was something we really did need to try to mitigate the risk of.

The FDA suggested some of those steps that made it a more rigorous test. We could see the wisdom of what they were requesting, so we did incorporate the suggestions, and then we were able to define a relationship between performance on the test (a test of functional vision) and visual acuity/visual fields (tests of visual function).

I think a very important point about the totality of the data we generated is that it was all internally consistent and it all hung together. For example, we actually had not anticipated from Phase 1/2 that the treatment would improve visual fields, but we kept it in as an exploratory endpoint. Then, in the larger Phase 3 study, we were able to show clearly that there was a big effect on visual fields, and that this improvement crossed the threshold that we had defined in the validation study for the mobility test, a threshold above which people with an inherited retinal dystrophy had a higher frequency of passing performances on the mobility test.

In fact, there were some limitations of the mobility test in that it had a ceiling effect. A number of patients, especially children, could, at the time of enrollment (pre-treatment) pass with a minimal number of errors at 4 lux, which is a pretty dim light level. And the dimmest we could go was 1 lux because at anything less than that, the video cameras couldn't record what was happening. What this meant in practice is that if a subject entered the test at 4 lux, they could only improve by one light level. It was impossible for them to improve any more than that – it was simply the end of the test. However, full-field light sensitivity, which was the first secondary end point, has a much greater

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dynamic range at lower levels of light. So we put those two pieces of data together, and this allowed us to show, for example, that even if there was a ceiling effect on the mobility test, the full-field light sensitivity might show a gain of two logs of light sensitivity. In other words, it extended the dynamic range of what we were able to detect as a result of the therapy.

Again, this is just to illustrate that all the data hung together and really reinforced the clinical findings. I think that made it more straightforward for the regulators to accept the novel endpoint. Novel endpoints can be difficult – I would certainly say that from my own experience in trying to get the paper describing it accepted, it's a daunting challenge.

Q As a former member of the FDA's Cellular, Tissue, and Gene Therapies Advisory Committee, what were your take-aways from the recent meeting to discuss the toxicity risks of AAV vector-based gene therapy products?

KH: I thought it was a really good meeting. It's always great when you have the FDA's participation in putting something like that together, because they have seen all the data.

A number of the problems that have emerged over the last couple of years have been in trials where people were using very high doses of AAV. I feel that we have now learned a lot about high doses – for instance, through some excellent clinical investigation in the Duchenne muscular dystrophy (DMD) gene therapy trials, we learned that very high levels of AAV can trigger complement activation in some programs, which leads to a whole series of related immune responses that were not seen at the lower doses used in hemophilia. I was really very impressed with the speed with which the teams conducting the DMD work deduced what was going on in terms of triggering complement activation.

Similarly, in the Audentes trial, very high doses of AAV were used in children who may not have had completely normal livers to begin with. This was an issue we wrestled with early on in hemophilia, because so many of the patients had a history of exposure to hepatitis B and hepatitis C. I remember very clearly first discussing this issue at a clinical advisory board with a group of hepatologists. In those days, we used a scoring system for severity of disease from hepatitis – the METAVIR scoring system, which went from 1 to 4. All the hepatologists agreed you should exclude people who were METAVIR 4, and all of them agreed you could include patients who were METAVIR 1 or 2. But when it came to METAVIR 3, a fight broke out! In general, I think the challenge with very high doses of AAV comes especially when there is underlying disease in the target organ.

So I thought that the symposium was timely. I don't know how others felt, but I felt more strongly than ever that high doses are not your friend. I think that in some situations, like DMD, people have understood what the challenges were and have learned to manage them. And they have not yet encountered any effects that they can't solve or mitigate.

Q What future trends in gene delivery and gene editing technology evolution do you foresee?

KH: Over the last two years, I have seen the first discussions of novel AAV vector capsids that are much more potent than the ones we have dealt with to date, and that can really be game changers in terms of allowing reduction of dose and therefore, reduced toxicity. I think that's a very exciting development, and again, some of this new work will be discussed at ASGCT this year, including by AskBio. It's too early to know for sure – not all of these approaches have been tested in humans yet – but the third-generation process involves screening large numbers of capsids in non-human primates, and heretofore at least, good performance in non-human primates has usually translated to good performance in humans. I think the advent of capsids that are much more potent than the ones we have been using to date is a key development.

I think that gene editing is going to be key in letting us extend gene correction to younger patients, whom we can't currently treat with AAV because their livers are still growing, and they eventually outgrow the therapeutic effect. The paper Intellia Therapeutics published in mid-2021 demonstrating they could do *in vivo* editing in human liver was very important.

Overall, my assessment is things are looking good for the field.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

Breaking new ground in the clinical application of CRISPR/Cas9

David McCall, Commissioning Editor, *Cell & Gene Therapy Insights*, **speaks to Laura Sepp-Lorenzino**, Executive Vice President and Chief Scientific Officer, Intellia Therapeutics.

LAURA SEPP-LORENZINO, PhD, has served as our executive vice president, chief scientific officer since May 2019. Prior to joining Intellia, Dr Sepp-Lorenzino was vice president, head of nucleic acid therapies, research leadership and a member of the external innovation team at Vertex Pharmaceuticals, Inc. from September 2017 to May 2019. From April 2014 to September 2017, Dr Sepp-Lorenzino was vice president, entrepreneur-in-residence and head of the hepatic infectious disease strategic therapeutic area at Alnylam Pharmaceuticals, Inc. Since October 2018, she has been a member of the scientific advisory board of Thermo Fisher Scientific, and in December 2020 she joined the board of directors of Taysha Gene Therapies, a biopharmaceutical company focused on developing treatments for monogenic CNS diseases. She is also a member of the BOD for Oligonucleotide Therapeutics Society and the Alliance for Regenerative Medicine. Dr Sepp-Lorenzino earned a professional degree in biochemistry from the Universidad de Buenos Aires in Argentina and a PhD in biochemistry from New York University.

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Q What are you working on right now?

LSL: At Intellia, we are building a full spectrum genome editing company. We are working on assembling and maintaining a toolbox of gene editing, as well as delivery modalities

that allow us to deploy the right combination of editing machinery to the right cell for a particular disease with precision and safety. We are applying this *in vivo*, delivering CRISPR/Cas9 as the drug, and *ex vivo* where we are using it as a tool to rewire cells with new instructions for a therapeutic purpose.

Our goal is to bring these potentially curative therapies to patients. The recent data we presented demonstrates CRISPR as a new therapeutic modality. Now, there are 15 human beings who have received CRISPR as a candidate medicine. We are seeing beautiful therapeutic and pharmacodynamic responses. We are very encouraged for what that means not only for the initial target disease, but for other programs in our pipeline and the field as a whole. It is a revolution in biology, and I am very proud to be leading the charge with my colleagues at Intellia.

On the pipeline side, we have a second *in vivo* program in the clinic: NTLA-2002 for hereditary angioedema. We are actively dosing patients, and we were going to release initial data on that program in the second half of the year. Behind that, there are a number of programs moving towards the clinic. With this approach, it is not only about knocking out genes, but also insertion of genes for loss of function diseases. We have two candidates for this: factor IX for hemophilia B, in partnership with Regeneron; and NTLA-3001, where we are inserting the wild-type form of alpha-1-antitrypsin for patients who are deficient. And on the *ex vivo* side, we recently announced that we are dosing our first patient with our autologous T cell therapy for acute myogenic leukemia, which is a big milestone for Intellia.

We have continued to work on developing an allogeneic platform, which offers a number of advantages over autologous cell approaches. We are applying it to *ex vivo* pipeline programs internally. We recently announced a wholly owned development candidate, NTLA-6001, an investigational allogeneic CAR-T therapy targeting CD30+ lymphomas.

In the platform development side, we continue to push forward on both gene editing and delivery technology fronts. On the gene editing side, we have canonical CRISPR/Cas9. We have a couple of enzymes that allow us flexibility. Additionally, we have a C-to-T base editor that we are applying. Then we have other modalities that we are also advancing – for example, a DNA writer that we are developing internally following our acquisition of Rewrite Therapeutics.

In short, we are firing on all cylinders on the three axes of our strategy – *in vivo* and *ex vivo* therapeutic candidates and platform development.

Q The positive interim data read-out from the Phase I trial of NTLA-2001 was arguably the standout story of 2021 in cell and gene therapy. What can you tell us about the ongoing study?

LSL: NTLA-2001 targets transthyretin, which is the causative agent of transthyretin (ATTR) amyloidosis. Last year, we showed interim data from the first two cohorts of part 1 of that study. Recently, we showed we completed part 1 for this study with four cohorts. The dose levels range from 0.1, 0.3, 0.7, and 1 mg/kg as a single IV infusion. Our findings reinforce and extend the observations we shared last year: we saw fast dose response and decline in serum TTR levels, which is a direct biomarker in the liver. Importantly, at the

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at 1.0 mg/kg dose level, treatment with NTLA-2001 led to 93% mean serum TTR reduction by day 28. We also saw consistency in responses for the patients in each of the cohorts, which is very important.

A new finding was that the reduction in TTR levels was quite durable. This duration ranged from two months to a year for the different patients in the different cohorts.

In addition, we saw an excellent safety profile. It was very well tolerated with most adverse effects being grade 1. We did have a severe adverse effect in one patient who had gastroparesis and vomiting. This is seen as part of the disease, as patients who have transthyretin (ATTR) amyloidosis often have gastrointestinal (GI) manifestations. This patient had prolonged vomiting, so he was kept in the hospital longer. However, we have not seen that with any of the other patients. We feel very comfortable with the safety. In addition, there were no laboratory abnormalities. There were some transient increases in AST that we solved quickly. Overall, we are very pleased with the performance of the drug and with how the trial is progressing.

Q Can you take us on a guided tour of recent gene editing platform evolution as you have perceived it - what are the key new directions for innovation, and what benefits can they deliver to the cell and gene therapy space?

LSL: CRISPR/Cas9 is a very precise mechanism to geolocate DNA sequences in the genome. Out of the three billion base pairs, with CRISPR/Cas9 you can hone in on a specific sequence. The canonical application of the technology is to introduce a double-strand break that you can then use either to create an indel to knock out the expression of a protein or to disrupt a particular sequence. It also helps to open up the DNA so that you can insert a gene or a piece of DNA to correct or to add functionality.

Due to the ease of use and specificity of this geolocation, you can modify CRISPR/Cas9 to have additional functionalities. There is an explosion of applications described in the literature as to the many different functionalities that range from changing nucleobases, to modifying epigenetic markers in the DNA, to regulating gene expression. Most recently, it has been shown to be able to write or to erase new sequences of DNA.

We have an opportunity to continue to push and think of different modalities that can be added. At Intellia, we are leveraging and realizing the opportunity of CRISPR firstly by using knockouts and insertions. We have invested since the beginning of the company in having additional modalities – we are working on DNA writing and other modalities. It is important to not just have a tool, but to make sure that tool serves a therapeutic purpose. This is combined with all the other elements you need to have to develop medicines.

Many publications present interesting data but many of those ideas are non-developable. At Intellia, because of the cross-functional and multidisciplinary team we have, we can take ideas and apply pharmaceutical development concepts from inception and bring them to fruition. For me, it is a fabulous place to be.

Q What are the key considerations for the clinical development of gene editing-based advanced therapies?

LSL: We have established a rigorous way of characterizing our clinical candidates. We are in the clinic in multiple geographies, including in the UK and New Zealand. We have talked to many regulatory agencies, and I feel comfortable that what we are doing has the quality and other attributes required to develop a drug.

Secondly, thus far, the clinical data looks superb. For patients undergoing gene editing, we have tons of preclinical data, which makes us confident this is going to be safe and efficacious. Ultimately, we need to see that in humans, and so far, everything looks as we expected. We are very encouraged by that.

The concern with some publications highlighting potential risks, is that they apply research-grade tools and conditions. Their findings are often not translatable to therapeutics, because there are different degrees of scrutiny for things you would use for a cell culture experiment versus a therapeutic. Understanding some of those caveats and how they can sometime lead to potentially wrong interpretations that can damage the whole field is important.

Q What future trends might we expect to see in gene editing platform evolution?

LSL: We are going to continue to develop multiple forms of CRISPR. The goal is to be able to mix and match, and use the right gene editing tool for the genetic change you want to elicit, for a therapeutic purpose. There is not a single tool that will serve for everything as there are different mechanisms and different changes. For us, we want to be

"...we are leveraging and realizing the opportunity of CRISPR firstly by using knockouts and insertions. ...It is important to not just have a tool, but to make sure that tool serves a therapeutic purpose."

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in a position where we have all the options available to make sure what we put forward to patients is the best modality and of the highest quality.

With regards to the field as a whole, right now we are all limited by delivery of the CRISPR modalities. It is as an area where I hope we and others will be making significant strides in opening new organs and new applications. We know how to modify the genome - the limitation today is how do you do that safely in humans. So far, we have seen it can be done for the liver. At Intellia, we have shared some data about doing bone marrow editing *in vivo*. We also shared some data a while ago on use by local administration to CNS. However, if the goal is to go everywhere for all therapeutic applications, we are not there yet. We do need to make advances in delivery to be able to realize that future.

Q Why was a lipid nanoparticle (LNP) platform selected as the delivery platform of choice for Intellia's *in vivo* gene editing therapeutic candidates – what are the advantages over the alternative options? And what are some of the specific considerations for LNP platform development?

LSL: LNP is a proven modality for the delivery of nucleic acid to hepatocytes in the liver. This is a smart decision that was made at the beginning of the company, years before I joined.

If we compare it with other delivery modalities, particularly with AAV for example, LNPs are non-immunogenic. There is no known immunity, so 100% of the patients will be amenable to receive your therapy. And if you need to re-dose, it is possible, unlike with AAV.

The second advantage is the unlimited cargo. LNPs are synthetic particles, so you can put large things there including different classes of RNA molecules.

Thirdly, we have designed these LNPs to be rapidly metabolizing. We know they can make it to the liver, deliver the cargo, and then it all goes away. This reduces any potential toxicity that could come from the LNP or the Cas9 enzyme.

Lastly, these are synthetic particles. In the manufacturing, you can do a lot of structural work to change properties, so they can be very versatile.

Q Turning to the ex vivo therapeutic setting, how is Intellia addressing the additional layers of complexity that a gene editing platform could bring to T cell immunotherapy product and process development?

LSL: For *ex vivo*, our goal is to be completely unlimited by the number of genetic changes we wanted to make. It is not just about putting a T cell receptor (TCR) or a chimeric antigen receptor (CAR) in a cell and letting it roll - it is making sure it is high quality and a homogenous product. We also want to be able to have other edits either to allow the use of allogeneic cells, and/or to introduce immune-enhancing edits to make the cells durable and more efficacious.

With that vision in mind, we looked at the state of the art in *ex vivo* gene delivery. Most people use electroporation. But with electroporation, the cells get beaten up. You take a hit on viability. Additionally, by itself, electroporation leads to random double-strand breaks. Additionally, we avoid the use of lentivirus that leads to random integration of the cargo, with a potential for insertional mutagenesis. Consequently, we have developed a platform where we use LNPs to deliver the cargo. We make our multiple changes, insertions, and knockouts in a sequential manner: we take cells, do edit number one, wait, then come back to do edit number two, and so on.

We showed data at the European Society of Gene and Cell Therapy (ESGCT) last year in doing five sequential edits – two insertions and three knockouts - with over 90% efficiency, ending up with homogenous, multi-edited cells. If you wanted to do this with electroporation, because you can only do it once, you would need to do it all at the same time. This would cause lots of translocations and a poor-quality product.

We are applying this approach to TCRs as well as CAR-Ts. We are happy with how efficient the product is, because it allows us to expand those cells and they have very good characteristics.

The other place we spend a lot of time is trying to get an allogeneic platform that is truly allogeneic. When people talk about allogeneic, there are three things you need to overcome: graft versus host disease, the elimination of T cells by the host CD8 and CD4 cells, and thirdly, if you have allogeneic cells that are missing Class I MHC, natural killer cells in the host will immediately destroy them.

At Intellia, we have found a way where we can avoid any of that happening. We believe these off-the-shelf cells will have the durability that will be required for adoptive cell therapies. You need to allow for the cells to outlast the tumor, making sure the tumor is completely controlled for the longest time possible. This is not what we are seeing in the clinic with other technologies. We are hoping that ours is going to be differentiated and superior.

Q You had a strong focus on RNA therapeutics in your previous career - can you reflect on the journey that RNA delivery platform innovation has taken over the past few years in particular, and share your thoughts on likely next steps and application areas for the field?

LSL: I remember when RNA came out and people thought it was going to be non-specific and toxic. Now, it is an approved therapeutic modality that gives beautiful data and benefit to people suffering from many diseases. I see many parallels with what we have learned through that journey that we are now applying to CRISPR. This is the evolution of technology – you build on the lessons learned from others. At the end of the day, we want to find the most efficacious and safe therapies for patients.

COVID has been an enormous disrupter of our lives, but it has also been a disrupter of science. Now, there are millions and millions of people vaccinated with a safe, quick, and efficacious LNP mRNA. This has shown us the value of investing in biotechnology, and continuing to react to new information in order to move the technology forward.

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AUTHORSHIP & CONFLICT OF INTEREST

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FASTFACTS

Accelerating adherent cell production for diabetes gene therapy using a scalable fixed-bed bioreactor platform

Tom Bongiorno, Field Application Scientist, Corning Life Sciences

Current technologies for adherent cell culture work well for small- to medium-scale applications, but producing large quantities of adherent cells or adherent cell products, such as gene therapies, is challenging due to limitations in space and labor. To help meet production requirements for larger disease populations, Corning has developed a novel fixed bed bioreactor (FBR) platform specifically designed for high yield production of cells and cell-based products from adherent cell lines.

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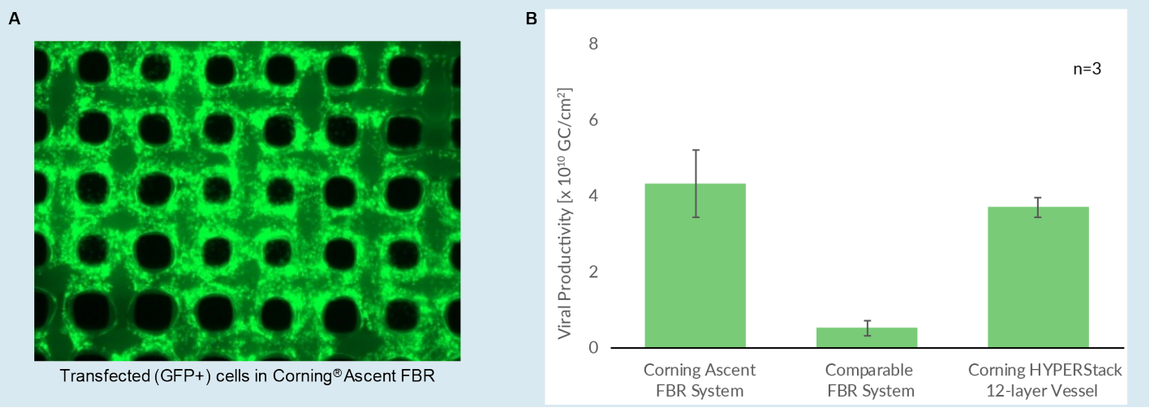
The Corning® Ascent™ FBR system is performance optimized for high cell density and productivity and provides linear scalability and improved yields that can drive significant cost reduction for gene therapy applications.

UNIFORM FLOW

The novel bioreactor of the Ascent FBR system utilizes a substrate of stacked PET woven-mesh disks, the geometry of which supports uniform fluid flow from the bottom to the top of the bioreactor. This uniform fluid flow promotes uniform cell distribution and growth throughout the bioreactor. As shown by GFP fluorescence in **Figure 1A**, the uniform mesh geometry of the Ascent system also results in uniform transfection, driving viral productivity. The 2.5 m^2 Ascent FBR system can achieve an eight-fold higher AAV2-GFP titer than a comparable FBR with a similar surface area **(Figure 1B)**.



The AAV productivity was comparable between the Ascent FBR system and the Corning HYPERStack® vessel, which is commonly used in the seed train leading up to Ascent. These data indicate effective scaling with minimal optimization needed when moving from existing planar protocols to the more 3D-like Ascent FBR system.

SCALABLE PERFORMANCE

The scalability of the system was tested by performing the same process in 1 m^2 and 5 m^2 Ascent FBR bioreactors. HEK293T cells were seeded into the Ascent FBR system at a density of 22,000 cells/cm^2 in both bioreactors. After 3 days of expansion, the cells were harvested at 152,000 cells/cm^2 in the 1 m^2 bioreactor and at 184,000 cells/cm^2 in the 5 m^2 bioreactor, representing seven- and eight-fold expansions, respectively. The scalability of the system is supported by the similarity of the expansion, cell viability, and cell recovery while the surface area increased five-fold **(Table 1)**.

ASCENT IN ACTION: GENE THERAPY FOR DIABETES

Hyperglycemia in diabetes can be caused by a deficiency of beta cells in the pancreas. AAV gene therapy can be used to reprogram alpha cells into beta cells, to help restore normal blood sugar levels. Researchers at the Gittes Lab for Diabetes and Pancreatitis Research at the University of Pittsburgh Medical Center scaled up production of one such gene therapy from small animal studies to primate studies, utilizing the Ascent system to produce AAV in HEK293 cells more efficiently.

Altogether, 3.5 billion genome copies (GC)/cm^2 were collected in the Ascent FBR system, representing a 48% higher viral productivity compared with a traditional 2D cell culture platform, at 2.36 billion GC/cm^2. The Ascent system allowed labor savings from automation, reduced footprint, and closed-system operations that enabled process steps to occur outside of a biosafety cabinet.

Table 1. Comparable yield and viability for HEK293T cells grown in 1 m^2 and 5 m^2 Ascent bioreactors.

	Ascent FBR 1 m^2 (n = 1)	Ascent FBR 5 m^2 (n = 2)
Seeding density (cells/cm^2)	22,000	22,000
Harvest density (cells/cm^2)	152,000	184,000
Fold-expansion	6.9	8.4
Days of expansion	3	3
Cell viability	96%	97%
Cell recovery	100%	99%

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

EXPERT INSIGHT

Engineered exosomes: a transformative therapeutic modality

Peter Jones, Dave Carter, David Lowe & Antonin de Fougerolles

Exploiting the therapeutic use of exosomes could revolutionize the pharmaceutical industry, by addressing major challenges such as enabling successful drug delivery to multiple tissues in a way that has a low immunogenicity profile and that allows repeat dosing. Exosomes are small, lipid-bound vesicles released by most if not all cell types. They play an array of biological roles and can carry a variety of cargo, which can be delivered into the cytoplasm of recipient cells. As 'nature's delivery vehicle' they can be engineered to carry different kinds of therapeutic cargo, from RNA to proteins and even viruses. At Evox, we are addressing many of the challenges of harnessing exosomes as therapeutics by optimizing them to maximize loading of a range of drug cargoes, by scaling up the consistent manufacturing of these exosomes, and by also engineering exosomes to specifically target cell types and surmount normally restricted biological and physiological barriers.

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INTRODUCTION

Exosomes are a type of extracellular vesicle (EVs) that are physically defined based on their size and method of cellular biogenesis. They are small nanometre-sized, lipid membrane-enclosed vesicles (approximately 30–150 nm in diameter) that are secreted by most, if not all, cells.

Originally identified in the 1980s, they were initially regarded as simple waste products being excreted from cells [1,2]. By the mid-2000s, it became evident that exosomes had a biological function and were in fact able to facilitate the shuttling of proteins and RNA between cells [3,4].

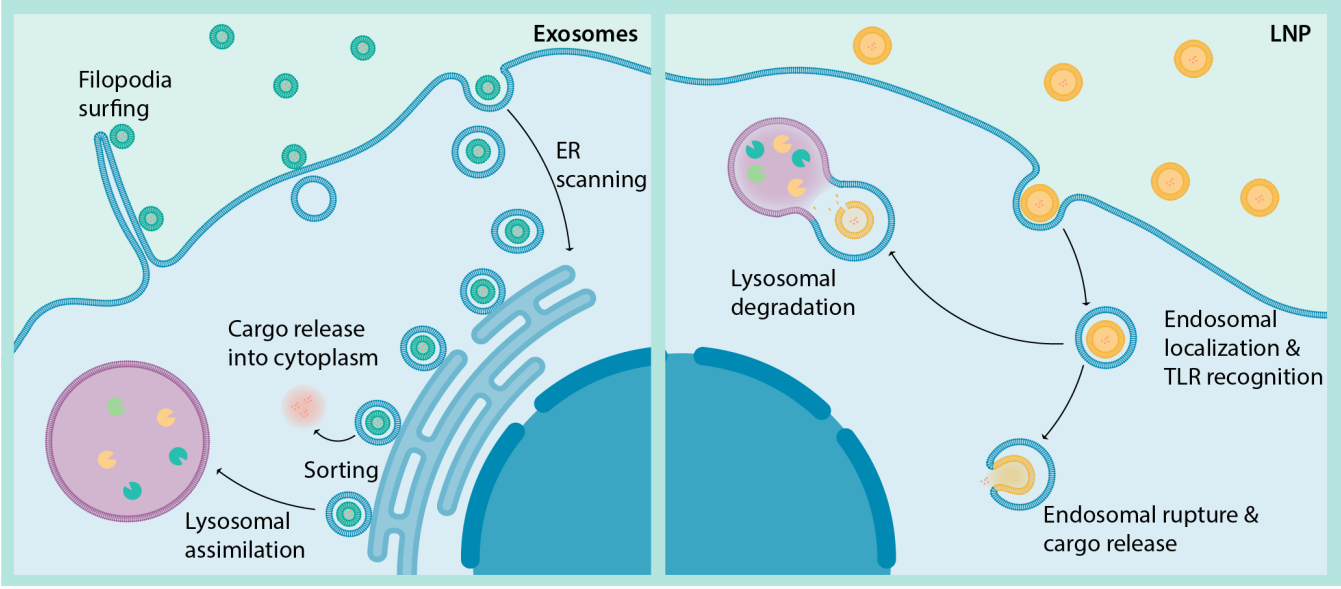
Exosomes (as well as other subsets of extracellular vesicles) represent a highly conserved and advanced system of intercellular communication, by which cells efficiently and safely exchange material [5]. EVs have been shown to be capable of naturally transporting a wide variety of cellular metabolic cargoes, such as proteins, lipids, transcription factors, miRNAs and mRNA, and it is this natural role of exosomes as 'nature's delivery vehicle' that has led to much interest in their potential exploitation for therapeutic use. Most of this therapeutic interest is focused on exosomes, but there is still much work to be done in characterizing the different classes of EVs from both a biological function, as well as a transcriptomic and proteomic perspective. Other classes of EVs, such as microvesicles, which are released through outward budding of the plasma membrane, or apoptotic bodies, may also hold potential for therapeutic development [6].

Exosomes are formed through invagination of the endosomal membrane to form an endosomal multivesicular body (MVB), which fuses with the plasma membrane, releasing the intraluminal vesicles as exosomes. The contents of EVs, their characteristics and cargo vary by the type and state of the cells involved in their generation. They can contain macromolecular material of the source cell including proteins, peptides, lipids, DNA, microRNA, long non-coding RNA and mRNA [7]. In contrast, other forms of EVs such as microvesicles/shedding particles and apoptotic bodies (both considered to range >100 nm in diameter) are released through outward budding of the plasma membrane or during apoptotic cell death, respectively [8].

Exosomes protect and deliver functional macromolecules intercellularly, including nucleic acids, proteins, lipids, and carbohydrates, transferring their cargo to recipient cells. As shown in Figure 1, the pathway by which exosomes deliver their cargo into recipient cells appears to be fundamentally different than other lipid-based nanoparticles

FIGURE 1

Exosome cellular uptake and internalization pathways appear to be distinct from lipid nanoparticles (LNP).



Adapted from Heusermann *et al.* [9].

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TABLE 1

Characteristics of different therapeutic modalities.

	Exosome therapeutics	mRNA therapeutics	RNAi therapeutics	Gene therapy (AAV)
Modality	Exosomes	Lipid nanoparticles (LNPs)	LNPs or conjugates	Virus
Safety	▶ Naturally occurring ▶ Non-immunogenic	Innate immune activation	Innate immune activation	▶ Pre-existing immunity ▶ Acquired immunity
Drug cargos	▶ Protein biologics ▶ RNA therapeutics ▶ AAV and gene editing ▶ Small molecules	mRNA	siRNA	Genetic material
Size limitations	No biological limit observed	None	None	~4 kb cargo limit
Repeat dosing	Yes	Unclear for >2–3 doses	Yes	No
Bioactivity	▶ Intrinsic intracellular access ▶ Broad organ distribution ▶ Targetable	▶ Delivery vehicle required ▶ Liver, myeloid cells/APCs	▶ Delivery vehicle required ▶ Liver, muscle, local	▶ Liver ▶ Tropism dependent

(LNPs) [9]. Exosomes seem to be taken up by filopodia as single particles and spend a significant amount of time interacting with the endoplasmic reticulum where it is hypothesized some cargo is released into the cell cytoplasm.

In contrast, LNPs, which are spherical man-made vesicles (composed of ionizable lipids, helper lipids, cholesterol and PEG lipids), are taken up by cells via endocytosis, and the ionizability of the lipids at low pH enables some endosomal release of the cargo into the cytoplasm [10]. Due to the inherent mechanism of action of LNPs, which involves localization to and disruption of the endosome, innate immune recognition sensors are often triggered requiring either pre-treatment of patients with corticosteroids and/or introduction of chemical modifications into any RNA drug cargo to reduce innate immune recognition. Although LNPs are efficient for mRNA transfer, it has been reported that exosomes are more stable and less immunogenic than LNPs [11].

Exosomes are conserved across different organisms, are produced by virtually all cells and therefore are found in all biological fluids [12]. As a result, exosomes are abundantly found in many routine medicinal products and procedures, such as blood transfusions and therapeutic plasma exchange [13], supporting their favorable safety profile. In addition, exosomes can deliver a variety of drug cargos including RNA without eliciting an innate immune response [14,15]. Due to their ability to transfer bioactive components and transverse biological barriers such as the cell membrane or the blood brain barrier, exosomes as shown in Table 1 are increasingly being considered as a therapeutic modality, enabling broader use of mRNA, RNAi and gene therapy (AAV) drug cargos. Multiple groups are also investigating the ability of exosomes to serve as a substitute for cell therapy [12,16,17].

There are over a dozen different clinical trials that have been performed or are on-going using non-engineered exosomes produced from different cell sources (for a recent review refer to [12]). To date these purified 'native' exosomes have demonstrated a promising safety profile in humans but have sometimes lacked sufficient efficacy in clinical trials [12].

As will be discussed in the next section, several academic groups and companies have been pursuing a variety of strategies to engineer exosomes to contain much higher levels of a desired drug (often increasing levels of

active molecules by several orders of magnitude). Importantly, from a safety perspective, these drug-loaded engineered exosomes continue to show excellent pre-clinical safety profiles [14,15,18] which is also corroborated so far by early clinical trial data [19].

ENGINEERING EXOSOMES AS POTENTIAL THERAPEUTICS

In order to develop robust exosome therapeutics, various groups (including Evox Therapeutics) are engineering exosomes to contain a diverse range of drug cargoes and to target different tissues. When developing an exosome therapeutic, an important consideration is the source of the exosome-producing cells [12]. At Evox, we have screened many potential exosome-producer cell lines and have identified several suitable human cell lines with known regulatory history. Our approach is to use a single human allogenic cell source for all patients with the intention that the same cell source might be used for multiple different disease applications.

Currently, Evox is developing proprietary cell lines based on CEVEC's amniocyte production cell line (CAP®) and human embryonic kidney (HEK293) suspension cell sources. Other groups are developing proprietary mesenchymal stem cell lines (MSCs) and induced pluripotent stem cells (iPSCs). An important consideration in sourcing a cell line, apart from its regulatory history, is to ensure it is well characterized and can be manufactured through a scalable process. In terms of manufacture, the selected cell line will constitutively secrete exosomes into the culture media (often several thousand exosomes per cell per day), from which the exosomes can be purified and placed in a vial for later administration to the patient [20,21].

An overview of Evox's exosome engineering platform and manufacturing process from drug loading through to final exosome purification is shown in Figure 2. One of the major breakthroughs in the development of exosome therapeutics has been the ability to engineer exosomes to contain high copies of drug cargoes. This can be done either through:

- Exogenous loading: purifying the exosomes upfront and afterwards loading existing drugs, such as siRNA and small molecules, directly into the exosomes. Loading of drugs into exosomes can be accomplished by a variety of means;

- Endogenous loading: where the cell making the exosome also makes the desired drug in a format whereby it is naturally loaded into the exosomes. Loading is achieved through genetic association of the drug to an abundantly expressed exosomal protein or fragment thereof.

At Evox, we have employed both approaches and have often been able to load hundreds to thousands of copies of a particular drug per exosome. As shown in Figure 2, dependent on the engineering approach used, these drugs can be loaded on the exosome surface (surface display), in the exosome membrane, or in the lumen of the exosome. Luminally loaded drugs can either be tethered onto the inner surface of the exosome membrane or the drug can be freely soluble within the exosome lumen, using an Evox proprietary pH-cleavage linker system. A more detailed review on exosome engineering and drug loading approaches will soon be published [Lowe D *et al.* 2022, *Drug Development and Delivery*, In press].

Multiple different drug types can be engineered into or onto exosomes covering nearly the entire breadth of drug modalities (Figure 3). It is also possible to load drugs of different classes into the same exosome, something that we are already employing when using CNS-targeted exosomes to deliver siRNA systemically to the brain and which we are now investigating in the gene editing context through loading of CRISPR-Cas enzymes into exosomes alongside guide RNA. Once drug-loaded exosomes are taken up into the desired target cells, the cargo is then released into the recipient cells.

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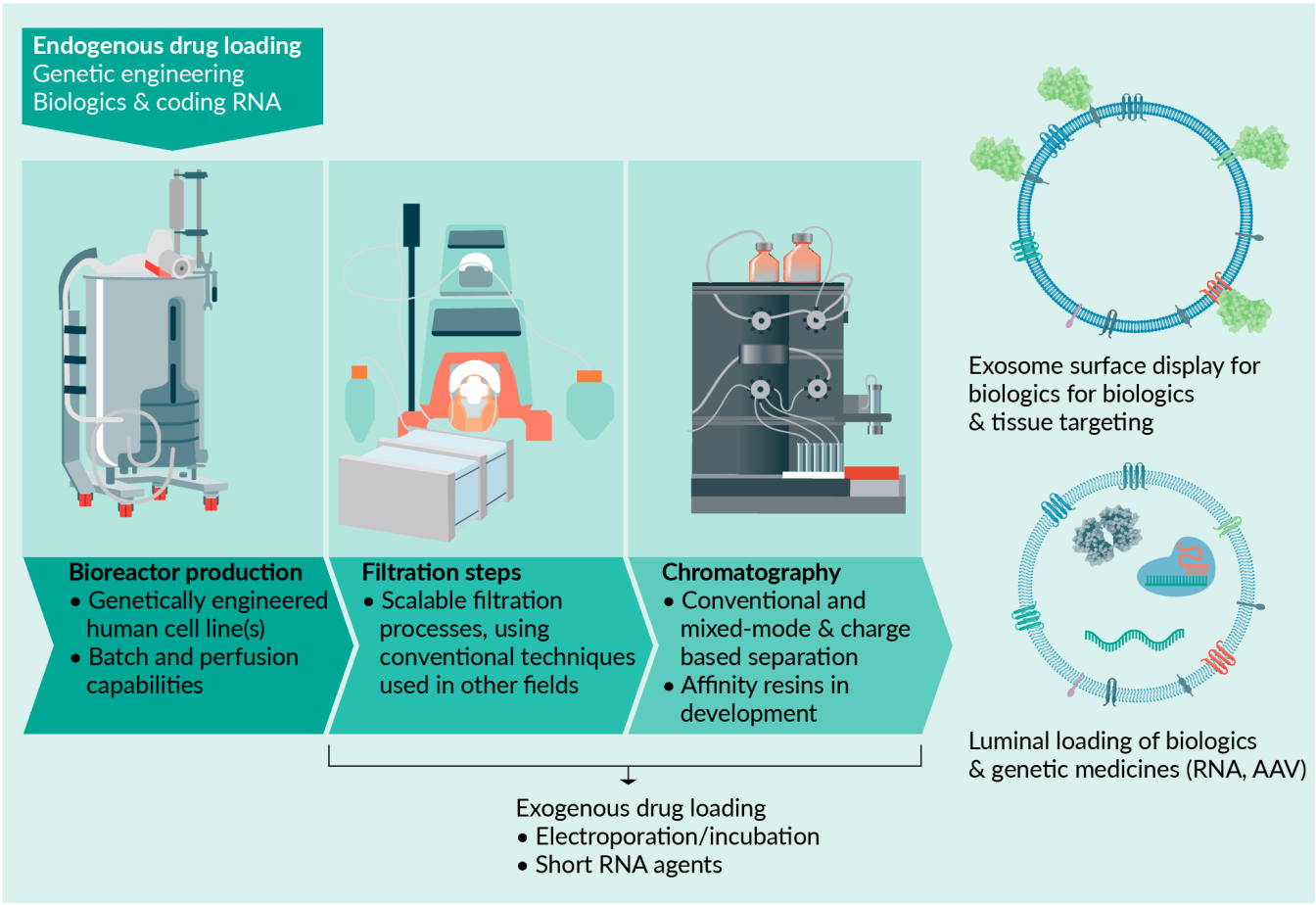
FIGURE 2

Evox's therapeutics platform - drug loading flexibility enabled by our DeliverEX™ platform coupled with a modular manufacturing approach.



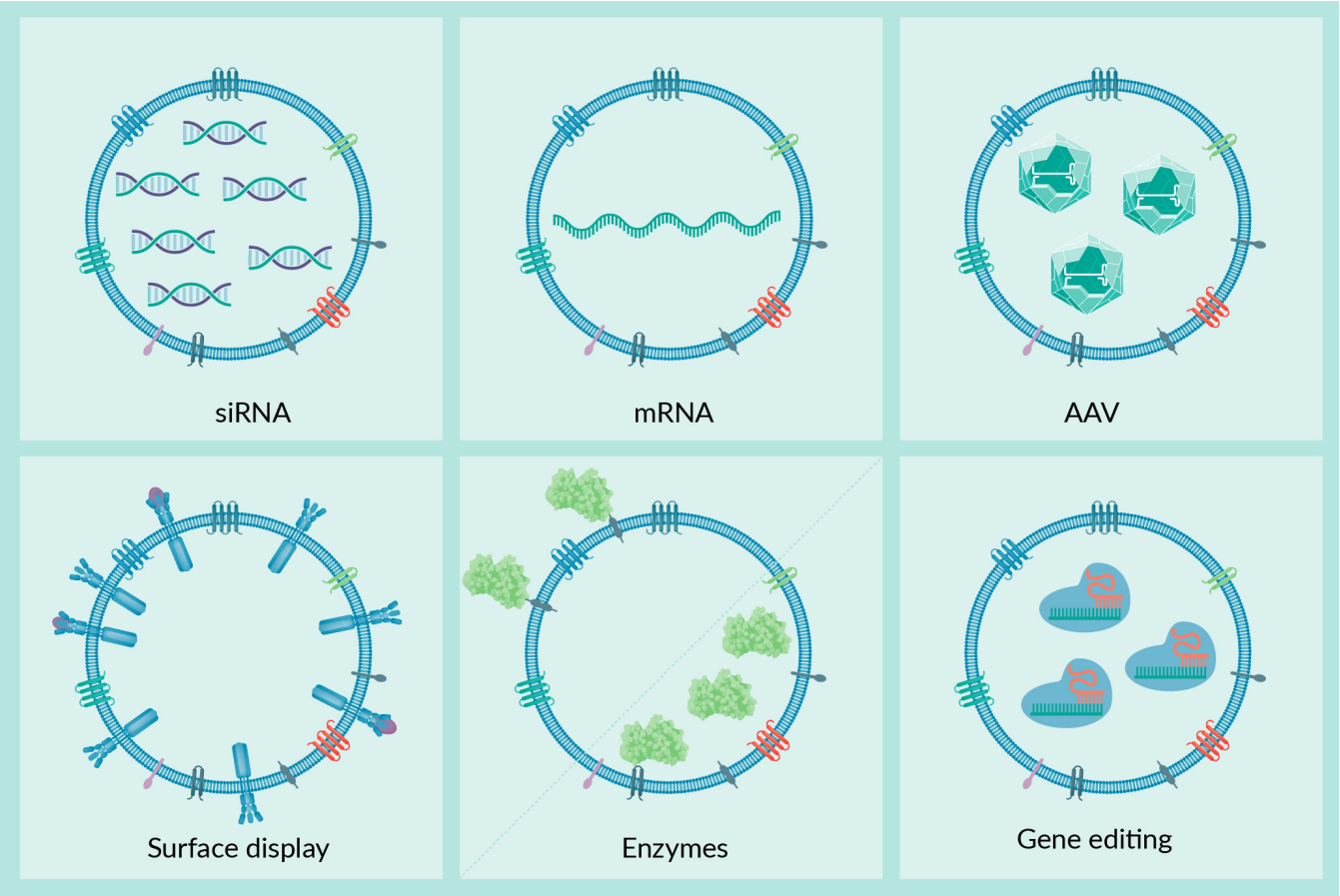
DISEASE APPLICATIONS OF EXOSOME THERAPEUTICS

The types of drug cargoes that can be delivered and have been shown to be effective *in vivo* in animal models includes RNA therapeutics (siRNA, anti-sense, microRNA, mRNA), proteins (such as receptors, enzymes, antibodies, and peptides) and small molecules. The engineering of exosomes to contain and deliver drugs *in vivo* was first reported by Professor Matthew Wood's group [22] and this approach has since been expanded upon by various groups to include delivery of different drug payloads to a variety of tissues (recent examples reviewed in [12]). Recent publications have highlighted the potential for exosomes to efficiently display therapeutic molecules such as IL-12 and TNF/IL6 antagonists on their surface [23,24]. Exosomes can also be engineered to display ligands on their surface in order to target exosomes to specific recipient cell types or to facilitate the crossing of physiological barriers as first demonstrated by Evox's co-founder Professor Matthew Wood and his group in a landmark *Nature Biotechnology* paper in 2011 [22]. The approaches being developed rely on multi-modular engineering strategies, where one exosomal protein is used to enable drug loading and another protein imparts targeting moieties on the surface of the exosome.

The exploitation of exosome-based therapeutics for the treatment of a wide variety of diseases could revolutionize the pharmaceutical industry by delivery of protein biologics and nucleic acid-based therapies into cells and tissues that are currently out of reach for other drug delivery technologies (Figure 4). Among the transformative therapeutic applications

FIGURE 3

Examples of potential therapeutic cargoes used with exosomes.



being considered for exosomes-based therapies is the ability of exosomes to deliver protein and nucleic acid across normally restricted barriers (such as the blood brain barrier) to enable access to the brain, the ability of exosomes to functionally deliver drugs such as antibodies into the cell cytoplasm, the ability of exosomes to allow repeat non-immunogenic dosing of AAV-based gene therapies, and the ability to expand the reach of RNA drugs to tissues beyond the liver and localized applications. With many of these newer advanced therapeutics such as RNA interference, mRNA, AAV gene therapy and gene editing platforms, the design of the drug is no longer the major limiting factor but is rather the inability to deliver them to the correct cellular or anatomic location. In this way, exosome therapeutics offer the possibility to dramatically improve a wide range of already established or readily available drugs.

An area where exosome-based therapeutics can make a transformative impact is in the treatment of rare genetic diseases. In recently published work, exosomes have been utilized to improve outcomes in many rare diseases [12]. As an example, it was demonstrated that exosome-mediated delivery of splice-correcting oligonucleotides could markedly improve production of dystrophin and lead to functional improvements in a murine model of Duchenne muscular dystrophy [18]. In this example, exosome-mediated delivery of a splice-correcting oligonucleotide resulted in 10–20% restoration of wild-type levels of dystrophin in *mdx* mice as compared to 1–2% correction when equimolar amount of the oligonucleotide alone were added.

Among the potential advantages of exosome therapeutics for the treatment of rare metabolic and lysosomal diseases are the ability to repeatedly dose exosome-loaded

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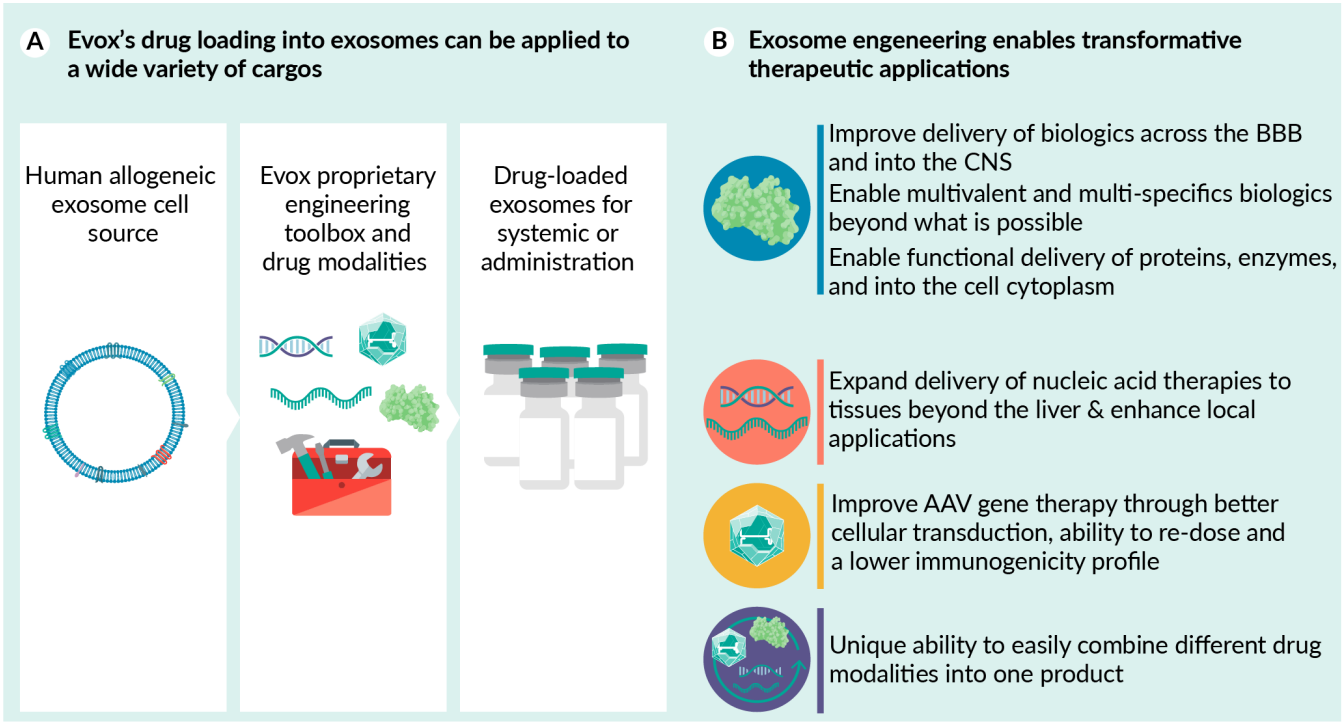
drugs, to dose titrate or withdraw drug as needed, to load a wide variety of drug cargoes regardless of size, to enable distribution to multiple different tissues or cell types at the same time, and to take advantage of the favorable safety profile to apply these to a broader set of rare diseases. These exosome-specific advantages to delivering drug cargoes are differentiated relative to other approaches to delivering gene therapy and gene editing. At Evox we are engineering exosomes to enable specific loading of therapeutic proteins, nucleic acids, or AAV-based gene therapy, with the aim of using these engineered exosomes as potential therapies in inherited metabolic and lysosomal diseases, such as phenylketonuria (PKU), argininosuccinic aciduria (ASA), Citrullinemia type I, and Niemann Pick-type C.

In addition, exosome-based therapeutics can also have broad applicability beyond rare diseases. Published *in vivo* pre-clinical work has already demonstrated that brain-targeted exosomes can be used to deliver RNAi drugs directed against α-synuclein and the Mu opioid receptor to the brain for treatment of Parkinson's disease and morphine dependency, respectively [25,26]. Other potential Parkinson's drugs such as redox enzyme catalase has been successfully delivered to the brain using exosomes [27]. In other applications, both non-targeted and tumor targeted exosomes have been shown to deliver RNAi drugs resulting in improved survival and tumor reductions in mouse models of pancreatic and prostate cancer [28–30]. Other published *in vivo* work has shown the ability for exosomes to enable improved delivery of small molecule drugs such as curcumin, doxorubicin and paclitaxel to tumors [31].

More recently, pre-clinical reports have suggested that exosomes may also be capable of delivering functional AAV capsids resulting in not only improved *in vivo* efficacy but also reduced immunogenicity [32]. AAV is commonly used in gene therapy due to its favorable safety profile, stability, and duration of transgene expression. One major limitation of

FIGURE 4

Engineering exosomes to create transformational drugs. (A) Evox's exosomes are engineered to enable loading of a variety of cargoes. (B) Applications and advantages of engineered exosomes.



BBB: Blood–brain barrier; CNS: Central nervous system.

AAV vectors is AAV neutralizing antibodies (nAb), which are present in many potential patients (preventing treatment) and develop in all patients following an initial dose (preventing re-administration). Encapsulation within exosomes (exoAAV) is a potential strategy to protect AAV from antibody-mediated neutralization, allowing efficient transduction and repeat dosing even in the presence of nAbs.

BENEFITS & CHALLENGES OF EXOSOMES AS A THERAPEUTIC MODALITY

The potential for using engineered exosomes as therapeutic delivery vehicles is vast. As summarized in Figure 5, some of these benefits include: the ability to deliver a wide variety of drug cargos at high concentration using the same underlying platform, the ability of exosomes to encapsulate and protect their drug cargos from harsh environments, and the ability to introduce a variety of different drug-like attributes through exosome engineering.

While exosomes are very versatile, sometimes there is a need to introduce additional drug-like properties into them. Some of these hurdles and potential mitigation strategies are discussed below:

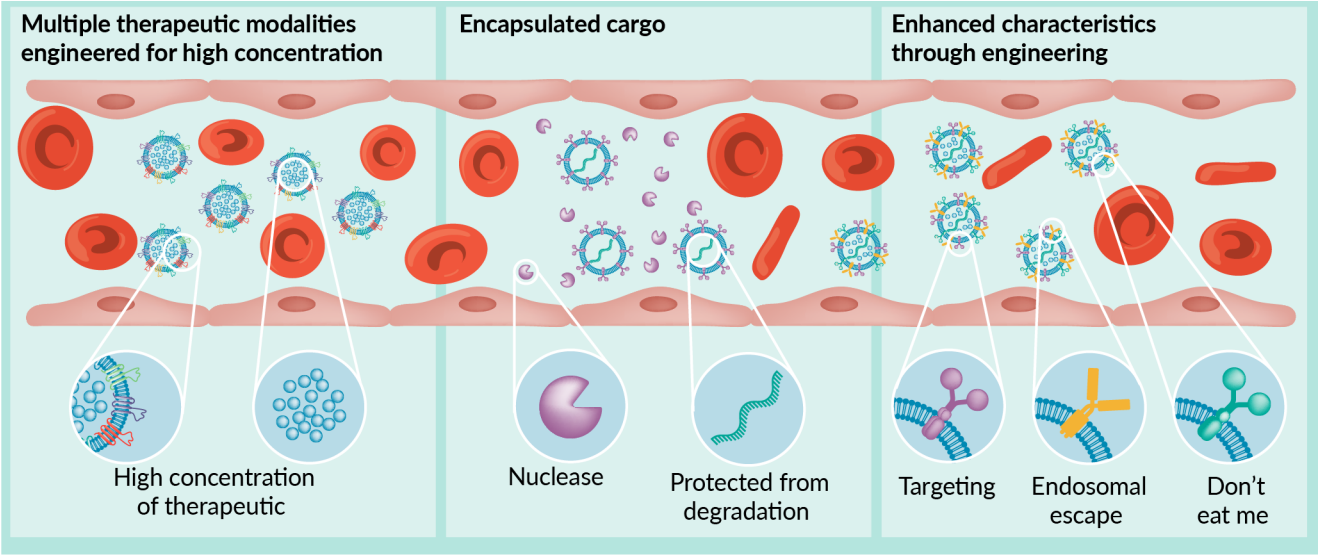
- Exosomes can protect their cargo from degradation in blood, but their half-life in circulation is relatively short due to rapid uptake into tissues and clearance. While this rapid clearance can often be an advantage, if long circulation time is desired, this can be addressed by engineering 'don't eat me signals' such as CD47 onto the surface of exosomes [33]. Localized delivery of exosomes can also be leveraged to increase persistence time of the therapeutic to the target cells of choice;

- The natural tropism of exosomes from different cell sources can be harnessed to target specific organs. However, EVs from commonly used clinical grade cell lines, such as HEK293, may not home to the desired target organ. To address this, the biodistribution of exosomes can be altered by engineering targeting moieties onto the vesicle [18,22,34];

- Exosomes are naturally occurring and have low immunogenicity. To further mitigate any immunogenicity risk, human protein sequences can be used where possible. A

FIGURE 5

Potential benefits of using engineered exosomes.



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protein 'corona' has also been shown to surround exosomes in plasma and this may provide an immunogenic cloak [35];

- For cargo that is luminally loaded, cytosolic delivery of the therapeutic product may require improved endosomal escape or disruption. This can be overcome by additional engineering of the exosome to include fusogens or other endosomal escape mediators.

- Exosome biogenesis is complex, and cells produce subpopulations of vesicles, which may vary in their incorporation of therapeutic cargo [36]. Improvements in understanding vesicle biogenesis could lead to optimized loading strategies. Use of affinity resins might also be used to separate therapeutic cargo-containing exosomes from 'empty' vesicles and other impurities.

However, as with any exciting new modality, there are still challenges to overcome. Many of these relate to technical considerations of general exosome work [37], including identifying the optimal isolation methodology for a given application of exosomes [38], the heterogeneity of extracellular vesicle preparations [39], and the availability of suitable methodology and equipment. As the exosome field has expanded in the past few years, tremendous progress has been made to address these challenges. We have greater understanding of how isolation methods impact on the characteristics of exosome preparations, ever-improving bespoke equipment for analyzing exosome number and content, and clearer insights into the biology of exosome production and heterogeneity [36].

Maximizing the inherent potential of EVs as therapeutics may also require the optimization of several EV characteristics. Depending on the clinical application and type of drug cargo to be delivered, these might include lengthening the biodistribution and pharmacokinetics of EVs, maximizing drug loading, improving intracellular delivery of cargo, and avoiding non-specific uptake by phagocytic cells.

SUMMARY

Engineered exosomes exhibit key characteristics that make them extremely attractive as therapeutics, particularly their ability to enable targeted delivery of a wide variety of therapeutic payloads, their benign safety profile, and their potential for low immunogenicity. As the exosome field continues to expand it is likely there will be other applications of this technology across an even wider spread of drug cargoes and diseases. While there are still obstacles that remain to be surmounted prior to the broader use of exosome therapeutics, we and others are addressing these remaining challenges. Through these efforts, the true potential of exosome-based therapeutics will be realized in the coming years.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

INTERVIEW

Evolution in gene therapy for the treatment of hemophilia

David McCall, Commissioning Editor of *Cell & Gene Therapy Insights*, **talks to Roland Herzog**, Riley Children's Foundation Professor of Immunology, and Director of the Gene and Cell Therapy Program at The Herman B Wells Center for Pediatric Research, Indiana University

ROLAND W HERZOG, PhD, is currently Riley Children's Foundation Professor of Immunology, and Director of the Gene and Cell Therapy Program at The Herman B Wells Center for Pediatric Research at Indiana University. He received a PhD in Microbiology from Auburn University in 1996, followed by postdoctoral training at the Children's Hospital of Philadelphia and faculty appointments at the University of Pennsylvania and the University of Florida. Dr. Herzog received multiple awards for his research, including from the American Society of Gene and Cell Therapy and the National Hemophilia Foundation. He currently serves as editor-in-chief of *Molecular Therapy*. His research team develops AAV-based gene therapies and immune tolerance protocols for hemophilia and defines immune response mechanisms to AAV gene therapy vectors and to therapeutic proteins used in replacement therapy for genetic disease.

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Q What are you working on right now?

RH: We work on various aspects of treatment of the bleeding disorder hemophilia, which is an X-linked inherited disease affecting boys and results in an inability of the blood to clot.

My lab is mainly focused on two aspects. One is to develop gene therapies for hemophilia and to further advance existing therapies to make them more efficacious. Secondly, we want to understand how the immune system interacts with the treatment, both in conventional protein replacement therapy as well as in more novel gene therapies. In some patients, there is a tendency for the immune system to reject the therapy for various reasons. We are trying to understand why that is, and how to avoid it happening.

Q How do you view recent progress in the hemophilia space, and what key challenges remain?

RH: Most of the clinical progress in the past few years has been on adeno-associated viral (AAV) gene transfer to treat both hemophilia A and B by transferring the functional gene into the liver of the patients. The liver cells then produce the functional clotting factor and secrete it into the bloodstream. Progress in this application area has been quite substantial and there are multiple vector products currently in Phase 3. For hemophilia B, which concerns efficiency in clotting factor IX (and for which there are fewer patients), progress is ahead of the curve and a couple of these products look particularly promising. Over the next couple of years, I would expect hemophilia B therapeutics to receive regulatory approval and become medicines.

Hemophilia A has been more challenging. Factor VIII, the protein lacking in hemophilia A, is trickier to express. There has been some success, but it has come with various challenges and in some trials, the gene therapy was not long-lasting and the factor levels declined over time. There has also been a certain degree of variability between patients, and it is not entirely clear why the levels of expression are not more stable. The field is now looking closely at these issues, so it has been disappointing yet encouraging at the same time.

"Most of the clinical progress in the past few years has been on adeno-associated viral (AAV) gene transfer to treat both hemophilia A and B by transferring the functional gene into the liver of the patients."

Q What does the recent Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC) meeting on AAV safety concerns mean for the field? And what learnings would you like to see the gene therapy space taking forward?

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RH: Especially in diseases that require very high doses of vector to be administered systemically to patients for various reasons, there are safety concerns. The discussion about the safety of these protocols shows that we still need to learn much about the basic biology of how the virus interacts with the host.

There are questions about how much of the virus integrates into genomic DNA and what the potential risk for insertion mutagenesis might be. Other questions include what causes the toxicities observed at high vector doses, and how much of the problem is related to the virus itself versus the underlying disease that may predispose the patient to certain adverse reactions. We need to learn more about the biology to improve these vectors so that they will hopefully be efficacious at lower doses. We need to see what we can change in the gene transfer protocol or design of these vectors to make them safer. It is also unclear what the impact might be of the various manufacturing platforms that different companies use in AAV vector production. There is also a need for standardization. It is difficult to compare one study to another at the moment, because the reported titers have not been independently verified or compared.

"We need to learn more about the biology to improve these vectors so that they will hopefully be efficacious at lower doses. We need to see what we can change in the gene transfer protocol or design of these vectors to make them safer."

At the same time, though, we are learning a lot from these clinical trials. Indeed, some of that knowledge can only come out of clinical trials, as there are limitations to what you can learn in animal or other preclinical studies. We have to be concerned and reflect on what has been found, but we also have to appreciate that a lot of the knowledge exists as a result of these trials. We need to know what is happening with patients and what can be changed to make the therapies safer. After all, in many cases, the patients are suffering from a disease for which there is no other good treatment, or at least not a cure. Gene therapy has a chance to treat diseases that are otherwise untreatable, and to cure diseases that can otherwise only be managed.

Q What is the state of the art in novel AAV vector design and engineering? How should we go about building improved vectors?

RH: We are starting to identify components of the vector that enhance immunity, or that are recognized by the immune system. We are starting to learn about how the early warning sensors within the innate immune system recognize the virus that we are injecting, and how that can link to the immune responses that we are trying to avoid. That would allow us to change the way the vector is designed or combine it with other therapies, such as immune modulatory therapies, to make the treatment safer and longer lasting.

Related to that, the field is developing methods to deal with preexisting immunity. Since AAV in different forms is present in the population, many of us have preexisting antibodies to the virus. Methods are being developed to be able to deliver the gene therapy medicine despite that preexisting immunity. The field is also continuously creating variations of the virus that are more efficient in transferring genes to particular organs.

Targeting is another big issue. How do you get the virus efficiently to the cells you want it in, and not to unwanted places? One approach is called molecular evolution. You have a library of viral capsids, and you screen for those that have the desired characteristics you are looking for. There may be more rational designs and ways to insert ligands into the surface of these viruses, or these viruses could be coupled to a ligand that will deliver it to the desired receptor of a cell. Similar approaches are being used to get to places that are hard to reach without surgery, thus avoiding these invasive procedures. For example, the field is working on ways to overcome the blood–brain barrier, so you can peripherally inject a vector and make it go to the central nervous system.

Another effort in the AAV field is looking at ways to overcome packaging limitations to be able to get larger genes into the vector. AAV vectors are constrained by how much DNA they can package. One way to potentially overcome this is to make two vectors in which the gene products could be spliced together to form a functional, larger protein.

The field is also looking at the structures of other related viruses. AAV is part of the family of parvoviruses, and there are other parvoviruses that have larger genomes. The structure of those viruses could be borrowed to make a vector that can accommodate larger genes.

Q What will be the ongoing trends in gene delivery technology evolution across the space?

RH: One key trend will be more efficacious and more targeted vectors, with less immunogenic designs. Combination therapies, such as combined gene and cell therapy, or gene therapy combined with immune modulation, are also a current trend. This can take the form of sequential treatment using different approaches earlier and later in life.

The other big development is utilizing gene editing to more precisely alter the genome, achieving lasting gene expression by inserting therapeutic DNA into a specific place where the gene is expressed in the way that you want. You can also turn endogenous genes that have a therapeutic value 'on' or 'off'. The related challenge to the field is how to deliver these more precise gene editing tools efficiently to the place where you want them to be, and how to do so in a way that ensures the components of the gene editing that should only be there transiently do their job, and then go away. You might not want them to stick around for different reasons, such as immune response.

Q What are your work goals and priorities over the next few years?

RH: We are trying to make progress in understanding why gene therapy for hemophilia A may not last in patients. We want to understand the underlying reasons for this, and what we can do to change it.

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The other big theme we are working on in the lab is the connections between the innate immune system and sensing of the virus and the viral infection, and how that relates to the types of immune responses that could eliminate our gene therapy. We wish to understand how the immune system senses and responds to the injection of virus. What exactly are those signal mechanisms, and how can we exploit that knowledge to avoid that whole process from happening? This will enable us to have more precise interventions.

Right now, much of what is going on in the field is using broadly immunosuppressive drugs, that are the same types you might find in organ transplantation or in the treatment of autoimmune diseases. Can we intervene more precisely and in a way that is more specifically tailored to our AAV gene therapy?

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

EXPERT INSIGHT

Unleashing the full potential of cell therapy through RNA engineering

Metin Kurtoglu, MD, PhD

The US Food and Drug Administration has approved Chimeric Antigen Receptor (CAR)-transduced T-cells (CAR-T cells) for the treatment of relapsed/refractory B-cell malignancies and multiple myeloma, ushering in a novel therapeutic modality that uses genetically engineered living cells as therapeutic drugs. CAR-T cell therapies on the market today are engineered by inserting CAR genes into the genome of cells using a virus. DNA-engineered cells maintain the therapeutic activity throughout their life because they are permanently modified. Further, their daughter cells carry the altered gene as the cells proliferate. In some types of relapsed/refractory blood cancers, this approach has been effective, though safety concerns remain. DNA-engineered cells carry a high chance of severe toxicity as there is not a natural 'off-switch' if the cells over-expand or otherwise exhibit off-target toxicity. Indeed, patient deaths have occurred due to uncontrolled proliferation of CAR-T cells. Therefore, all patients receiving CAR-T cell treatments must be strictly monitored for both short-term and long-term toxicity. This restricts the use of DNA-engineered cells only to patients with imminently fatal diseases like relapsed cancer that is refractory standard of treatment. A solution is to engineer cells with RNA rather than DNA. RNA does not integrate into the genome and has a measurable natural half-life, effectively limiting the duration and magnitude of therapeutic exposure. A defined half-life also informs the clinical protocol design for adjusting the dose and schedule of treatment to minimize toxicities. Therefore, RNA Cell Therapy is expected to provide safer alternatives without requiring cumbersome monitoring programs, and to allow the expansion of engineered cell therapies into indications beyond advanced cancer. In fact, RNA engineered CAR-T cell therapies are already being tested in an autoimmune disease (i.e., NCT04146051) as well as newly diagnosed cancer (i.e., NCT04816526). Other indications will quickly follow as safety experience with RNA Cell Therapy accumulates and new technologies make it possible to transfect billions of primary cells with one or more RNAs. In particular, the ability to engineer various cell types with multiple RNAs will lend itself to the design of novel combination products, a capacity that will be highly useful to treat complex diseases. This Expert Insight article reviews historical milestones that paved the way to the development of RNA Cell Therapy products now in clinical trials. Three examples of active clinical trials are described to show the transformative possibilities of engineering cells with RNA.

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Today, there are six genetically engineered adoptive cell therapy products on the market, all approved for the treatment of advanced B-cell malignancies or relapsed/refractory multiple myeloma [1]. Cells are engineered with a virus that carries a gene encoding a Chimeric Antigen Receptor (CAR), wherein the gene is integrated irreversibly into the cell's genome. [2]. Therapeutically a single infusion is often sufficient because cells can proliferate without losing CAR expression, a feature useful for eliminating large tumors. These CAR-T cells can also persist and surveil for tumor for extended periods. For example, 24% of patients with relapsed/refractory Diffuse Large B-cell Lymphoma treated with the first approved CAR-T treatment, Kymriah, had a lasting response about three years later [3].

In the same trial, 74% of the patients experienced Cytokine Release Syndrome (CRS), a potentially fatal toxicity that occurs secondary to proliferation of CAR-T cells [3]. Furthermore, in 23% of the patients, the severity of CRS increased to grade 3 or 4, requiring admission to an Intensive Care Unit (ICU) for several days. Now patients treated with the CAR-T cells on the market are required to follow a strict CRS monitoring program for up to 4 weeks following infusion [4,5]. Monitoring policies, which can require prophylactic admission to the ICU, are inconvenient, costly, and a potential cause of serious nosocomial infections. Kymriah's retail price is $475,000, and the American Society for Transplantation and Cellular Therapy recently advised hospitals administering CAR T-cells to bill $2 million to $2.5 million to cover the costs of monitoring and treating potential toxicities [6]. Furthermore, this cost does not take into consideration the risk of DNA-engineered cells to destabilize the cell's genome and transform into cancerous cells themselves due to irreversible and random integration of the CAR gene product into the cell's genome by virus-mediated gene transfer [7]. Taken together, the risk:benefit profile of DNA-engineered CAR-T cells are likely to foreclose use of this promising technology outside of advanced cancers.

One solution to make cell therapies suitable for treating diseases beyond oncology is to engineer the cells with RNA rather than DNA. RNA does not integrate into the genome and naturally degrades over time, thereby preventing the cell from behaving out of control, e.g., CAR+ cells do not proliferate indefinitely. The definable kinetics of RNA expression also allows for preclinical pharmacokinetic (PK) and pharmacodynamic (PD) studies to inform clinical protocol design, propose a dose escalation scheme, minimize exposure-related toxicities, and maximize efficacy. Safety monitoring can be tailored according to the half-life of RNA, which can reduce unnecessary visits or laboratory tests. These features render RNA Cell Therapy suitable for clinical investigation in indications in which a significant risk of life-threatening acute toxicity would be unacceptable, such as newly diagnosed cancers and non-oncological chronic diseases like autoimmune disorders.

While RNA cell therapy sounds elegant in principle, it has proven to be difficult in practice: despite 20 years of attempts since the first clinical trial of an RNA-transfected cell [8], no FDA-approved RNA Cell Therapy is available in 2022. Past failures have been ascribed to the rapid intracellular degradation of artificially introduced mRNA and to the difficulty in generating meaningful numbers of high-quality RNA-transfected cells. Here we describe some ways these challenges are being overcome, and the new possibilities that result.

MILESTONES IN RNA CELL THERAPY: HISTORICAL CHALLENGES & CURRENT OPPORTUNITIES

RNA cell therapy emerged as a new field after demonstration of efficient protein translation from *in vitro* transcribed mRNA in 1990 [9]. Initial studies focused on using *in vitro* transcribed (IVT) mRNA to express antigenic

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epitopes in dendritic cells for generating an immune response against cancer [8,10].However, clinical studies using IVT mRNA-transfected dendritic cells did not show a benefit. The largest randomized clinical trial was in 462 patients with metastatic renal cell carcinoma, where IVT mRNA-transfected dendritic cells showed no survival benefit over standard of care [11]. As adoptive T-cell therapy gained momentum in the early 2000s, efforts began to engineer T-cells with RNA. The first successful transfection of mRNA into T-cells was demonstrated in 2004 by Smits *et al* [12]. In 2006, Rabinovich *et al* reported the first anti-CD19 CAR-T cell engineered by mRNA transfection [13]. Following preclinical proof of concept, clinical trials testing CAR-T cells engineered via mRNA in various relapsed refractory oncology indications were initiated in 2010s [14–16]. At the time, the maximum production scale was $1–2\times10^{10}$ [9] CAR-T cells.[17] Two clinical studies using anti-mesothelin CAR-T cells showed that mRNA-modified CAR T-cells could be administered safely at doses up to 1×10^{10} [9] cells per infusion [16.18]. No CRS was observed despite the abundance of antigen for the CAR-T cells to engage, even though mesothelin is expressed on tumor cells and normal cells. Thus, unlike engineering cells with DNA, RNA-engineering can restrain CAR-T cells from multiplying out of control.

The initial wave of interest in RNA-engineered CAR T-cells receded in the 2010s as several studies in refractory oncology indications failed to show efficacy. Please see Foster *et al* [19] for a review of these studies. The primary culprit was manufacturing: sufficient numbers of viable engineered cells could not be produced. Even today, commercially available technologies can transfect only up to $1–2\times10^{10}$ [9] cells. Keeping cells healthy and functional through RNA transfection is challenging. However, recent developments in biotechnology have begun to overcome this challenge, and new novel platforms can already generate lot sizes that are an order of magnitude higher than the previous scales (see below for examples). Thus, with the right molecular engineering and transfection methods, the manufacturing scale of RNA-engineered CAR-T cells is no longer a barrier to clinical utility.

Another major historical limitation has been ascribed to the short half-life of mRNA. The median half-life of mRNA in human cells is about 10 hours [20]. However, not all mRNAs have a short half-life. By modifying the critical domains of IVT mRNAs such as the 5' cap, untranslated regions (UTRs) and poly A tail, cells can translate IVT mRNA for several days [21,22]. A major determinant of IVT RNA half-life comes from its ability to evade the intracellular RNA-degrading machineries that protects the cells from foreign RNAs [23,24]. Toll-like receptors in T-cells and cytosolic receptors in non-immune cells such as retinoic acid-inducible gene I protein (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), have evolved to recognize, inactivate and degrade non-native RNA sequences [23–26]. Toll-like receptors also stimulate interferon production and prompt host immune cells to remove RNA-transfected cells [27,28]. However, these hurdles can be overcome by incorporating modified nucleotides into IVT RNA such as pseudouridine, 2-thiouridine, 5-methyluridine, 5-methylcytidine or N6-methyladenosine, hereby increasing the half-life of IVT RNA and the resulting cellular phenotype [29–31]. Circular RNAs may also have longer-lasting translation capabilities than some linear mRNAs [32]. These advances in RNA synthesis mean that the RNA half-life is no longer a barrier for producing a highly effective cell therapy product.

Taken together, the historical limitations against producing high quality, non-immunogenic RNA and transfecting it into living cells at a large scale, are being overcome. The capacity to dose billions of high-quality cells repeatedly, each transfected with one or more IVT mRNA and each capable of expressing a protein for days or weeks, opens up a transformative range of possibilities to treat an array of indications. Below are three innovative examples of RNA Cell Therapy that are in Phase 1/2 trials.

RNA CELL THERAPIES ENABLE TRANSFORMATIVE POSSIBILITIES

Example 1:

RNA Cell Therapy can be combined with standard of care to specifically target the cause of treatment failure. For example, newer drugs for the treatment of multiple myeloma, such as proteosome inhibitors and immunomodulatory amides, result in deep, durable remissions, but almost all patients with high-risk genetic characteristics continue to have residual disease that relapses and is eventually fatal. A clinical trial is evaluating the ability of a BCMA-targeting RNA-engineered CAR-T cell (Descartes-08) therapy in combination with first-line induction therapy (NCT04816526) to eliminate treatment-resistant residual myeloma cells. Cells are infused without lymphodepletion chemotherapy (a common preconditioning treatment for conventional DNA-based cell therapies) since RNA-engineered CAR-T cells will not proliferate; removing an extra layer of toxicity that is required by DNA-engineered therapies.

Example 2:

Descartes-08 is the first CAR T-cell product to enter clinical trials for an autoimmune disease (NCT04146051) in generalized Myasthenia Gravis (gMG). This is a disorder of neuromuscular transmission caused by pathogenic autoantibodies that target critical components of the nicotinic acetylcholine receptor (nAChR) or other proteins supporting the receptor's function.[33] The antibody-driven nature of the disease strongly supports a central role for pathogenic autoantibody-producing long-lived plasma cells (LLPCs) in the disease process. However, all currently approved treatments target biologic pathways that are either upstream or downstream of LLPCs. Off-label use of CD19/CD20 targeting agents that directly target B-cells have shown limited success,[34] suggesting that BCMA+ LLPCs may be the main source of secreted autoantibodies. Descartes-08 can eliminate BCMA+ long-lived plasma cells and the inherent safety of the product seen with multiple myeloma supported its use in gMG.

Example 3:

There is no cargo limit in RNA Cell Therapy. Strategically designed RNA-engineered cells can translate therapeutic proteins that synergize to produce a highly potent clinical benefit, even in treatment-resistant diseases. An example of this is Descartes-25, an off-the-shelf, human umbilical cord-derived mesenchymal stem cell (uc-MSC) product engineered with three novel mRNAs to deliver a cargo of potent, synergistically active anti-myeloma therapeutics locally. These mRNAs encode a secreted BCMAxCD3 bispecific antibody, the effector molecule; secreted single-chain IL-12 (scIL-12), a potent cytokine that amplifies bispecific antibody activity and primes anti-tumor T-helper 1 polarization; and a homing protein that targets the cell to the tumor microenvironment for local delivery of the therapeutic cargo. Combining BCMAxCD3 bispecific protein and IL-12 is significant: IL-12 is highly synergistic with TCR/CD3 stimulation to produce IFN-γ, a pleotropic cytokine that can induce strong anti-tumor immunity[35]. Homing of the cells locally is expected to increase the safety margin of the product. This strategy is expected to result in deep and long-lasting responses in patients with multiple myeloma and autoimmune diseases, and Descartes-25 is currently enrolling patients with multiple myeloma in a Phase 1/2 study (NCT05113342).

CONCLUDING REMARKS

For many purposes, RNA Cell Therapy combines the best attributes of conventional RNA therapeutics (i.e., nanoparticle-based Covid vaccines and other therapies) and conventional DNA-engineered adoptive cell therapies. Compared with conventional RNA Therapeutics, RNA-engineered cells are minimally immunogenic and enable repeat dosing, and

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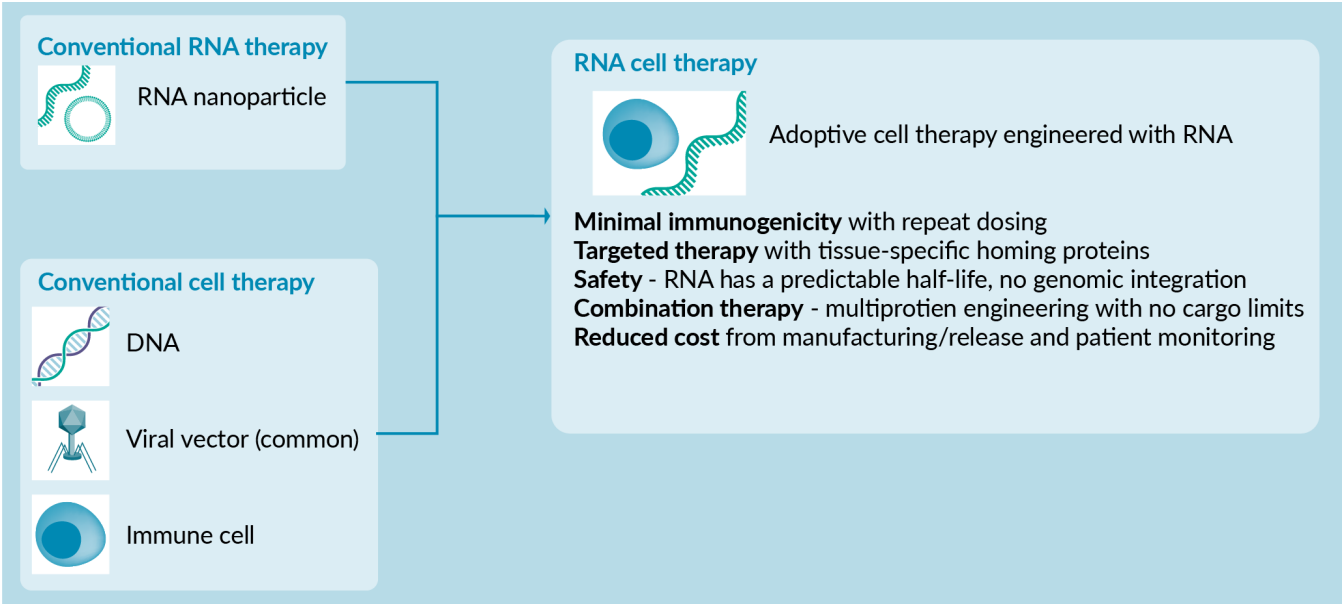
cells can be targeted delivery agents by engineering RNAs that encode homing proteins. Compared with conventional DNA-engineered adoptive cell therapies, RNA-engineered cells are safer due to RNA's predictable half-life without risk of genomic integration, and enable true combination therapy with essentially no cargo limits. With these five advantages, RNA Cell Therapy will find utility in a range of frontline oncology indications, and for many diseases beyond oncology.

TRANSLATION INSIGHT

RNA Cell Therapy is becoming an exciting field with products being tested in clinical indications that were previously uncharted territory for cell therapy. RNA Cell Therapy has five attributes that distinguish it from conventional (nanoparticle-based) RNA therapy as well as conventional (DNA-based) adoptive cell therapy (Figure 1). Compared to conventional RNA therapy, RNA Cell Therapy is less immunogenic and can be engineered to target a diseased tissue. Compared with conventional adoptive cell therapy, RNA Cell Therapy is safer, land unconstrained by cargo limits, such that it is a true combination therapy. The capacity to use the cell as both the protein production factory and a delivery vehicle opens up innovative possibilities in cell-based combination therapy. Multiple RNAs can be selected to deliver a combination of therapeutic proteins locally to virtually any diseased tissue. As such, RNA Cell Therapy can unleash the reach and potential of cell therapy.

FIGURE 1

RNA cell therapy combines the best attributes of conventional RNA therapeutics and adoptive DNA-engineered cell therapies.



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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING PLATFORM EVOLUTION

SPOTLIGHT

COMMENTARY

Therapeutic mRNA delivery with targeted lipid nanoparticles: next-generation transformative medicines

Umar Iqbal & Jagdeep K Sandhu

Messenger RNA (mRNA) has recently emerged as a new class of genetic drug for the prevention and treatment of various diseases. The rapid development and clinical deployment of COVID-19 vaccines worldwide has highlighted the potential of mRNA-based technologies as useful tools for the treatment of emerging infections. The clinical translation of mRNA therapeutics has been enabled due to the recent advances in drug delivery systems, including encapsulation of mRNA in lipid nanoparticles (LNPs) and improved intracellular delivery strategies. Therapeutic mRNA can also be leveraged for the treatment of genetic disorders, rare diseases and even cancer. However, broad application of therapeutic mRNA is limited due to its preferential accumulation in the liver. In this article we discuss strategies that can be employed to direct LNPs away from the liver and precisely deliver therapeutic mRNA to target cells of interest. The goal of delivering therapeutic mRNA *in vivo* represents a significant opportunity and a future of many new possibilities.

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mRNA-based therapeutics have emerged as a new category of drugs that have revolutionized the development and clinical use of the two COVID-19 vaccines authorized for emergency use. Prior to the COVID-19 pandemic, mRNA-enabled technologies were mainly limited to academic laboratories. The potential of mRNA was explored more than

30 years ago by Katalin Kariko and others [1], however it had proven difficult to advance mRNA as a drug product, mainly due to its ability to induce strong immune responses and rapid clearance or degradation in the body upon administration. The recent use of lipid nanoparticles (LNPs) as delivery vehicles for mRNA against the SARS-CoV-2 spike protein [2] has advanced mRNA as a drug product that has changed the course of the COVID-19 outbreak.

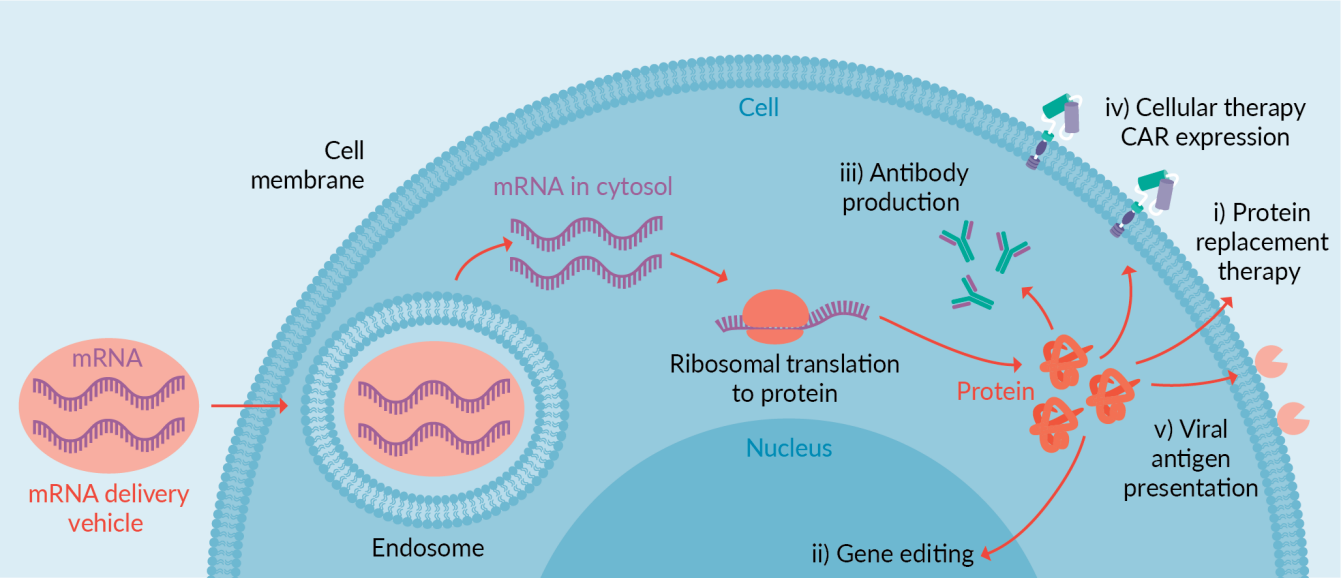
mRNA-BASED THERAPEUTICS

mRNA is a single-stranded molecule of RNA that corresponds to the genetic sequence of a gene. Upon entry into cells, exogenously delivered mRNA is transiently expressed in the cytoplasm using the ribosomal translation machinery and then converted into functional proteins (Figure 1). Therapeutic mRNAs are produced from linearized DNA in a cell-free system using an *in vitro* transcription reaction. Therapeutic mRNAs can be custom designed to encode certain peptides, proteins or antibodies for the purpose of providing a disease-specific treatment. Currently, therapeutic mRNA is being developed for a wide range of applications, including: (i) protein replacement therapy - replace a defective protein for the treatment of rare diseases; (ii) genome editing – deliver gene editing machinery such as CRISPR/Cas9; (iii) antibody production - *in situ* production of therapeutic antibodies and/or intrabodies inside cells; (iv) cellular therapy - introduce new functionality into cells of the hematopoietic system, lymphoid cells such as T-cells for chimeric antigen receptor (CAR)-T therapy or myeloid cells such as macrophages for tumor targeting; (v) viral vaccines - present new antigens to theimmune system (for example against SARS-CoV-2 spike protein). Of these applications, we will primarily discuss specific targeting of the cells of the hematopoietic system.

Eukaryotic cells are equipped with a diverse array of extracellular and intracellular innate immune sensors that can recognize

FIGURE 1

***In vitro* transcribed mRNA therapeutics.**



mRNA encapsulated in a delivery vehicle is taken up by cells via an endocytic vesicular pathway. The delivery vehicle is engineered to escape the low pH environment of the endosome and release its mRNA cargo into the cytosol. Inside the cytosol, the mRNA interacts with the protein translational machinery to become a genetically engineered protein designed to carry out a specific function, including i) protein for replacement therapy, ii) enzyme for gene editing, iii) antibody against a specific antigen, iv) protein destined for insertion in cell membrane for surface expression and v) viral protein antigen for presentation to the immune system.

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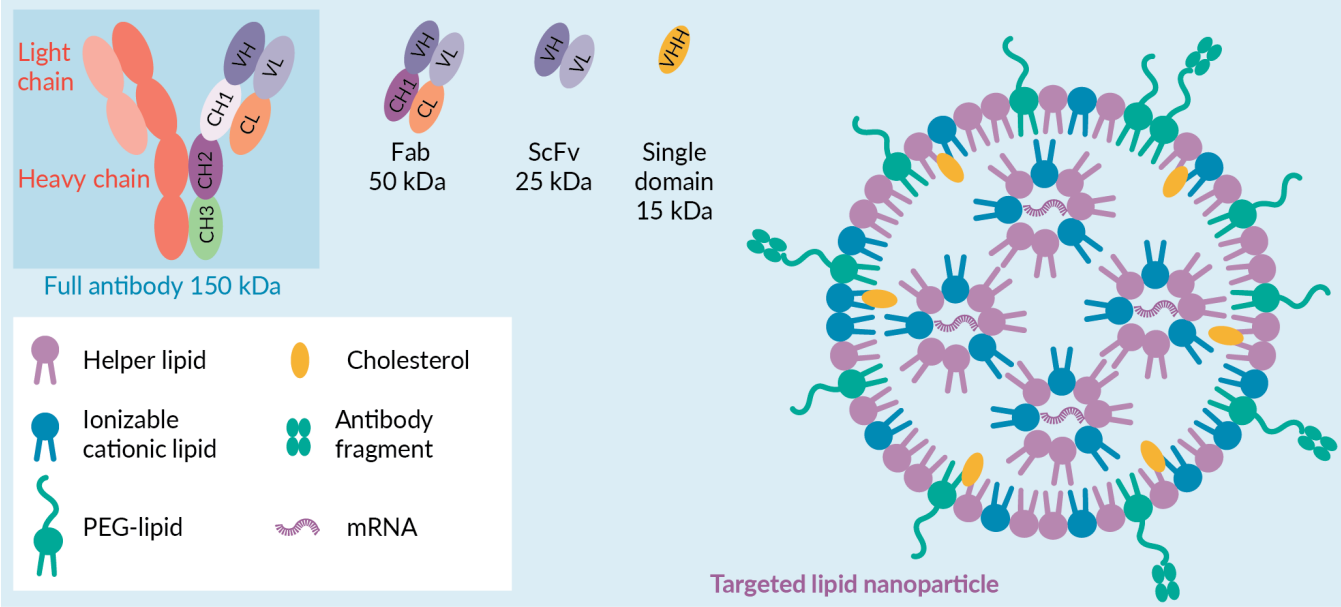
FIGURE 2

Targeted mRNA lipid nanoparticles.



Structure of a full antibody and various antibody fragments namely, fragment antigen-binding (Fab), single-chain Fv (scFv) and single domain antibodies (sdAb) are shown. The full antibody or antibody fragment can be linked to the external surface of mRNA containing lipid nanoparticles (LNPs) to create targeted LNPs (t-LNPs). LNPs consist of four main lipid components: helper lipids, ionizable lipids, PEG-lipids (lipid attached to polyethylene glycol) and cholesterol. Lipids are mixed in specific ratios with mRNA to create a unique and compact structure with mRNA encapsulated within its core.

mRNA as a danger signal and pose a major hurdle for therapeutic mRNA delivery [3]. To address this challenge, i*n vitro* transcribed mRNA is engineered to be similar in structure to endogenously produced mRNA by including a number of critical features: 5' capping, an open reading frame flanked by untranslated regions, poly-A tail and inclusion of modified nucleosides [4]. Overall, these important mRNA structural features serve to maintain stability, lower immunogenicity and increase expression inside cells. Systemic delivery of naked therapeutic mRNA is not feasible due to its ability to induce strong immunogenic responses, short circulation half-life (<5 min), vulnerability to degradation, inability to cross cellular membranes and almost negligible internalization by most cell types. Viral delivery of mRNA is an option, but it suffers from poor biodistribution, immunogenicity and toxicity issues. Therefore, in order to use therapeutic mRNA *in vivo*, it has to be first protected from RNAases in the blood, delivered specifically to the cell of interest and produce sufficient amounts of proteins to achieve therapeutic effects. Non-viral delivery vehicles, such as the LNPs, have recently emerged as leading nanocarriers for the encapsulation of mRNA-based therapeutics. The mRNA encapsulated inside LNPs is protected from extracellular nucleases, increasing stability and also facilitating cellular uptake and endosomal escape.

mRNA DELIVERY USING LIPID NANOPARTICLES (LNPS)

The most advanced and clinically relevant nanoparticles for nucleic acid delivery are LNPs (Figure 2) [2]. LNPs are able to extend the plasma half-life of nucleic acids in systemic circulation, increase stability and improve accumulation into tumor tissues via the enhanced permeability and retention (EPR) effect [5]. LNPs have proven clinical success in delivering nucleic acids such as

small interfering RNA (siRNA) [6]. The first-of-its-kind LNP-based siRNA drug, Patisiran (Onpattro, Alnylam Pharmaceuticals), was approved by the U.S. Food and Drug Administration for the treatment of hereditary amyloidogenic transthyretin-mediated amyloidosis (hATTR), a disease characterized by neurodegeneration from the overproduction of mutant transthyretin (TTR) proteins in the liver [6]. In addition, LNPs have revolutionized mRNA delivery, evident by their use in delivering mRNA encoding the SARS-CoV-2 spike antigen as COVID-19 vaccines developed by Moderna/mRNA-1273 and BioNTech/Pfizer BNT162b1 [2]. LNPs are able to both protect mRNA in the blood and deliver it efficiently into the cytoplasm of cells [3]. The standard mRNA-containing lipid nanoparticle (mRNA-LNP) formulation consists of four main lipid components: a helper fusogenic phospholipid, cholesterol, polyethylene-glycol (PEG)-lipid and an ionizable cationic lipid that can be rapidly mixed with mRNA using various rapid mixing techniques (Figure 2) [7]. Each lipid has an important structural role, but of critical importance are the PEGylated lipids that help to prevent aggregation and prolong blood circulation. The ionizable cationic lipids entraps the mRNA during particle formation and is critical for endosomal escape and mRNA release into the cytosol for protein translation. Incorporation of cholesterol increases the stability of LNPs by modulating membrane integrity and rigidity. The main limitation of LNPs, when given systemically, is the predominant localization of the LNPs in the liver [3]. For diseases of the liver, this is desirable, but in order to expand the utility of LNPs to other cell types, re-targeting strategies are required. The use of targeting moieties, such as ligands, antibodies, antibody fragments or peptides has the potential to direct the LNP away from the hepatic site and toward specific cells of interest that are accessible, such as cells of the hematopoietic system (i.e., blood stem cells and immune cells).

EMPOWERING LNPS USING CELL-SPECIFIC ANTIBODY TARGETING LIGANDS

Antibodies or immunoglobulins (Ig) are Y-shaped glycoproteins found in vertebrates and responsible for carrying out a variety of immune related activities with the goal to bind and neutralize foreign antigens (i.e., viruses or bacteria). Immunoglobulin G (IgG) represent the dominant class of human antibodies and have a structure consisting of four polypeptide chains: two identical heavy chains and two identical light chains connected via disulfide bonds forming a Y-shaped structure. At the amino-terminus of the heavy and light chain is the variable region or the antigen-binding region. At the carboxy-terminus, there is a conserved constant region [8]. Novel antibody fragments including F(ab')2 and Fab (antigen binding fragments), scFv (single chain variable fragment) and sdAb (single domain antibodies) can be isolated, engineered and produced by precisely dismantling the full antibody structure [8]. Each type of fragment retains at least one antigen binding domain, which is required for antibody targeting. Being smaller than the full antibody, antibody fragments are currently being exploited as precision warheads for targeting nanoparticles to specific cell types [9]. The antibody fragments have natural advantages compared to full antibodies, especially when considering attachment to LNPs, including lower immunogenicity [10], smaller size and site-specific engineering [11]. Alternatives to antibody fragments are also possible, which include ligands and peptides [9, 12]. A commonly used antibody conjugation site for LNPs is present on the external side of functionalized PEGylated lipids, which is introduced during formulation [9]. Careful consideration of the antibody attributes is warrented, as each unique antibody has the potential for improved targeting of nanoparticles to specific cell antigens of interest. Of utmost importance for targeting is also the selection of the cells' antigen of interest. For successful antibody targeting of

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LNPs, both the antigen and antibody should satisfy a list of key criteria, which are summarized in Table 1.

One potential set of antigens that possesses the necessary criteria for antibody targeting are the antigens present on the plasma membrane of cells of the hematopoietic system, including T cells, NK cells, macrophages and blood stem cells. In the future, we envision the use of an antibody targeted mRNA-LNP (t-LNPs) to genetically engineer patient's immune cells *in vivo*. These t-LNPs could one day replace the difficult to manufacture *ex vivo* cellular therapy technologies (i.e., CAR generation in immune cells or gene editing of hematopoietic stem cells (HSCs) *ex vivo*). An *in vivo* mRNA delivery approach would represent a more widely accessible, safer (transient mRNA versus more permanent DNA) and affordable alternative. Accordingly, a recent preclinical study has reported the success of using t-LNPs for *in vivo* targeting of $CD4^+$ T cells to lymphoid organs in order to achieve specific gene editing [13]. If the t-LNP approach was capable of producing comparable clinical results to their *ex vivo* counterparts, the technology would be disruptive, as it would allow for immediate treatment of a large number of patients who may be eligible for cellular therapy (monogenetic diseases, hematological cancers and possibly solid tumors). *In vivo* t-LNP delivery has several advantages: (i) a substantial time advantage for cancer patients who can't always wait for *ex vivo* manufacturing; (ii) access advantage as *in vivo* t-LNPs have access to a larger number of cells within a patient's body compared to *ex vivo*, where extraction of sufficient number of immune cells from a sick patient is more challenging; (iii) potential to be more cost-effective. Moreover, the scale-up of LNPs has been proven with the production of COVID-19 mRNA vaccines in an expanded list of countries when compared to the more exclusive and advanced pharma manufacturing capabilities currently required for *ex vivo* cellular therapies.

IN VIVO CHIMERIC ANTIGEN RECEPTOR (CAR) GENERATION USING TARGETED-LNPS (t-LNPS)

Chimeric antigen receptor (CAR) T cell therapy has emerged as a novel form of immunotherapy where patient T-cells can be reprogrammed to express disease-specific CAR for precisely targeting and killing tumor cells. Although CAR T cell therapies for hematological cancers have been approved by the FDA, the complex procedures and high production costs remain significant obstacles for their use as a mainstay cancer treatment [14]. Current methods of manufacturing CAR T cells require multiple laborious steps: T cell isolation from patients, modification *in vitro*, selection and expansion of modified cells followed by their infusion back into patients, which can only occur in very specialized centres at a very high cost. To achieve nucleic acid delivery into T cells, novel antibodies that target the multimeric protein complex, cluster of differentiation (CD3) and trigger rapid internalization would be needed (Figure 3). The CD3 protein complex is a distinct identifier of the T-cell lineage, therefore anti-CD3 antibodies

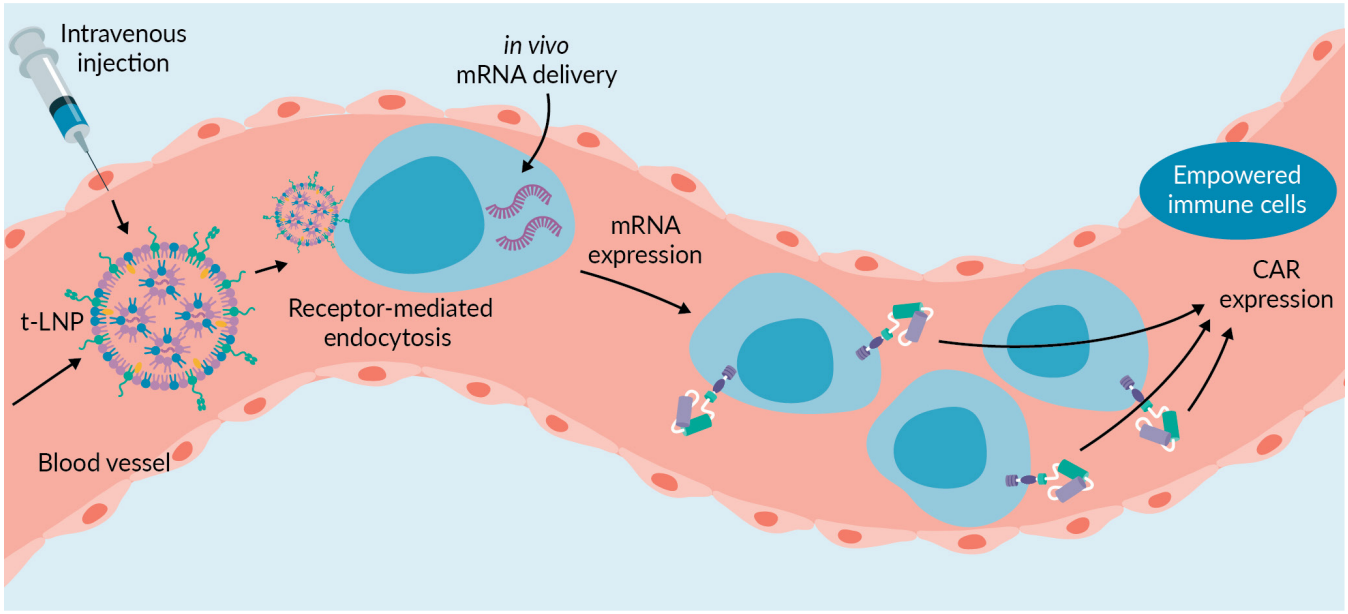
TABLE 1

Key criteria to be considered when developing an antibody against a cell surface target

Key criteria for antigen selection	Key criteria for antibody selection
Antigen must be specific to cell of interest	Antibody should have at least low nanomolar affinity for antigen
High antigen expression on cell surface	Antibody should be close to neutral in charge
Antigen and cell must be easily accessible to LNPs *in vivo*	Antibody must trigger internalization of the antigen upon binding
Antigen should have fast internalization potential	Antibody should be attached in a site-specific manner and not significantly increase LNPs size or cause aggregation
Antigen should be able to recycle back to the surface to avoid impact on regular physiological functions	Antibody fragments, which lack the Fc unit and have an overall smaller size, would be preferred

FIGURE 3

***In vivo* delivery of mRNA with t-LNPs.**



Upon intravenous injection, t-LNPs containing chimeric antigen receptors (CAR) mRNA can home to cell-specific antigens on immune cells in the blood or accessible tissues and deliver mRNA to the cell cytosol. The mRNA is translated inside the cell, leading to expression of CAR on the surface of the immune cell. The newly modified CAR immune cells are empowered to target a specific antigen on a particular cancer or diseased cell and have the ability to destroy it.

have been effectively used as T cell markers [15]. In a pioneering proof-of-concept study, it has been shown that circulating T cells can be modified *in vivo* with leukemia-specific CARs using DNA encapsulated into polymeric nanoparticles [16]. The CD3-targeted nanoparticles bound to approximately one-third of all T cells within 4 hours of infusion and inhibited tumour growth in a mouse model. The results of this study were comparable to conventional, *ex vivo* CAR-T cell treatment when tested in a B-cell acute lymphoblastic leukemia (B-ALL) mouse model using an anti-CD19–41BB CAR [16]. In another report, anti-CD4 antibodies conjugated to mRNA-LNPs were specifically targeted to $CD4^+$ T cells (up to 60%) in mouse spleen [13]. Together, these studies point the way towards the ability, at least in mouse models, to specifically deliver nucleic acids to T cells for application in both gene addition (i.e., CAR mRNA) and gene editing (i.e., Cas9 mRNA with small guide RNA). Furthermore, similar *in vivo* CAR delivery using t-LNPs could be applied to other promising anti-tumor immune cell types, including NK cells [17] and tumor-associated macrophages (TAMs) [18], both of which demonstrate better penetration in solid tumors than T cells.

IN VIVO MODULATION OF TUMOR-ASSOCIATED MACROPHAGES (TAMS) USING t-LNPS

Solid tumors consist of tumor and non-tumor cells, including stromal cells, tumor vasculature and infiltrating immune cells to form the tumor microenvironment (TME). The TME is a highly heterogeneous milieu in which tumor cells have evolved to create complex networks in which they communicate with tumor and non-tumor cells via cell-cell contact and secreted factors. The TME poses a series of challenges to immune cell penetration, trafficking and function due to the presence of immunosuppressive molecules, such as transforming growth factor β (TGF β) and interleukin-10 (IL-10) which can disable

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antitumor immune responses. In addition, physical barriers (i.e., tumor stroma, disrupted vessels, and interstitial fluid pressure), acidosis, hypoxia and functional inhibition via cell-cell contact can also contribute to immune escape [19]. This complex cross talk results in a highly immunosuppressive TME, which play a crucial role in immune evasion and compromise the efficacy of T cell immunotherapy for solid tumours. Therefore, there is a need for innovative solutions to facilitate immune cell penetration and increase the efficacy of immunotherapies for the treatment of solid tumours.

Tumor infiltrating macrophages are the key regulators of the TME and orchestrate complex interactions not only with tumor cells but also with other infiltrating immune cells [20]. Based on their *in vitro* phenotype, macrophages can be divided into two subtypes, M1 and M2. M1 macrophages play an important role in inflammation and anti-tumor immunity [21], while M2 macrophages (also known as TAMs) promote tumor progression [22]. TAMs are major innate immune cells that comprise of up to 50% of the TME population [20] and most studies have shown a positive correlation between TAM infiltration and poor prognosis in many human tumors [23]. Targeting TAMs with t-LNPs is a highly desirable therapeutic avenue with the potential to modulate M2 macrophage-mediated immunosuppression and allow for improved cellular immunotherapy for solid tumors. To this point, it has been shown that a single dose of LNPs carrying mouse interleukin-12 (IL-12) mRNA delivered intratumorally were able to induce local expression of IL-12, promoting infiltration of $CD8^+$ T cells and interferon-γ (IFN-γ) dependent responses that correlated with TME transformation. The induction of IFN-γ responses was associated with tumour regression in various mouse models [24]. In another study, intravenous administration of polymeric nanoparticles formulated with both mRNAs encoding interferon regulatory factor 5 (IRF5) in combination with its activating kinase IKKβ, was able to reverse the immunosuppressive nature of TAMs. This treatment was associated with the phenotypic switch of macrophages to anti-tumorigenic, which correlated with increased survival in a mouse model of ovarian cancer [25]. Together, this data supports the notion that targeting mRNA-LNP to TAMs could result in a more specific and efficient uptake, similar to antibody targeting approaches for T cells. However, widespread depletion of TAMs may not be an ideal treatment scenario, as TAMs consist of different subsets, including some with tumor-suppressive capabilities that slow tumor progression. CD163, a transmembrane scavenger receptor, is highly expressed on immunosuppressive TAMs. Cancers with the highest density of intra-tumor CD163-positive TAMs have been shown to be associated with poor survival rates [26]. Specific depletion of CD163-positive TAMs showed a marked tumor growth inhibition as compared to a pan-depletion of TAMs [27]. A key feature of the CD163 receptor is its ability to be rapidly internalized upon binding to anti-CD163 antibodies, which was harnessed to target an anti-inflammatory drug, dexamethasone to CD163-positive TAMs [28]. Although, anti-CD163 targeted antibodies conjugated to drug-loaded nanoparticles [27] and antibody-drug conjugates [28] are promising immunotherapies, a t-LNP approach, which can use mRNA to modulate macrophages (rather than deplete or alter them permanently) warrants investigation.

GENE EDITING OF HEMATOPOIETIC STEM CELLS (HSCS) USING t-LNPS

A number of monogenic diseases which are caused by variation in a single gene can be potentially cured by gene therapy of hematopoietic stem cells (HSCs) [29], including immunodeficiencies and β-hemoglobinopathies. Studies carried out in preclinical rodent models and in human patients have shown that a defective gene can be corrected

by *ex vivo* genetic modification of HSCs using lentiviral or retroviral vectors, followed by their infusion back into patients [30,31]. However, several challenges remain with the HSC-based gene therapy which include cost of biomanufacturing, insertional mutagenesis and the difficulty in obtaining HSCs from diseased patients [32]. With the advent of programmable nuclease technologies such as CRISPR/Cas9, the development of novel strategies to perform *in vivo* therapeutic genomic editing are on the horizon [33]. In a small study of six patients with hereditary ATTR amyloidosis, LNPs were able to successfully deliver Cas9 mRNA and a sgRNA targeting TRR to hepatocytes that resulted in lowering of serum TRR levels [34]. In the future, it may be possible to achieve specific targeting of HSCs by using anti-CD34 antibodies, which are readily endocytosed into HSCs. CD34 antigen is highly enriched on the surface of HSCs [35]. Using this approach, t-LNPs could deliver the CRISPR/Cas9 machinery specifically to HSCs for gene editing of specific mutations either *ex vivo* or possibly *in vivo* delivered via an intravenous or bone marrow injection. A safer, accessible and lower cost treatment for editing HSCs *in vivo* could be the next generation of therapies for monogenetic diseases.

CHALLENGES

In the case of COVID-19 vaccines, LNPs not only were able to deliver the mRNA-encoded immunogen, the lipids also acted as adjuvants and contributed to enhanced immune responses [3]. Although these immune responses were advantageous for prophylactic vaccines, they could represent a safety concern in the case of protein replacement therapies and genome editing applications. The safety profile of LNPs depends on lipid properties and the mRNA molecules. The charge of lipids used is important as repeated use of some lipids might activate host immune responses [36]. Following systemic delivery, complement family of proteins or innate immune receptors, such as toll-like receptors (TLRs) on the cell surface may be activated by LNPs, leading to NFκB activation and production of type I interferons and proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β [37]. Following cellular internalization, mRNA-LNP complexes are directed into the endosomal system, where LNPs disrupt the endosomal membrane leading to endosomal escape of mRNA. The translation of mRNA in the cytosol could activate the innate immune sensors, such as the NOD-like receptor (NLR) family, pyrin domain containing protein 3 (NLRP3) inflammasome and retinoic acid-inducible gene I (RIG-I)-like receptors [3]. This risk can be mitigated by substitution of 1-methylpsuedouridine into the RNA sequences that evades recognition by the innate immune sensors [38] and choosing lipids that are non-immunogenic and biodegradable [39]. In addition, PEG-associated immunogenicity especially in patients with pre-existing anti-PEG antibodies could impact the safety and efficacy of mRNA-LNP-based therapies, an obstacle to clinical translation [40].

The delivery of mRNA by LNPs involves complex mechanisms that may vary in different cell types and have not been thoroughly investigated. LNPs can also be exocytosed by cells resulting in inefficient delivery. mRNA can also be packaged into extracellular vesicles that can not only be transferred to neighbouring cells but also to distant organs and produce new copies of the protein that may result in undesirable effects [41]. Although promising results have been obtained and LNPs currently represent the gold standard for therapeutic mRNA delivery, selective accumulation of LNPs in the liver and extra-hepatic organs remains a major roadblock for the treatment of systemic diseases. Advances in the development of biocompatible and biodegradable LNPs and targeted mRNA-LNP nanoformulations will ultimately expand the application of mRNA-based therapeutics to the treatment of a wide range of diseases.

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CONCLUSIONS & LOOKING FORWARD

mRNA represents a novel modality to deliver therapeutic proteins that hold a great promise for the treatment of a wide variety of diseases and LNPs represent the most advanced mRNA delivery platform.

Targeted mRNA-based therapeutics will be developed as one of the most important next generation medicines for the treatment of other indications. Due to the success of the mRNA vaccines, biopharmaceutical companies could be racing with their clinical pipelines and many might be shifting their strategic directions. It is anticipated that there will be a high demand for mRNA and lipids and companies need to be prepared to address these critical manufacturing bottlenecks to meet future demands.

Although large-scale production of all the components required for the manufacturing of COVID-19 vaccines has been successful, the manufacturing of t-LNPs with encapsulated mRNA adds more complexity. Since mRNA manufacturing is carried out in a cell-free system, traditional manufacturing in mammalian cell culture facilities would not be ideal, and companies need to be equipped with dedicated equipment and specialized facilities with GMP compliance. Furthermore, biopharmaceutical companies need to develop expertise and capacity across the entire mRNA workflow, ranging from securing supply of raw materials, large-scale manufacturing, which will position them at the forefront of this technological revolution.

The development of mRNA-LNP vaccines for COVID-19 at an unprecedented pace has paved the way for the development of mRNA-LNP encoded therapeutics not only for emerging infectious diseases but also for genetic disorders and chronic diseases, such as cancer. We envision that targeting LNPs for delivering therapeutic mRNA specifically to diseased cells will prevent off-target effects. This would lead to the development of safe and affordable treatments for incurable diseases that could change the landscape of health care. Empowering LNPs with antibody-based precision targeting to cells of the hematopoietic systems has potential to be a disruptive step in future mRNA medicine. The t-LNPs have the capability of both widening the patient population eligible for treatment and improving health outcomes for difficult-to-treat diseases, like cancer or monogenetic disorders. With the advent of small antibody fragments and highly efficient and site-specific conjugation to the nanoparticle surface [42], the targeted nanoparticle field is poised to develop rapidly. At the same time, the continual evolution of mRNA technologies, including the incorporation of miRNA target sites within the mRNA therapeutic to eliminate expression in non-specific cells (i.e., hepatocytes), but maintain efficient expression in the cell type of interest [43] will also contribute to game-changing advancements in selectivity and safety. Finally, a remaining challenge for the field will be the regulatory approval, scale-up and manufacturing of more complicated targeted nanoparticle (for example, mRNA-LNPs conjugated to antibodies). Due to increase in structural and chemical complexity, more emphasis would be required on advanced characterization and standardize potency assays to help satisfy regulatory requirements. Scale-up and manufacturing has been successfully achieved separately for antibodies and for mRNA-LNPs, but combining the two with additional chemistry would present other hurdles in reproducibility and increased costs. However, with the potential for disruptive future medicine for t-LNPs, it is expected that industry, academia, and government will use their respective resources to carve out a path forward.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING
PLATFORM EVOLUTION

SPOTLIGHT

VIEWPOINT

Overcoming the immune-related shortfalls of AAV

Genine Winslow
Chameleon Biosciences, Inc

"To achieve safe and effective repeat dosing, and to treat patients with preexisting antibodies, we have developed EVADER™ platform technology."

VIEWPOINT

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On March 30th 2022, David McCall, Editor, *Cell and Gene Therapy Insights* spoke to Genine Winslow about overcoming the immune-related shortfalls of AAV. This article has been written based on that interview.

A JOURNEY TO CHAMELEON

I started my scientific career studying immunology at UC Berkeley, specifically T cell activation in James Allison's lab. I loved trying to figure out how systems work together as a whole picture. After graduate school I went back to industry, and I worked on some of the very early gene therapy technologies. With such a new field there was still a lot of discovery and learning to do, even though we were doing product development.

I have worked in gene therapy for almost 30 years now. What I have always loved about science is that I am in the rare position to be able to potentially help people.

I founded Chameleon 5 years ago. I had been working in the lab for a long time, and then managing groups and programs, and I was looking for a challenge. At the time, in the field of gene therapy, we were starting to see clinical trials showing Adeno Associated Virus (AAV) technology worked and promised to durably 'cure' a genetic disease with a single dose 'one-and-done.' It is hugely powerful technology, but clinical trials were still in early stages and the industry was still relatively immature.

I was working at a startup and noticed a lack of communication between the immunologists and the gene therapy scientists. I also met the parent of a child who suffered from a fatal genetic disease and saw the anguish and devastation that these types of diseases bring to families. I decided that I wanted to do all I could to work towards lifelong treatments to help these kids. These families deserved the best that we could give them.

I ended up leaving that job and diving into the scientific literature with the idea that I might be able to improve on the current AAV technology by considering the immune response to AAV. I reasoned that better potency and the ability to safely repeat dose a gene therapy could open the doors to treating many more children and many more disease types. With the 'one-and-done' dosing strategy, only non-dividing or slowly dividing cells would result in long term, durable disease correction. This means that there are many more genetic diseases that can't be treated with the first generation AAV technology than there are that can be.

I wanted to offer all children suffering from severe genetic diseases the potentially life-saving or life-changing option of gene therapy. That is what drove me to try starting this company.

I am still working here 5 years later, and we have raised around $17 million.

LIMITATIONS OF AAV

To reverse these genetic diseases, we have to get the correct version of a gene into patients' affected cells. The best way to get genes into the nuclei of patient cells is to use a virus as a shuttle. AAV is a relatively safe virus which does not cause any disease symptoms. We therefore use a recombinant version of AAV to deliver corrective genes to specific cell types.

However, our patients' immune systems cannot tell that recombinant AAV (rAAV) is a 'good virus.' From the outside, rAAV gene therapies look like the native form of the virus and are recognized and attacked by the patients' immune systems. When patients receive a single dose of current gene therapies, they generate antibodies to the recombinant virus. If we try to give them a subsequent dose, the AAV particles are flagged by antibodies and eliminated.

This is why a 'one-and-done' strategy was tried. A single dose is administered to deliver maximal therapeutic effect because subsequent doses are not efficacious and risk immune responses.

The 'one-and-done' strategy works for some diseases and some patient populations, but not all. For example, in hemophilia, adult livers are mature and don't grow in size, so it was thought that a single dose of gene therapy targeting liver cells could provide disease correction for the lifetime of the patient. In contrast, as children grow, their livers double in size between infancy and adolescence. As their liver cells divide, the clotting factor gene that reverses disease

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symptoms will become diluted, losing disease correction over time. These children would need subsequent doses to maintain clinical efficacy, but second doses are not available to them with current technology, so children are excluded from gene therapy treatment for hemophilia. Indeed, even in adults where we thought a single dose of gene therapy would last a lifetime, disease correction seems to be waning with at least one product in clinical trials.

As the gene therapy industry has matured and more products have progressed through clinical trials, we have seen other drawbacks to current technologies emerge. There have been clinical holds due to safety concerns and tragically, even patient deaths where very high doses were administered to achieve effective disease correction. These clinical adverse events are largely thought to be caused by the massive immune responses to some of the very large doses needed to provide disease correction.

Another drawback to the first generation AAV technology is that patients who have been exposed to the native version of the virus will have already developed pre-existing antibodies that can recognize and clear out the gene therapy before it has a chance to deliver a therapeutic gene. This population can represent up to 50% of potential patients, depending on the type of AAV being used.

I don't want to have to exclude any children or adults from a potentially life-saving treatment. Our goal in exploring the immune response to the AAV virus was to somehow neutralize the host immune system, or trick it into not responding, allowing us to get disease correction without risking severe adverse events, and to safely and effectively administer more than one dose to be able to treat more patients.

CHAMELEON'S PLATFORM & APPROACH TO ADDRESSING AAV-RELATED ISSUES

What differentiates Chameleon is that we think we should be able to treat patients who have been previously exposed to AAV. Our technology seems to have strong resistance to neutralizing antibodies in animal models. So we potentially do not have to exclude any patients who may have pre-existing antibodies. We now have data in animals showing we can successfully give a subsequent dose that is as effective as the first dose and has a significantly reduced immune response compared to the current AAV technology.

The ability to effectively repeat dose a gene therapy combined with our projected resistance to pre-existing antibodies opens the door to consider using our technology to treat many more types of diseases and patients. With these advantages we can consider expanding the use of gene therapy to treat all types of diseases.

We now know that AAV viral particles are made with an exosome surrounding a percentage of the virus particles that are secreted by producer cells. Casey McGuire, a co-founder of Chameleon, has called it exosome-associated AAV or enveloped AAV. He has been characterizing these particles for 11 years, and has shown that all different serotypes of AAV can be found with these envelopes.

Enveloped AAV delivers more copies of the therapeutic gene into target specific cell types than you would see with the non-enveloped version, or the naked AAV that is currently used on the market and in clinical trials. This indirectly starts to solve our problems: it means we can lower doses slightly thereby reducing the number of antibodies being produced by the host immune system after the first dose.

To achieve safe and effective repeat dosing, and to treat patients with preexisting antibodies, we have developed EVADER™ platform technology. An EVADER particle is an AAV particle enveloped by an exosome, into which we have engineered two checkpoint immune suppressing molecules or ISMs. The checkpoint ISMs that we are using have been studied for years in the context of oncology, so we know that they work well in humans. They are receptor and ligand pairs that deliver a suppressive or potentially tolerogenic signal to immune cells when they engage their

counter receptors on immune cells. The immune cells receive a signal telling them to not respond to EVADER particles.

Our manufacturing method is well established; we have been producing similar pseudotyped, enveloped virus particles (lentivirus like particles) for 30 years. It is relatively easy to engineer some types of exogenous molecules into the envelopes of AAV and lenti-based virus particles. In fact, our EVADER production is similar to how lentiviral vectors are made for use in CAR T therapy.

What distinguishes Chameleon's EVADER technology is our aim to treat unique patient populations that current technology can't treat. These include patients with preexisting antibodies and children whose target tissues are still growing. We have achieved significant resistance to neutralizing antibodies and effective repeat dosing in animal models. Based on these models, our immune suppression appears to be very strong. An added benefit is that we see about a tenfold increase in efficacy in animal models, potentially significantly reducing our cost of goods and increasing safety in patients.

R&D PIPELINE

Our lead program is in hemophilia B. Starting this summer we will be raising a round of funding to generate clinical data in adults with higher levels of pre-existing antibodies. I am really excited about our clinical trial design. We are anticipating a very different type of Phase I/II clinical trial design for EVADER gene therapies. We are proposing a two-dose strategy. This will allow for dose escalation and a more traditional PK/PD analysis.

We are hopeful that this model could potentially be a safer paradigm for gene therapy clinical trials and result in increased benefits to patients. We also have three other pipeline programs, including hemophilia A, which we are going to reserve for ourselves for later.

We plan to initially develop programs in-house and then license and partner strategically. Again, the overall goal is simply to treat more patients.

In addition to the hemophilia B and A, we are targeting the childhood disease Niemann-Pick type C (NPC). NPC is a metabolic disorder where cells build up cholesterol, and the children with the most severe form (early onset infantile NPC) start losing brain cells immediately. They slowly lose brain function until around adolescence, then they pass away. There is currently no treatment.

We are very hopeful that our technology could help these children. Another disease we are working on is one that affects the mitochondria called mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Both of these diseases are rare multisystemic diseases that have no current treatment. With our ability to repeat dose, we can potentially dose titrate for each patient and have a better chance of delivering therapeutic levels of corrective genes into all target tissues.

Once we have large animal and human proof of concept data, we will seek to expand the use of this platform. Our manufacturing is virtually plug-and-play with existing mammalian cell manufacturing systems. Taking Zolgensma® as an example (a gene therapy drug marketed for spinal muscular atrophy), we could use the Zolgensma drug creator Avexis' (acquired by Novartis) production plasmids in our platform, and potentially treat the patients who received a suboptimal dose in early clinical trials to improve their disease correction.

NEXT STEPS

My vision is to make gene therapy more accessible – to lower the cost of goods (COGs), and to make it available to treat other types of diseases. Because we anticipate that we will be able to safely and effectively repeat dose EVADER gene therapies, we could use our technology platform to broaden the range of treatable disease types, to include severe genetic diseases and diseases that are not of genetic origin.

The cost of goods for EVADER products are projected to be lower than the current

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COGs for AAV gene therapies. As we further develop the platform and our pipeline, we predict that the cost of goods will go down even more.

I want Chameleon to become a center of excellence for gene therapy, with superior manufacturing as well as superior knowledge of the immune-related consequences associated with AAV-based gene therapies. Through partnerships, licensing, and our own efforts, we will be developing programs to treat genetic diseases and expand to treating all kinds of diseases, not just those of genetic origin.

BIOGRAPHY

GENINE WINSLOW is the CEO and co-founder of Chameleon Biosciences, a biotechnology company she founded in 2017 focused on safer, more effective gene therapies. At Chameleon, Genine combines her backgrounds in both immunology and gene therapy to engineer a next generation AAV based vector for systemic repeat dosing for children and adults who today cannot be treated with conventional gene therapies. Prior to Chameleon, Winslow has led gene therapy scientific initiatives at multiple companies including Audentes Therapeutics, BioMarin, BlueBird Bio, and Cell Genesys.

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CELL & GENE THERAPY INSIGHTS

GENE DELIVERY/GENE EDITING
PLATFORM EVOLUTION

SPOTLIGHT

Process & analytical insights for GMP manufacturing of mRNA lipid nanoparticles

Emmanuelle Cameau, Peiqing Zhang, Shell Ip, Linda Mathiasson & Katarina Stenklo

The successful development and rapid deployment of the messenger RNA (mRNA) vaccines against SARS-CoV-2 virus during the COVID-19 pandemic has catalyzed the industry to look even more closely at the technology beyond their potential use for novel vaccines to enable breakthrough treatments for cancer, rare diseases and more. Indeed, the mRNA and lipid nanoparticles (LNP) technologies that underpin the COVID-19 vaccines have far-reaching potential to transform modern medicine. However, as a relatively new technology, there remain barriers to successful industrialized manufacture of LNP-encapsulated mRNAs (mRNA–LNPs).

The manufacturing of the mRNA–LNP drug product can be broken down into five key steps (Figure 1): DNA template manufacturing, mRNA drug substance synthesis and purification, mRNA–LNP formulation and purification, fill/finish operations, and analytical testing. This article will first examine each step and discuss challenges and opportunities pertaining to the process itself and for the manufacturing facilities.

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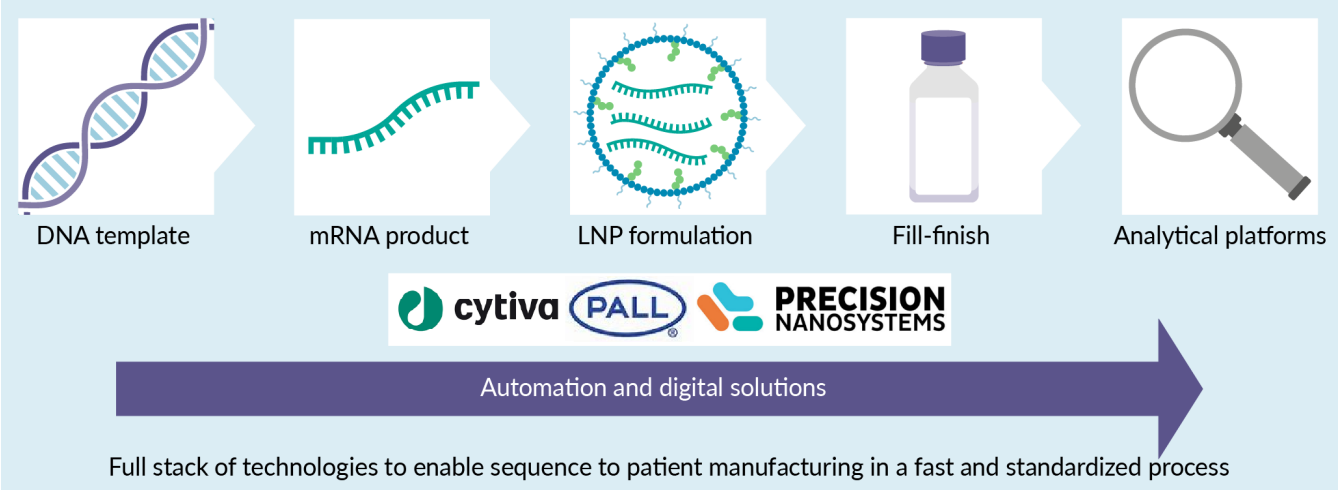
INTRODUCTION

The past decade has seen an extraordinary leap in our knowledge of the human genome and its role in health and disease, which has driven exciting advancements in precision genomic medicines that are now poised to revolutionize ways to improve human health. The wealth of sequencing and bioinformatics data has provided deep insight into genomic and epigenetic factors that contribute to the underlying molecular causes of diseases. Combined with decades of research in drug development and manufacturing, this perfect storm of innovation has made it possible for pharmaceutical innovators to develop novel nucleic acid-based

FIGURE 1

mRNA–LNP therapeutic production process global overview.



drugs that can act upon the genetic instructions defining the disease itself.

Among genomic medicine modalities, RNA-based therapeutics comprise a rapidly expanding category of drugs accelerated even further by the clinical success of the mRNA COVID-19 vaccines to combat the SARS-CoV-2 virus. The mRNA and LNP delivery technology that forms the foundation of these vaccines has gained tremendous attention recently but is built upon years of research and groundwork by dedicated scientists [1–3]. mRNA as the technological basis of therapeutics and vaccines offers a great flexibility with respect to production and application. The mRNA backbone's physicochemical characteristics are unaffected by changes in the encoded antigen, allowing for the establishment of product-agnostic manufacturing platforms that can be standardized and easily adapted to new sequences [4] – a game-changing technology well-suited for rapid pandemic responses.

Additionally, while the recent focus has been their use for vaccines, RNA-based therapeutics have immense potential for similar approaches in the fight against cancer and other diseases. Researchers are investigating the therapeutic potential of mRNA beyond infectious diseases, including replacement therapy for genetic deficiency, as cancer vaccines, or as adjuvants for cancer drugs [5].

Data from early phase clinical trials shows dosage levels of mRNA–LNP therapeutic drug products ranging from single to double digit milligrams per injection [6–8], two to three logs higher than the COVID-19 vaccines. The lack of long-term clinical and real-world experience warrants caution and due diligence to assess the safety/toxicity of such high doses of mRNA as therapeutics. Next-generation mRNA vaccine designs that utilize self-amplifying RNA (saRNA) or circular RNA (circRNA) molecular formats could decrease the required dosage levels or elicit a higher and more durable antigen expression. This may improve both the performance and cost of existing prophylactic mRNA vaccines and may prove useful to ease dosage requirements for other therapies. Researchers are also exploring other classes of RNA molecules such as small interfering RNA (siRNA) and microRNA (miRNA) in silencing or regulating gene expression or acting as guide RNAs as therapeutic options for a variety of diseases (reviewed in [9, 10]).

To meet the increased demand for mRNA vaccines and other RNA-based therapeutics, with the potential to range all the way from pandemic response to truly personalized treatments, cost-effective manufacturing processes at the right scales coupled with well-defined product characterization will be needed to bring forward promising new solutions to treat and cure diseases [11].

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DELIVERY METHODS FOR GENOMIC MATERIAL INTO HUMANS

The goal of genomic medicines is to deliver genetic information to a target cell, either to replace a defective function (monogenic disease), or to introduce an additional function to treat (as in cancer) or to prevent (as in a vaccine) disease. Gene delivery vehicles for the introduction of such genetic material (i.e., DNA, RNA, oligonucleotides) are called vectors. There are broadly two categories of vectors: viral and non-viral. The choice of delivery method depends on the intended application, technology, target tissue, and indication, but there is certainly a place for both in the genomics toolkit, each with their advantages and disadvantages (reviewed in [12]).

Viral vectors

Viruses have an innate ability to invade cells and can efficiently transduce specific cell types and tissues *in vivo*. Contemporary viral vectors are based on retroviruses (lentivirus), adenoviruses (Ads) or adeno-associated viruses (AAVs) modified to disable their replication capability. As pathogens, viruses are naturally immunogenic and can still induce significant immune responses, which not only reduce vector penetration and treatment efficacy, but can also have severe adverse health consequences. Researchers have taken steps to reduce the immunogenicity of viral vectors. Engineering viral vector capsid proteins to make them 'invisible' to the human immune system or incorporating 'suicide genes' are potential methods to alleviate this risk [13].

Non-viral delivery methods

Traditional non-viral delivery methods rely on physical methods like electroporation, passive, and ballistic delivery. The concept relies on delivering naked DNA or RNA using high voltage electroporation to increase the permeability of cell membranes to promote the entry of genetic material into the cell, but these methods are limited to *ex vivo* usage. *In vivo* delivery of RNA is particularly challenging since naked RNA is quickly degraded by extracellular RNases and is not internalized into cells efficiently (reviewed in [14]). A great deal of work has been put towards developing transfection reagents that can protect RNA from degradation and facilitate its cellular uptake. A prime example are the LNPs, which were utilized for the COVID-19 vaccines. The LNP encapsulates the mRNA within a protective shell, protecting it from nuclease degradation. The lipid composition of the LNP facilitates entry into the target cells by endocytosis where RNA is released into the cytosol via endosomal escape mechanism (reviewed in [15]).

LNPs have demonstrated a promising record of safety and tolerability for repeat treatment. Billions of doses of the mRNA–LNPs have been administered during the COVID-19 pandemic with mostly brief and mild adverse events reported after two doses [8]. Additionally, clinical trial data for Onpattro™, an siRNA-LNP drug, reported comparable reactions with the placebo from repeated infusions every 3 weeks for 18 months [16]. Being non-viral, the risk of genome integration is also low. This is attractive for genome editing applications, particularly since LNPs also can package multiple RNA payloads within the same formulation, allowing for Cas9 mRNA and single guide RNAs (sgRNA) to be packaged together [17]. Co-formulation of multiple RNAs reduces the pharmacokinetic and regulatory complexity of such drugs. LNPs can package and deliver large payloads such as 11 kb single-strand self-amplifying RNA, which promises to increase RNA potency by orders of magnitude. LNPs can encapsulate RNA that encodes for any protein antigen with minimal change to chemical characteristics, easing the burden of multi-product manufacturing. This platform technology provides opportunities for lipid raw materials to be pre-purchased, while common equipment and analytical methods can be used to produce RNA drugs

across vastly different indications, thus shortening the drug development duration.

As a relatively new technology, innovative solutions, expertise, and out-of-the-box thinking will need to coalesce to address challenges and bottlenecks in manufacturing to truly realize the transformative potential of this disruptive technology. Below, we summarize the process for manufacturing an mRNA drug product and examine important factors and considerations for both process and analytical development.

PROCESS & GMP MANUFACTURING

1. Process overview

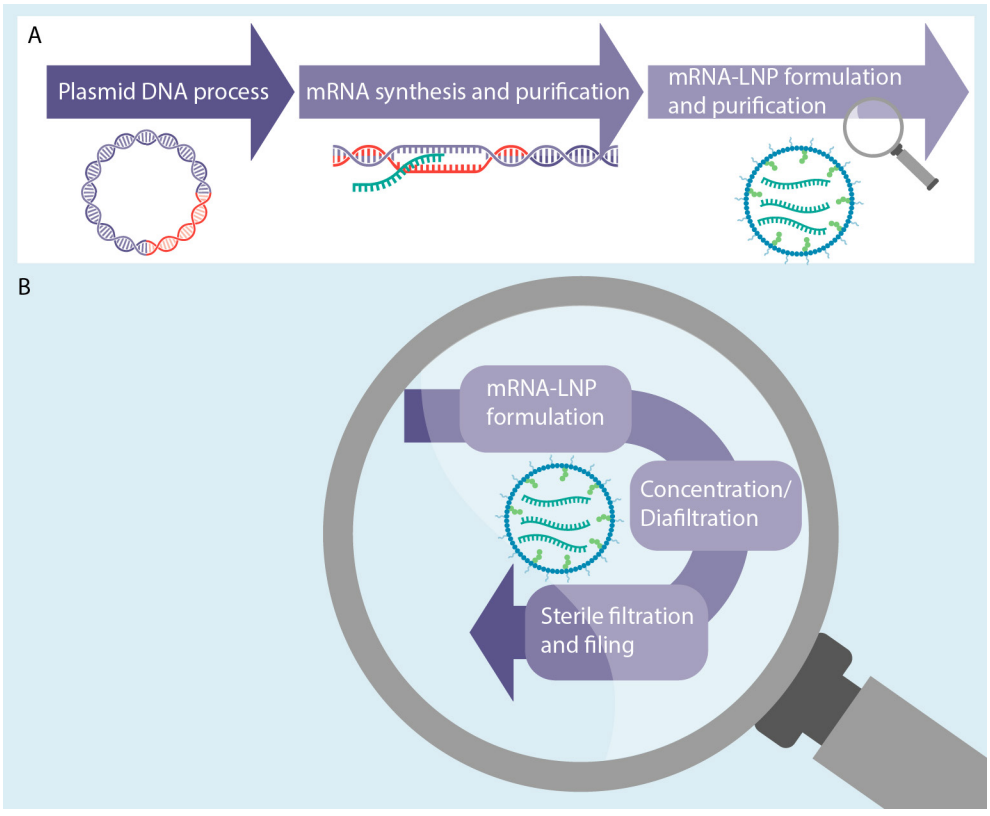
The manufacturing process of mRNA–LNPs is composed of three different key sub-processes: plasmid manufacturing, mRNA synthesis and purification and mRNA–LNP formation and purification (Figure 2 A & B).

1. **Plasmid (pDNA) manufacturing:** Production of pDNA is a microbial process utilizing *E. coli* fermentation. Cell lysis is required to release the intracellular pDNA, which is followed by a series of downstream purification (DSP) steps to remove impurities and host cell contaminants (e.g., endotoxin) to achieve high purity and quality [18]. This is especially important because these attributes can impact the overall yield of the following mRNA cell-free synthesis step.

2. **Cell-free mRNA synthesis:** Frequently known as the *in vitro* Transcription (IVT), this step relies on a

FIGURE 2

mRNA–LNP manufacturing process sequence from pDNA production to mRNA–LNP bulk filling.



A: Overall mRNA–LNP process sequence; **B:** Detailed process steps of the mRNA–LNP formation and purification process.

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series of enzymatic reactions. First, the pDNA needs to be linearized to act as a template for mRNA production and capping. After mRNA synthesis, purification usually includes tangential flow filtration (TFF) steps using either flat sheets or hollow fibers. A capture step follows TFF, using resin or membrane chromatography in either bind/elute or flow-through mode. Formulation (concentration/diafiltration with TFF) and finally, sterile filtration (0.2 µm) prepares the bulk drug substance, which can then move to LNP encapsulation [19].

3. **mRNA–LNP formation:**
This process usually consists of three steps: rapid mixing of the mRNA and lipid solutions to create encapsulated mRNA–LNPs, a concentration/diafiltration TFF step to remove residual solvents and concentrate the mRNA–LNPs drug product in the desired buffer formulation, followed by a final 0.2 µm sterile filtration. The product can also be further formulated as required and processed through filling operations [19].

2. Important process considerations

Despite the success and large-scale production of mRNA vaccines for COVID-19, mRNA technology is not yet mature and there is no single, standardized manufacturing workflow. Many of the technologies used are designed for other processes (e.g., monoclonal antibody production), which can be a challenge to reconcile with the unique requirements of mRNA–LNPs manufacturing [20]. However, it is promising to see that there are initiatives amongst the solution providers to develop tailored, scale-appropriate products specifically for mRNA therapeutics production. The absence of standardized protocols means manufacturers must develop and optimize their process, leading to a considerable number of process variations in both upstream and downstream processes. There are many variables and decisions throughout the production workflow that will greatly impact the equipment selection, setup, batch cost, and throughput. In this article we discuss a few of the more challenging steps, but optimization is key to the whole process, from IVT to final drug product.

Capping strategy

The choice of the mRNA capping strategies is one of the key process decisions. The cap is a methylated guanosine at the 5′ end of the sequence, is essential for mRNA maturation, and allows the ribosome to recognize the mRNA for efficient protein translation. The cap also stabilizes mRNA by protecting it from nuclease digestion. The cap can be added in two ways, either co-transcriptionally, or enzymatically as a separate reaction from the IVT. Co-transcriptional capping is less expensive and faster than enzymatic capping since it occurs during the IVT step, in the same reactor mix. However, capping efficiency and yield are typically lower and can lead to the formation of non-capped impurities or cap analogs incorporated in the wrong orientation.

Enzymatic capping is achieved in a separate reaction after mRNA purification from the IVT mixture. This reaction usually uses a vaccinia virus-capping enzyme to add the capping structure to the mRNA. While enzymatic capping has a very high capping efficiency, it is more expensive and requires an extra unit operation. This results in a longer process, which can decrease the total process yield, increase the consumables used and therefore impact the overall process cost.

mRNA–LNP formulation

The formulation method to create mRNA–LNP molecules involves mixing lipids dissolved in an organic solvent with RNA in an acidic buffer to induce spontaneous self-assembly. This is governed by complex intermolecular interactions between the RNA and four different lipids species. Since physical properties of the LNPs such as size and morphology are intricately tied to their biodistribution and

function, fine control over both the chemistry and the mixing environment in which self-assembly occurs is vital to ensuring the uniformity and quality of the particles [21]. Highly specialized expertise is required to design and optimize the right combination and proportions of lipid species, buffers, and solvents, to effectively deliver the RNA drug substance for a defined therapy. A reproducible method for mixing RNA and lipids is also necessary to ensure a uniform population of particles and batch-to-batch consistency. Additionally, a scalable process is desired to minimize process redevelopment when translating from bench to clinic. Both T-junction and microfluidic mixing have been reported extensively for LNP production. T-junction mixing is a continuous process suitable for large scale production, with typical flow rates of 40–60 mL/min. However high flow rates and high minimum volumes make small scale production for formulation screening and development more challenging. Emerging technologies based on microfluidic mixers offer access to non-turbulent, well-controlled mixing environments and ensure the scalability from lab to manufacturing scale. The staggered herringbone mixer (SHM) has been used extensively for preclinical development (reviewed in [22]). With flow rates on the order of 10's of mL/min SHM is well suited for preclinical scale production. Multiple mixers have been arrayed in parallel to increase throughput for larger scale production [23]. Recently, Precision NanoSystems have developed a toroidal microfluidic mixer that is scalable from tens of mL/min to hundreds mL/min to enable scalable manufacturing from RNA screening [17] to clinically relevant scales [22, 24]. Ensuring that LNPs can be manufactured at bench scale and scaled up to commercial scale, at high quality and yield, will be crucial to support the industry as it looks to translate mRNA medicines beyond vaccines to more advanced therapies.

Formulation considerations

Decisions made during the final TFF formulation and sterile filtration steps may also impact production outcomes, since the encapsulated mRNA–LNP intermediates are shear sensitive. The choice of TFF consumables such as the selection of hollow fiber or flat sheet cassettes, the molecular weight cut off threshold and the sterile filter membrane type needs to be carefully evaluated together with the processing conditions of each of operation to maximize process efficiency while minimizing impacts to product quality (i.e., LNPs size and average size distribution). The time it takes to execute the process may need to be balanced by the stability of the product or examined in the context of overall facilities usage. Ultimately, good process knowledge and planning for future demands early in process development can mitigate risk and enables cost and time-efficient decisions.

Capital equipment

Today, many capital investments are made while products are still in the early-stage process development. As mRNA technologies are still evolving, the key for mRNA manufacturers is to build in flexibility with modularized single-use equipment. This can mitigate risk and enable rapid reconfiguration to accommodate different manufacturing scenarios for optimized facility utilization across different products and at different scales. While not required for process development, employing single-use equipment for cGMP production may be beneficial to expedite technology transfer and scale up to manufacturing for clinical use.

Filling operations

There can be a strategic benefit to having an in-house filling platform that can solely support advancement of drug candidates within your own pipeline instead of being reliant on outsourced organizations who are juggling the priorities of many clients. As mRNA therapeutics move towards the personalized scale there will be a greater need for process control and risk reduction. Regulatory agencies place greater emphasis on process control as the scale decreases.

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Filling machines can generate particles that can contaminate the fill containers used, which is especially challenging with a translucent product as with mRNA–LNP formulations where visual product checks aren't possible. As well, conventional filling solutions often have issues with tipping or broken vials requiring manual intervention, which presents opportunities for product contamination. Robotic filling systems capable of closed, aseptic operations mitigates the risk of particulate contamination and need for manual correction, while also enhancing process control, and provide flexibility for multi-products. Moreover, the current mRNA–LNP formulations require storage at -80 C to extend their shelf-life, therefore the final container and closure combinations must be capable of maintaining their integrity under these conditions. There have been also efforts to enhance mRNA–LNP stability when stored at 2–8 °C or even at ambient temperature, including lyophilization strategies [25], which is expected to reduce the cost on cold-chain logistics and drug storage.

As important as flexible and scalable platform technologies are to the rapid development of mRNA-based therapeutics, they would not be possible without rapid, robust, accurate, sensitive, and scalable analytical technologies. In the next section, we discuss key requirements and considerations for analytical technologies used for process development and in-process and product-release testing.

3. Analytics

However controlled and reproducible the manufacturing process, confirmation of the critical quality attributes (CQA) is an essential part of batch release and similar information informs process decisions at the critical control points (CCP). As such, fit-for-purpose analytics needs to be demonstrated to have the required performance characteristics for the intended use. This is essential to provide crucial information with respect to process performance and product quality during manufacturing to ensure the quality, purity, potency, safety, and stability of mRNA therapeutics. Where there is a choice of analytical method, the merits of in-line, at-line, and off-line testing can be balanced against the impact of the proposed assay on the process flow. For example, off-line assays may be slow but may provide greater accuracy or sensitivity and are more acceptable for final batch release than in-process testing. Whereas the analytical method chosen for in-process measurements may prioritize turnaround time given comparable sensitivities, especially for process operations that cannot proceed until the analytical results are available.

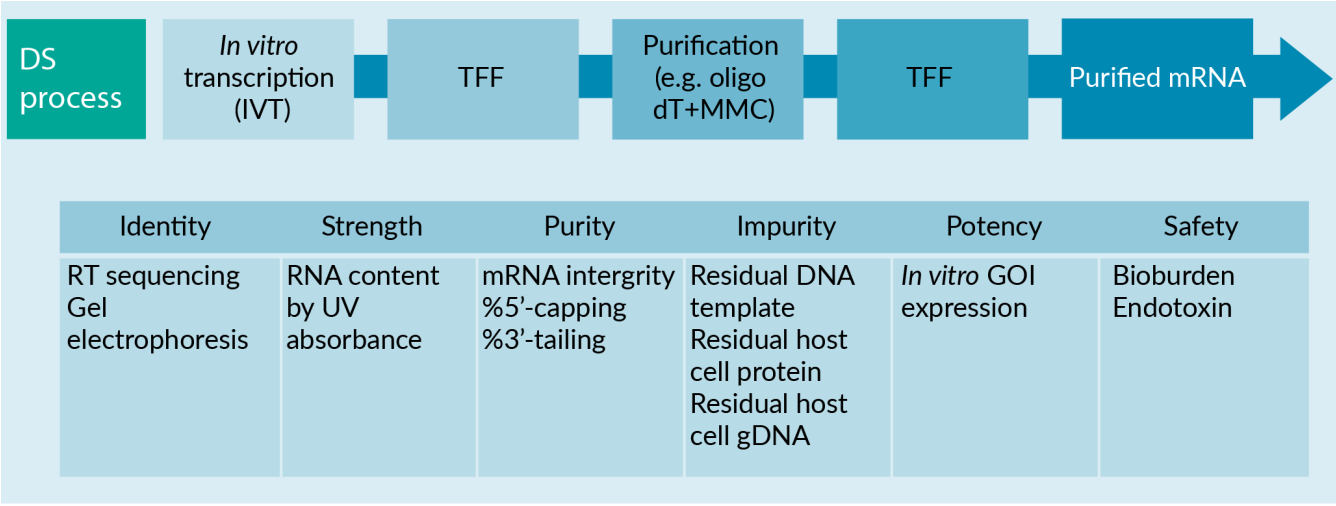
The manufacturing process of mRNA-based modalities involves linearized plasmid DNA as starting material, purified mRNA as drug substance, and formulated mRNA–LNP as the drug product, all of which require analytical testing for in-process controls, product release, and stability programs. Plasmid DNA and mRNA are large molecules and LNPs are complex nanostructures. As a result, a suite of complementary tools and technologies are required to cater to the wide range of product quality attributes testing with the resolution and speed needed in a manufacturing setting. Figure 3 and Figure 4 below depicts examples of analytical methods that are typically required for drug substance and formulated bulk processes.

Analytical methods for the drug substance process

During the drug substance process, mRNA molecules are synthesized via enzymatic reactions resulting in a mixture of product variants, including different 5′-cap structures (i.e., Cap0 vs Cap1), variable 3′-polyA tail length, and truncated mRNA transcripts. Other notable impurities include double-stranded RNA molecules and residual plasmid DNA templates. Additionally, the incorporation efficiency of modified nucleotides, if used in the IVT process, should be checked. Despite purification steps to remove these unwanted

FIGURE 3

Analytical methods for drug substance process (i.e. mRNA IVT and its purification).



Identity	Strength	Purity	Impurity	Potency	Safety
RT sequencing Gel electrophoresis	RNA content by UV absorbance	mRNA intergrity %5'-capping %3'-tailing	Residual DNA template Residual host cell protein Residual host cell gDNA	*In vitro* GOI expression	Bioburden Endotoxin

byproducts, a small portion may be carried through the process. Therefore, such product quality attributes should be characterized during in-process and lot release testing of the purified mRNA.

Analytical methods for the formulated bulk process

During the formulated bulk process, lipids are introduced into the process stream to be combined with the purified mRNA molecules. During formulation, the mRNA will be encapsulated into the LNPs forming a large and complex nanostructure, with different physical and chemical characteristics compared to the individual parent mRNA and lipid molecules. Advanced particle analytical assays are required to assess particle sizing and polydispersity index (PDI), which can impact final biological function. In addition, the surface charge on LNP has critical impact on the gene expression. Analytical assays that characterize the surface charge, such

FIGURE 4

Analytical methods for formulated bulk process (i.e., mRNA encapsulation and formulation).

Identity	Strength	Purity	Impurity	Potency	Safety
mRNA ID Lipid ID	RNA content by florescence assay	Encapsulation efficiency LNP size (mean and PDI) Lipid purity	Residual DNA template Residual DNA template Residual host cell gDNA Double-stranded RNA Lipid-related impurities	*In vitro* GOI expression	Bioburden Endotoxin

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as TNS assay and Zeta potential assay, should be included in the product characterization package [26].

In-process analytics

In mRNA processes, product concentration determination is required to quantify the performance of chromatography and TFF steps, to ascertain the load conditions, and to calculate the step yield. Spectroscopic methods to determine concentration determination, such as UV absorbance or fluorimetry are relatively rapid and easy analytical procedures. On the other hand, quantitative profiling of purity-related product quality attributes is more complex due to the large size and near-identical physical and chemical characteristics of the mRNA molecules and their variants. For example, mRNA transcripts with Cap0 and Cap1 on the 5′ end only differ by 14 Da in their molecular masses [27]. As a result, high resolution analytical technology is needed to resolve such subtle differences.

For nucleic acid analysis including pDNA and mRNA, separation sciences such as capillary electrophoresis (CE), high performance liquid chromatography (HPLC), and coupled technologies such as liquid chromatography-mass spectrometry (LC-MS), are powerful tools for high resolution analysis. Different analytical methods can be implemented on advanced CE systems [28], depending on the separation mechanism (i.e., capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF)). CE with UV or laser-induced fluorescence (LIF) enables separation of molecules based on their hydrodynamic radius. Generally, pDNA for clinical applications should be > 80% supercoiled (vs. open or circular) [29]. CE-LIF has been demonstrated to be an automated and reproducible method for the rapid quantitative analysis of pDNA purity and to distinguish pDNA isoforms. Similarly, CE-LIF can be used to analyze the size variants of mRNA transcripts (i.e., intact, or truncated transcripts, poly-A tail length profiling). Advanced LC-MS technologies [28] combine the capability of high-resolution separation by LC and accurate mass detection by MS to offer a multi-purpose analytical platform well-suited for mRNA characterization. This includes mRNA 5′-cap analysis, poly-A tail analysis, and lipid identification based on accurate mass of both the intact molecular ions and their associated fragment ions. It should be noted that mRNA drug substance and formulated LNP samples have significant differences in the characteristics of their sample matrices. Therefore, assay optimization is required to ensure fit-for-purpose performance of the advanced analytical technologies.

It should be also noted that mRNA–LNP based therapeutics are a novel class of advanced therapies. Thanks to the COVID-19 pandemic, several mRNA vaccines have been brought to the market via Emergency Use Authorization (EUA) and later received fully marketing authorization. Given the relative novelty of the technologies and accelerated development timelines, we have limited experience with respect to their CMC, non-clinical, and clinical performances, product quality analysis and specification. While there are sporadic reports on the quality aspects of some mRNA assets [30], there is a lack of regulatory and industry-level guidance and standardization for the specific critical quality attributes to be targeted, release criteria and specifications setting strategies, which is still an actively evolving area. That said, establishing a comprehensive suite of analytical technologies and testing strategies to provide critical insights into product quality and to inform process development and manufacturing decisions, will continue to benefit the advancement of new development programs.

4. Data management

With the relatively short processing time for mRNA there is a potential to have high throughput workflows with many batches per year in the manufacturing line. As a result, data management and batch release can become a potential bottleneck, regardless of the

production scale. Investment into automated data management solutions can support efficient batch release and enable readiness for future production capacity. Manufacturing equipment with integrated software that can log data electronically can be implemented into existing workflows across a continuum, from discrete islands of automation, to fully integrated and connected platforms. Resource planning systems, electronic production records, process control systems, data historian, Manufacturing Execution System (MES) systems and centralized data repository (and off-site backup) are key technologies for GMP manufacturing. They also enable robust data management to support process development, characterization, and efficient tech transfer. These digital platforms can provide on-demand access to process data that can streamline product manufacturing, testing, and release.

5. Facility considerations

The sensitivity of mRNAs to RNase contamination dictates that the mRNA manufacturing should be separated from other cell-based processes. If possible, it might be beneficial to build a dedicated manufacturing environment for mRNA altogether to eliminate the risk. Multi-product, -process designs can be incorporated if there is a need to manufacture multiple mRNA products in the same facility/manufacturing line, that can support efficient changeover between products as well as reducing the risk of contamination can increase facility flexibility.

As an important side note, the mRNA–LNP is formulated using a solvent injection technique, and thus manufacturing facilities need to be designed to handle this specialized process as well as the volumes of reagent needed for the intended manufacturing scale. Be aware that different global regulations apply, making it necessary to work with the local authorities to get the correct approvals.

mRNA based vaccines have a great potential for pandemic preparedness and localized vaccine manufacturing. Modular prefabricated facility designs have become a consideration for these initiatives either as stand-alone or nested into existing facility space. They offer a pathway to decentralized vaccine production and to bring it to the point of need with speed, flexibility, and predictability. Several modular solutions have been announced lately in collaboration with vaccine manufacturers to improve global production of the COVID-19 vaccine. However, as the applications for RNA therapeutics continues to broaden, the most flexibility will be achieved with an end-to-end technology provider that can cover the entire process from IVT to filling and that is agnostic to what product will be manufactured in the facility.

WHAT'S NEXT?

As stated earlier, mRNA–LNPs are manufactured using technologies that were developed originally for traditional biologics such as mAbs and viruses. Manufacturing technologies designed specifically to meet the production scale and maximize productivity of mRNA–LNPs are needed as demand continues to increase for vaccine development and other modalities. Drug developers and solution providers will need to work together to understand the emerging needs to find the best fit solutions.

Notably, IVT is currently the most cost-driving step in the mRNA process where the raw materials (i.e., RNA polymerase and nucleotide triphosphates) represent 60–65% of the total cost of goods. Alternatives to batch production with different vessel designs, or eventually a continuous reaction flow, could be of interest to potentially increase the overall process productivity and result in the lowest utilization of costly reagents.

The pDNA template is an essential raw material for IVT, which is also used in viral vector applications, another growing area of biologics. The converging demand of pDNA has strained the supply of GMP quality pDNA, which is creating bottlenecks

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for manufacturing. One possible solution to overcome this limitation would be to use a cell-free technology, such as rolling circle amplification, to generate the DNA template. Such technologies offer a faster, simpler, and cleaner process that may be of interest for personalized therapies as well.

LNPs represent another area of focus for further development and optimization to define formulations to improve thermostability. Currently available mRNA vaccines must be stored frozen, resulting in a complex supply chain that limits their utilization in low-income countries. In addition, engineering tissue specificity can improve targeted mRNA drug delivery with the potential to reduce off-target effects, dosage levels to better manage drug safety/toxicity [12]. LNP surface functionalization, including antibody conjugation, may also present future opportunities for improving tissue targeting of mRNA–LNP for their broader applications in various therapeutic areas. Developing the LNPs systematically, by screening libraries and optimizing parameters, and then modeling the full unit operation at small scale can facilitate these goals and de-risk future manufacturing.

Some leading mRNA companies are also partnering with artificial intelligence (AI) companies to employ rational design approaches often used in biomolecular engineering to mRNA design. Sequence design, prediction modeling, manufacturability analysis, and other metrics are bringing insights and opportunities for example in the development of a personalized cancer vaccine/ immunotherapy, where tumor genome sequencing is used to create a patient-specific drug regimen. In this case predictive algorithms compares the genomic sequence of the tumor to healthy tissue and identifies the set of tumor-specific neoantigens that would elicit the strongest anti-tumor immune response with the lowest side effects for the patient. The drug manufacturer can then use this information to develop RNA vaccines and bring truly personalized medicines to realization. Another example of partnerships is between RNA companies and machine learning and cloud providers to efficiently and quickly be able to design research experiments, find insights, automate laboratory and manufacturing processes, simplify technology transfer and more easily comply with regulations during production and testing of vaccine and therapeutics candidates. This has been demonstrated in an ongoing collaboration between Moderna and Amazon Web Services (AWS) [31].

Finally, from a regulatory perspective, because of the novel composition of the RNA vaccines, regulatory criteria and standards have yet to be fully defined. Regulatory agencies are constantly modifying their guidance on both the manufacturing drug product requirements. To overcome this, more effort in product characterization and analytics are needed. A globally harmonized standard and implementation of manufacturing control strategies is crucial for the wide adoption of mRNA–LNP as a novel modality. Experience from biologics modalities reveals some noticeable divergence of interpretation of ICH guidelines related to control strategy [32]. Future mRNA–LNP development should leverage the experience learned from the accepted regulatory framework established by the COVID-19 vaccines as part of the EUA, to accelerate regulatory approvals across different countries and agencies.

CONCLUSION

There are numerous tools in the genomic medicine toolbox, and it will be crucial to identify the right tool for a given application (disease to be treated, tissue to be targeted, duration of expression needed, level of reactogenicity) while balancing the benefit and risk to the patient and cost burden to the health care system. mRNA–LNPs represent a promising addition to the expanding repertoire but, with the relative newness of the technology there is still uncertainty surrounding the best approaches to process development and manufacturing with the best process economics. We have highlighted

some key challenges and areas for improvement for mRNA–LNP manufacturing and emphasize the value of partnering with end-to-end solution providers who can provide evolving and tailored strategies to support changing industry needs. As manufacturing challenges are resolved, we will truly see influence of mRNA technology in the future trajectory of vaccine development, oncology, and personalized medicine.

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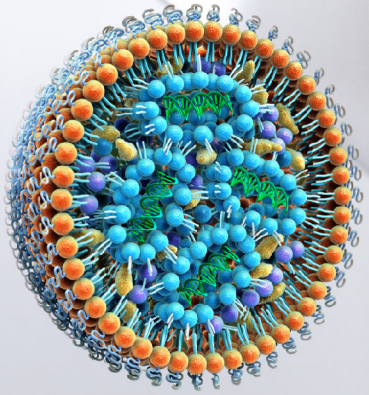

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INTERVIEW

The quest for the Holy Grail in AAV chromatography: empty–full separation

Ratish Krishnan & Oliver Rammo

Ratish Krishnan and **Oliver Rammo** share their thoughts on the need for a more nuanced understanding of AAV capture and empty–full separation, and the trends driving innovation in this area.

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Q What are the challenges in AAV empty–full separation?

OR: From a market perspective, there is not just one serotype, one cell type, or one empty–full capsid ratio. These all vary largely between cell lines, with the ratio falling

anywhere between 10 and 90%. This generates a lot of complexity, so it is difficult to have a one-fits-all approach.

Most AAV serotypes are the same size and only differ slightly in density and isoelectric changes. Even though these variations are very small, there is a range of analytical methods, such as ultracentrifugation and capillary electrophoresis, which enable high-resolution separation of the species. However, there are limitations in scale, throughput, and cost.

Alternatively, anion exchange chromatography has been widely explored for this application, due to its scalability, high throughput, and low need for sample preparation. When using anion exchange chromatography, the devil is in the details. With current methods, you can either achieve a good separation efficiency between full and empty capsids at low process yields, or a higher overall process yield with low separation efficiency. Even with preexisting process knowledge, time-consuming process development work is needed to finetune parameters such as salts and process additives.

A further complication is that AAV can show aggregation under certain process conditions; for example, AAV2 is more hydrophobic than other serotypes.

RK: The therapeutic role of empty capsids, or even partially full capsids that are formed during AAV packaging, is not fully understood. The general understanding is that empty capsids provide no therapeutic benefit. However, they may increase the innate or adaptive immune response in gene therapy patients.

Cesium chloride and iodixanol-based ultracentrifugation are commonly used in academia and for small-scale preps and first-generation processes. However, in larger-scale manufacturing, a chromatography approach using anion exchange is more desirable due to its scalability.

Q How well understood is empty–full capsid separation in the gene therapy industry? What are some of the misconceptions?

RK: The short answer is that it is not very well understood. The need to remove empty capsids is understood at a high level – they do not contain the gene of interest, so they are considered an impurity. The exact percent of full AAV capsids needed is vague. The acceptable level is based on clinical experience, and there is a debate as to whether they are a process-related impurity or if they have a biological function during treatment. It has also been suggested that empty capsids can act as decoys to reduce neutralizing antibody burden. We need to further understand the immune response to empty capsids.

"The general understanding is that empty capsids provide no therapeutic benefit. However, they may increase the innate or adaptive immune response in gene therapy patients."

- Ratish Krishnan

Our customer interactions over the last few years have led to discussions for process development groups involving a wide range of targets for percent full capsids. Some aim

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close to 100%, with others aiming considerably lower. Some customers are hoping for the FDA to set the limits, but a regulatory body would have difficulty setting a limit without understanding the clinical impact of these empty capsids. The overall goal is to maintain a consistent number of full capsids across the clinical phases of your program, with an emphasis on robust preclinical and clinical trial designs to understand the tolerability of varying levels of purity in patients.

"It has become critical to have future tools that enable the study of individual serotypes to ensure the safety and future of drug development and administration of AAV."

- Oliver Rammo

OR: Some customers believe that we need 100% full samples of AAV. This concern arose in 2020 when issues with high dosing with AAV were reported, including progression to liver dysfunction. This raised some fundamental safety concerns for the use of AAV vectors. It has become critical to have future tools that enable the study of individual serotypes to ensure the safety and future of drug development and administration of AAV.

Q Olli, how is Merck tackling the AAV capture and empty–full separation challenge?

OR: From a capture perspective, we currently have ongoing development programs that enable the purification of specific serotypes. We are looking at a universal approach from an academic point of view. The universal approach offers greater process flexibility and a one-for-all solution. While the approaches for single serotype affinity purification tend to enable better process economics, higher yield, and higher impurity reduction, they are only applicable for one specific serotype.

Our development program during an EU-funded project called DiViNe for Nanofitins® in collaboration with Affilogic is based on this approach. These Nanofitins are small affinity ligands, discovered in organisms that live in America's Yellowstone National Park geysers. As these organisms are suited to living under extreme conditions, the resultant stability of the Nanofitins rendered them very attractive for therapeutic applications. We optimized the scaffolds for chromatographic purposes.

From an empty–full separation perspective, we are exploring both resins and membranes. This includes small resin particles based on anion exchange chemistry, such as our Fractogel® resin family. These resins hold promise when applied to a hybrid gradient in finding the balance between process yield and separation efficiency for full and empty capsids. We have plans to further optimize these processes through additional collaborations, including looking into a multi-column approach.

We are also working on membrane absorbers in collaboration with a group at the University of Mannheim. This group holds great experience in process modeling originating in the monoclonal antibody market, which we want to transfer to AAV processes.

RK: In terms of other partnerships, our teams – including the process development services across the globe, manufacturing science and technology, and customer application teams – are all heavily engaging with our customers in solving this issue. We are focusing on a two-pronged approach – developing new products and generating data on existing products.

Q How can analytics drive innovation in this area?

OR: Having the right analytical tools is essential to match the pace and speed of current innovation of AAV therapies. To advance product development and keep up with the speed of manufacturing, we need to have novel process analytics that combine high resolution and high throughput. This is currently not available in the market, and end-users need to combine multiple analytical methods to achieve good impurity profiling or empty–full ratio determination. There is a need for innovation for an all-in-one method that gives information for all relevant parameters, such as capsid titer, empty–full ratio, and aggregation level.

The novel methods that hold promise for this include size exclusion chromatography multi-angle light scattering. However, this will require upfront investment and consideration of user experience. We are partnering in the NIIMBL project that started last year, supporting the development of a microfluidic chip. This innovative concept enables the determination of full–empty capsids on a single, portable device in a simple format.

RK: As a downstream scientist, I fully agree that there is no process without analytics. Innovation in analytics is critical to finding the root cause of this process issue.

Q Looking to the future, how do you expect to see technology for AAV capture and empty–full separation evolve over the next 5 years?

RK: As an optimist, I am confident we will be very close to solving this challenge. Empty–full separation is not always an isolated downstream issue, so tackling the production of more full capsids upstream inside the bioreactor is a commonly available solution.

Improvements in HEK production systems are expected. The use of baculovirus expression systems could also be a valid answer, where percent full capsids are generally higher at the end of harvest. However, there are other considerations to this approach. Even though insect cells may give higher percent full titers, there is a need to deal with higher levels of feed impurities that then shift the bottleneck back to the downstream route. The size of the payload or gene of interest that is used might also affect the separation efficiency.

From a downstream lens, innovative products and creative processes, such as hybrid salt and pH gradient elution on anion exchange chromatography resins, have been attempted with varying degrees of success.

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I also hope there will be more transparency in the data that is made available. There is so much work happening on empty–full separation in process development labs across the globe, but we hear very little about it.

Q How will future trends in AAV capsid design and production affect purification?

RK: Historically, we have used AAV serotypes 1 through 10. We are now seeing the era of hybrid serotypes emerging through novel capsid design, artificial intelligence, and machine learning, which are customized to target organs of interest with higher transduction efficiencies than currently available serotypes. For example, novel serotypes such as AAV-DJ, AAV-DJ/8, rh10, and AAV-PHP, are shown to target the central nervous system well.

The purification focus though will stay constant, so we need to be consistently delivering a product that meets the release criteria guiding patient safety. Moving to templated chromatography processes is desirable, though this will likely be via a case-by-case approach depending on the complexity of fine separation with these new serotypes.

OR: To add, we need to have orthogonal tools. We see a lot of invention in this field with respect toward novel serotype development, but it remains challenging to predict future targets for large affinity development projects. Therefore, we need to consider a more universal approach. We also recently saw some great results with an orthogonal tool for plasmid affinity.

BIOGRAPHIES

Ratish Krishnan, Senior Strategy Consultant, Novel Modalities, Merck

Ratish Krishnan is a Senior Strategy Consultant in the Novel Modalities BioProcessing group for the Americas. A Process Development Scientist by background, he has over 13 years of experience in vaccines, monoclonal antibodies, and viral vector modalities from pre-clinical to late-stage process characterization, validation, and commercialization activities such as BLA authoring. Before joining Merck, Ratish managed process development teams at Novartis and Pfizer. Now, he serves as a global subject matter expert for viral vector manufacturing and provides strategic guidance to internal stakeholders and key customers. He holds a master's degree in biotechnology from Pennsylvania State University.

Oliver Rammo, Director, Chromatography R&D, Novel Modalities, Merck

Oliver Rammo is leading the Novel Modality segment in the Purification R&D Group responsible for the development of novel purification strategies for the Viral and Gene Therapy market. Prior to his current role, he worked as Laboratory Manager and Application Engineer for the Merck Life Science division. He joined Merck KGaA in 2012, working for Performance Materials Advanced Technologies in the field of printing formulations for photovoltaic applications. Oliver holds a BSc in Applied Life Science from the University of Kaiserslautern and an MSc in Molecular Biotechnology from the University of Frankfurt.

AUTHORSHIP & CONFLICT OF INTEREST

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Applications and benefits of the CTS Rotea Counterflow Centrifugation System in cell therapy workflows

Carl Dargitz, R&D Manager, Thermo Fisher Scientific

The Gibco™ CTS™ Rotea™ Counterflow Centrifugation System is a closed cell processing system designed for cell therapy workflows. It has the flexibility to perform a variety of cell processing applications across several cell types. Here, we provide some key data to highlight the Rotea system's ability to isolate white blood cells (WBCs) starting with leukopaks, as well as perform high speed T cell wash & concentrate steps and efficient cell washing.

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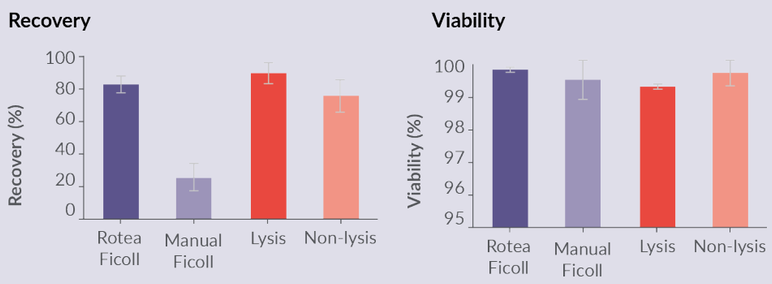
WHITE BLOOD CELL ISOLATION

Starting with a leukopak, WBCs can be isolated using Ficoll in the Rotea system with red blood cell (RBC) lysis buffer or without lysis (Leukopak wash), as shown in Figure 1. All three techniques yield high recovery and viability of WBCs and reduce RBC content. Both Rotea Ficoll and Rotea Lysis methods reduce RBCs to well below 10%. These protocols can be tweaked to satisfy other needs, such as including extra wash steps to reduce platelet burden.

Figure 1. White blood cell isolation from Leukopak using various methods on the Rotea system.

Cell types	Gating strategy	Frequency (%) of cells			
		Rotea Ficoll	Manual Ficoll	Rotea lysis	Rotea non-lysis
Leukocytes	CD45+	90.4	96.4	91.4	80.5
T cells	CD45+, CD3+	46.5	45.1	57.1	58.2
B cell	CD45+, CD19+	14.8	15.7	18.2	14.2
Monocytes	CD45+, CD14+, CD16-	18.8	18.9	17.9	19.8
Neutrophils	CD45+, CD14-, CD16+	20.4	25.1	18.4	18.1
NK cells	CD45+, CD56+	7.79	8.71	6.51	6.04
Dendritic cells	CD45+, CD11c+	4.05	4.86	14.3	17
Platelets	CD41a+	4.31	32.7	1.71	6.01
Red blood cells	CD235a+	6.46	5.58	8.7	22.9



HIGH SPEED T CELL WASH AND CONCENTRATE

A further application of the Rotea System is in cell wash and concentration steps. Using higher flow rates (100mL/min) with the Rotea System provides an opportunity to decrease process time resulting in shorter cell processing workflows.

Post-Rotea processed cells show recoveries greater than 95%, alongside improvement in cell viability (Figure 2). Cells returned to culture post-processing doubled every two days between day 12-14 and day 14-16 of expansion. Pre- and post-Rotea processed cells showed no significant change in relative CD4 and CD8 expression.

WASHING EFFICIENCY

The CTS Rotea system can be programmed to perform effective washout of media and buffer components. This protocol provides an easy, one-step alternative to the arduous process of several centrifugation steps. Wash buffer can be washed through the fluidized cell bed, enabling over 95% removal of original medium components with minimal cell loss and maintained cell viability (Figure 3).

Figure 2. Wash and concentrate steps run at high speeds on the Rotea.

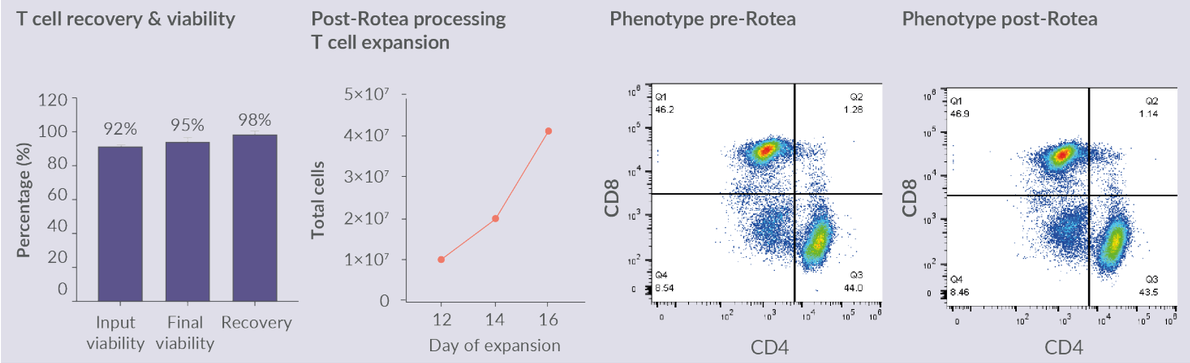
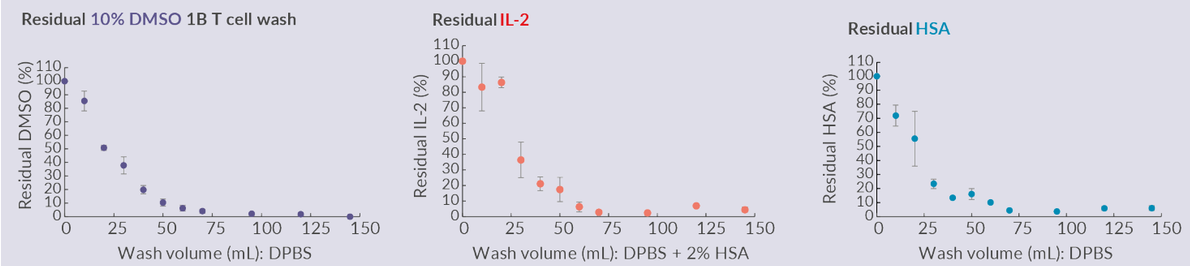


Figure 3. Washing efficiency of the CTS Rotea System.



Wash volume (mL)	Removed DMSO (%)	Wash volume (mL)	Removed IL-2 (%)	Wash volume (mL)	Removed HSA (%)
0	0	0	0	0	0
10	14.64	10	16.72	10	28.01
20	49.19	20	13.68	20	44.49
30	62.18	30	63.54	30	76.65
40	80.12	40	78.89	40	86.64
50	89.62	50	82.61	50	83.89
60	93.85	60	93.73	60	89.94
70	95.97	70	97.25	70	95.62
95	97.91	95	97.62	95	96.35
120	98.21	120	93.11	120	94.11
145	100	145	98.78	145	94.02

A pan-affinity resin for efficient AAV purification: a CDMO perspective

Vincent Ravault, DSP Expert, Process Development and Industrialization Department, Yposkesi

Over the last decade, the number of clinical trials involving recombinant adeno-associated viral (AAV) vectors has dramatically increased, the diversity of serotypes has expanded, and the demand for highly purified material manufactured to cGMP standards has rocketed. For contract development and manufacturing organizations (CDMOs) like Yposkesi, the key manufacturing challenges are centered around flexibility, robustness, and productivity, especially with regard to purification. The availability of universal tools to address any serotype with minimal process adjustments is therefore critical. Yposkesi conducted a series of experiments to evaluate the POROS™ CaptureSelect™ AAVX resin, a pan-affinity tool for universal capture of AAV vectors.

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FLEXIBILITY IN SEROTYPES

The POROS CaptureSelect AAVX Affinity Resin uses a ligand derived from a heavy-chain antibody that can bind AAV serotypes 1–9 and synthetic or recombinant AAV vectors **(Figure 1)**, making it possible to standardize a purification platform for several AAV serotypes with only a few adjustments.

DYNAMIC BINDING CAPACITY

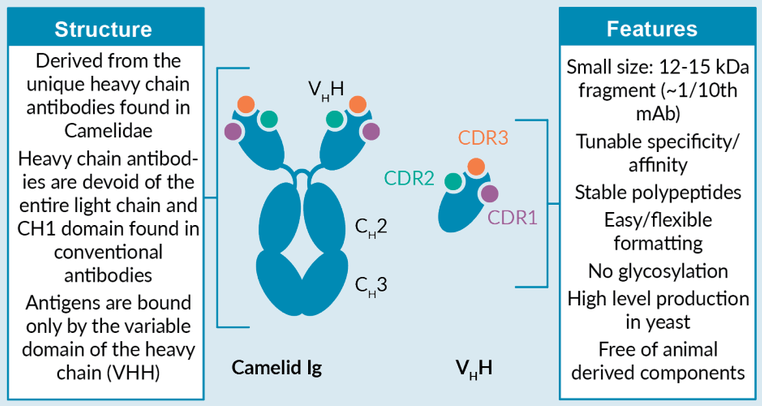
The binding capacity was assessed using 1 mL-prepacked columns, packed with either POROS™ CaptureSelect™ AAV8 or POROS CaptureSelect AAVX, at 1- and 3-min residence time on two feedstocks with different initial virus titers. Clarified supernatant containing AAV8 vectors was directly loaded on the affinity columns until a 10% breakthrough in AAV8 was observed in the flowthrough.

Multiple fractions (column volumes – CV) were collected at the outlet of the column during the loading phase, and the quantity of capsids was determined by ELISA assay in each collected fraction. The results for 3 mins residence time are presented in **Figure 2**, and results for 1 min were similar. For both residence times, there was no breakthrough on AAVX, with loading volumes up to 2,500 CV.

The results from these binding capacity studies led to three main conclusions:

- The AAVX resin has a better AAV8 binding capacity than the AAV8 resin
- Binding capacity increases with harvest titer
- Residence time has no significant effect on the binding capacity

Figure 1. Key features of camelid-derived, recombinant expressed ligands used in CaptureSelect™ Affinity Resins.



SCALE-UP OF THE CHROMATOGRAPHY STEP

Another series of experiments established good scalability from lab scale development to the 10 L scale for both AAV8 and AAV2 serotypes. The final yields of the capture step were close to 100% for both AAV8 and AAV2 serotypes **(Figure 3)**. The purity of AAV vectors captured with AAVX resins was high, with an impurity reduction of over 99% in the purified product after capture on AAVX for each serotype.

CONCLUSION

This long-term study with POROS CaptureSelect AAVX resin highlights several advantages of AAV capture using POROS CaptureSelect AAVX resin. Yposkesi concluded that this resin is a successful tool to improve purification processes in terms of quality, cost, and standardization, due to its flexibility in serotypes, high yield, low level of impurities, and compliance for large-scale GMP AAV manufacturing. In future, Yposkesi plans to implement this resin for the purification of other AAV serotypes.

Figure 2. Binding capacity at 3 mins residence time and 10% breakthrough for POROS CaptureSelect AAV8 and POROS CaptureSelect AAVX at AAV titers of 4.3×10^{10} caps/mL (left) and 4.9×10^{11} caps/mL (right). Blue bars: capsids per mL of resin at 10% breakthrough. Orange dots: column volumes that lead to 10% breakthrough.

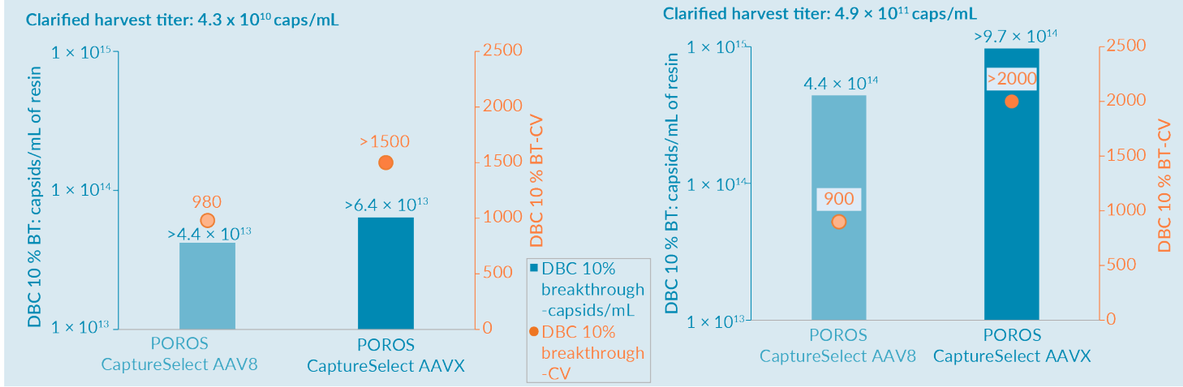
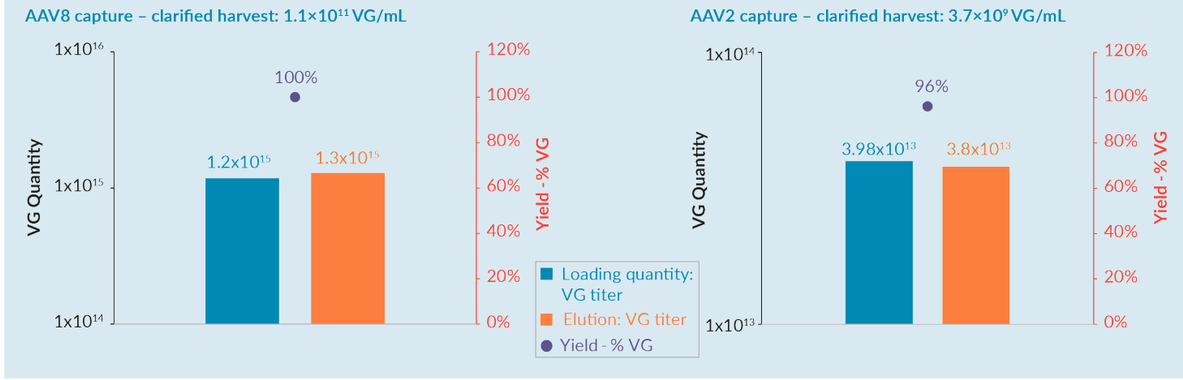


Figure 3. AAV capture on POROS CaptureSelect AAVX – yields for AAV8 (left) and AAV2 (right). Blue bars: product quantity loaded on each column. Orange bars: quantity of purified product recovered during the elution. Pueple dots: yield.



To explore the full study design & results, along with an author Q&A, watch the webinar or read the article

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May 2022
Volume 8, Issue 4

INTERVIEW

VIEWPOINT

INNOVATOR INSIGHT

-

FAST FACTS

Characterization of advanced therapies: leveraging advanced analytics to avoid FDA holds

Dana Cipriano & Alexei Saveliev

745

INTERVIEW

AAV vector characterization best practices

David McCall, Commissioning Editor, *Cell & Gene Therapy Insights*, speaks to **Gaël Stephant,** Senior CMC Project Leader, Vivet Therapeutics.

GAËL STÉPHANT graduated from the Arts et Métiers CNAM, Paris III in 2008 as Engineer in biology, after working a few years as a technician. M. Stéphant joined innovative cell therapy company, Celogos in 2005 to develop an animal component free cell culture media designed to increase cell growth as well as reducing risk for patient. In 2008, he set up the cell culture laboratory Xentech (CRO company) for preclinical *in vitro/ex vivo* cancer model development. In 2012, he was hired as head of vector bioproduction unit at Theravectys. He then started in 2015, a first CMC Project leader rôle at Yposkezy (formerly Genethon CDMO) followed by 3 years at Gensight Biologics, a biotech company where he was in charge of the production of AAV vector batches to be used in phases I and II clinical trials, as well as the preparation towards commercial phase. He then changed environment and joigned the pharmaceutical company Servier, to lead the CMC team of the CART cell/gene therapy development. Since 2021, M.Stéphant works for Vivet Therapeutics, a biotech company, where he manages the CMC activities for recombinant proteins and gene therapy products.

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Q What are you working on right now?

GS: Vivet Therapeutics is a biotechnology company focused on the development of the next generation of adeno-associated viruses (AAVs) for the treatment of

rare liver diseases, with headquarters in Paris, France and a research team in Spain. Vivet has several strategic partnerships in place, including with the Fundacion para la Investigacion Medica Aplicada (FIMA, University of Navarra).

I have worked as chemistry, manufacturing, and controls (CMC) project leader for a year now. Vivet, has a portfolio of five gene therapies for targeting five different liver rare diseases and is also working on the understanding and means to control the patient's immune response to AAV vectors.

I have more than 20 years of experience in biology and have focused on the cell and gene therapy (CGT) area for the past 15 years. During my time working in CGT, I have worked in wet labs in analytical development, process development, and bioproduction. For the last 7 years, I have worked as a CMC project manager. Manufacturing and regulatory compliance are two of the biggest challenges in the gene therapy area.

Q How are cutting edge analytics making a difference to your role and Vivet as a whole, particularly in terms of their capabilities to improve measurement of critical quality attributes (CQAs), accelerate processing, and reduce costs?

GS: There are many approaches in the analytical area regarding characterization of AAV products. The number increases each year, as do the needs and requests from regulators. In Europe, there is specific documentation regarding advanced therapy medicinal products (ATMPs), and there is documentation from the US Food and Drug Administration (FDA) of course. However, in France, there are no specific guidelines at the outset of development. For each new product, the CMC team has to prove to the regulators from scratch that the product is the right quality and safe for the patients.

As AAV products are complex, some of the raw or starting materials are also complex. This complexity is based on the fact that living cells are used to produce such products. The first step to ensure safety is to have complete control from the beginning, through manufacturing, to the end. The CMC lead has to prove and be confident in the process and environment by choosing the right contract development and manufacturing organization (CDMO) to manufacture the product.

Raw materials used as part of the process, such as the affinity resin, can result in more impurities than expected. Quality control (QC) testing for impurities in the final product has increased over the years. This led to us increasing the purification steps of our process to ensure the end product will have low parts per million (ppm) or billion (ppb) of residual material.

A key goal right now is to find new targeting methods in order to be more specific when identifying impurities. At the beginning, ELISA was used to work out the total level of host-cell protein (HCP) or PCR to know the bulk quantity of residual plasmid. Right now, I am trying to be more accurate in qualifying our product, by working out a more specific and precise method.

In addition, I also work on optimizing the characterization of the AAV product itself. 20 years ago when I started, experts were only focused on the sequence of the transgene injected in order to be sure it would produce the right proteins. But today, regulators want to know

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the full sequence of the AAV product, to understand if there is any risk of expression of other parts of the DNA.

Next-generation sequencing (NGS) can also help in detecting the identity of the AAV, to help ensure 100% of a product is safe, and each particle is comparable. With NGS, it is possible to focus on a single particle of AAV. Within the last five or ten years, many papers have focused on the fact that when you try to integrate your transgene into your AAV, there is some change, and some of the AAV particulates have more or less genes than expected. I have to prove that the extended DNA incorporated in the AAV poses no risk of expressing something else.

The next goal is to have a complete vision of the particle. The further one goes into detail, the greater the risk of finding differences. Biological differences are normal for this kind of production, but they need to be explained and risk assessed.

The most recent evolution for the characterization of product is focused on the capsid of the AAV. there are many serotypes for AAV, and some of them are specific to organs. For each product, there are one or two different serotypes that allow better targeting of the brain, or the liver, or other organs in the body. There have been recent requests from regulators for better characterization of the capsid. An ELISA test is not sufficient to do that, as it is not accurate enough for these small quantities. However, other methods, such as mass spectrometry, can help identify a discrepancy between the capsid construct and protein sequence. It is possible to evaluate the percentage of this type of capsid and conducting a risk assessment.

In terms of acceleration of bioprocessing, when I am confident in the product, I do not look to accelerate the bioprocess. Instead, I prefer to better characterize the final product and improve the method, to be sure of the safety and purity. Currently, my ongoing work is not related to the process itself, but rather on the in-process controls (IPC). I am not currently thinking in terms of acceleration, when I am at the beginning of the manufacturing development stage. I am working on the complete design of the clarification, chromatography, and tangential flow filtration (TFF) to be sure I have no big surprises at the end.

In terms of cost, I think a lot about the cost of goods and the final price of our product. It is desirable to reduce the costs and keep the same quality of product. The price of the future commercial product shall take into account the price of manufacturing and the price of starting material.

For me, reducing costs in terms of QC is not the priority. I have to work on scaling up our process to produce more from a single batch. In addition, I must also evaluate the cost of raw materials, working with CDMOs and purchasing more plasmid quantity to decrease the cost.

"The next goal is to have a complete vision of the particle. The further one goes into detail, the greater the risk of finding differences. Biological differences are normal for this kind of production, but they need to be explained and risk assessed."

Some big pharmaceutical or biotechnology companies decide to internalize some production in order to reduce costs.

Q What is your assessment of current tools and methods for measuring empty-full capsid ratio?

GS: There are a few methods that can allow us to evaluate the ratio between full and empty capsid. Some of these methods are sufficiently accurate to identify if it is a partially full vector. The most common method is electron microscopy - to observe the differences between full and empty through imaging. Other methods are more accurate, but electron microscopy is sufficient to give an overall proportion of capsids. I know at the end it will be impossible to have 100% full particles. The difference between empty and full particles is link to the inclusion of the DNA trangene, and techniques used to separate them during the purification process are not 100% accurate.

To manage this challenge, in your purification process, you have to be sure the proportion of empty-full is mostly full. At the beginning of the production, you are able to have only 10% full particles and 90% empty particles. It is complex, and it is more and more of a question mark for the safety of the patient. When injecting a patient, the dose is expressed as a transgene titer, for example. When you have only 10% full and 90% empty, if you inject 1×10^{14} vg/kg particles, you are injecting 90% waste. The more empty capsids, the greater the risk for side effects to the patient, because you have to inject a greater total quantity of particulates.

Sometimes, with 40–50% full alongside the right purification method, you are able to reach 80–85%. It is not easy, and it is also dependent on the AAV serotype. Some AAV serotypes produce many empty particles, so you have to evaluate your serotype before production and purification in order to avoid additional surprises. I do not try to have an accurate empty/full readout of ±1%, as this does not have a huge impact in terms of product quality.

Q Is there a consensus in the field as to what constitutes a minimum amount of CMC data in the ultra-rare disease setting?

GS: There is specific minimum testing for such products. For each new product, you establish your CQA, and which qualified method you will use to characterize your product. For each new Product, there is a minimum QC for safety and to characterize the identity of the product, though, as previously discussed. There isn't a big difference between the EMA, US FDA and French regulators in this regard. For all of them, The CMC team must find a good balance between the minimum QC testing and over-testing that will not be supportive for the safety or the efficiency of our product. I need to find a right way to be confident with data and lower the level of risk for the patient. Even if you have a rare or ultra-rare disease, there is no impact in terms of the quantity of QC.

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The difference is more linked to the phase of your project. When you are in Phase 1, you guarantee the product is safe, and you have to characterize impurities or identity of the product. When you reach the pivotal and commercial trials, you must share better characterization of your product. However, it is not always in the final certificate of analysis (CoA) for each new batch, because it is a time-consuming approach.

For me, whatever disease we target, I ensure the same level of quality for our products. With ultra-rare diseases companies are seeking interaction with the regulators on these aspects, but that does not change the way I work on the CMC side. I always characterize in the same way, and when I have questions, I focus on new characterization methods and new techniques in order to keep our products of transparent good quality.

Q Can you share any insights or best practices in terms of how to prepare for success in the area of potency assay development?

GS: The potency assay is a complex challenge. There is no easy way to assess activity to ensure consistency from batch to batch of such product. My recommendation is that when you start your project, work out an independent strategy for the development of your potency assay. It is rare for a Phase 1 clinical trial to have a fully developed and qualified potency, as it can be complex to establish.

For evaluation of the potency of AAV products during manufacturing, I have to work on in vitro live cell methods. This is complex with many parameters and high variability during a run or between multiple runs. My advice is to start as early as possible. It is mandatory to work in parallel with a surrogate assay, to prove to the regulators that you have a parametric approach to prove your product is efficient. Some surrogates are focused more on the mRNA expression, and others on protein expression. You have to create a parallel way to decrease the risk and prove your method in vivo with preclinical animal studies. However, for each batch, it is unfeasible to launch a new in vivo study.

You have to stay focused on your potency activity, and work with a good CRO in order to ensure you have a good reproducible method alongside a surrogate assay. At a certain level, you will have the first potency data, but your method will not yet be fully qualified. The surrogate is always there to support the potency and to tell authorities that the product you will inject into the patient is efficient. Only after two or three years with a potency assay can you discard the surrogate.

The potency assay for gene therapy is a very big challenge. For each disease in your portfolio, you go back to zero for each potency. There is no bridge between potencies. It is always a new approach, and a new mechanism of action of the transgene and the protein. The methods, the device, and the readouts

"The potency assay is a complex challenge. There is no easy way to assess activity to ensure consistency from batch to batch of such product."

will all be changed. You also need to show there is a dose response to your product, and that at each concentration, there is an improvement of the response. The ultimate challenge of potency is to have a range of concentrations to reassure regulators there are increasing effects of your product. In terms of cost, the potency assay and its surrogate take up a large part of the final budget of QC development.

If you have not developed your potency assay in-house, you need to outsource it. The choice of the CRO is also important - you have to find the right CRO company that can support you during development to solve any issues that arise. It is likely you will meet many issues during your potency development.

That is important, and I have had previous issues where I had to go to new vendors and CROs to manage the development and qualification of the potency assay. You need a scientific background and support internally, and you need to work as a team to find the right method and simplify the potency assay.

Q What are some key priorities for your own work and for Vivet as a whole over the next few years?

GS: The potency assays are important for me. I have to work, at early-stage, on the proof of concept to know which option or strategy we will go with.

Regarding the process itself, there are many CDMOs proposing AAV products. It is a big challenge to find the right one to support the development of the process and to manufacture the various non-GMP and GMP products on time. You must take time at the beginning to find the right one. The challenge for us at Vivet revolves around the capability of the CMO to manufacture high-concentration vectors. Priority number one is to have the highest possible ratio of full capsids, so I chose our collaborators based on that.

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CELL & GENE THERAPY INSIGHTS

VECTOR CHARACTERIZATION

The present hurdles and opportunities in our knowledge of AAV basic biology

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VIEWPOINT

"The ultimate goal is to understand the basic biology of both wild-type AAV and AAV vectors to create more targeted, efficient gene therapy platforms."

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RECENT ADVANCES IN VIRAL VECTOR ENGINEERING

For the past 15 years, the viral vector-driven gene therapy field has been consistently advancing on all fronts, but particularly in terms of vector optimization. Vector capsids and therapeutic payloads have both been engineered to increase specificity, efficacy, and safety.

There are different ways of engineering the adeno-associated virus (AAV) capsid. One of the most commonly used technique is capsid shuffling. In this approach, pre-existing capsids are shuffled to get a library of capsids which are then tested on cell/tissue of interest, both *in vitro* and *in vivo*. The capsids that are most efficient in transducing the target cells are then selected, manufactured, and characterized individually. These capsids can deliver the therapeutic payload more efficiently for a given target disease.

Optimized factor IX in hemophilia is a good example of how a therapeutic payload can be engineered. The activity of this version of the factor IX gene is significantly higher than the normal one. Gene regulatory elements such as tissue-specific promoters have also been developed, with some currently in clinical trials. Gene regulatory elements work well to prevent off-target effects in tissues that are not of clinical relevance, thus improving safety.

In addition to increasing specificity and efficacy, these approaches can also reduce the effective vector dose and even increase the number of disease targets, allowing a move away from the monogenic diseases that have dominated the AAV gene therapy field to date. With novel genome engineering technologies emerging, one may envision multiple and more complex diseases being targeted with the same AAV vector platform.

IMPLICATIONS FOR THE CHARACTERIZATION OF NOVEL VECTORS

When characterizing novel vectors, one must go through the standard FDA-approved regulatory steps. Safety is a big issue, of course, so all parameters must be met. Most of the key characteristics are the same across all vector platforms – e.g., purity, optical density, *in vitro* and *in vivo* potency, genome titer, pH, viral contaminations and so forth. The guidelines must still be met, meaning not much will change in this regard for the novel vector platforms emerging.

However, novel engineered vector platforms do come with differences of their own. For example, each capsid when produced reaches a different titer. This will be important in manufacturing large quantities of clinical-grade vectors for injection into patients. The greatest impact on characterization of these new vectors will relate to titer and how they interact with the host cells.

CURRENT GAPS IN UNDERSTANDING OF THE BASIC BIOLOGY OF EMERGING VECTOR ENGINEERING PLATFORMS

When it comes to the basic biology of engineered AAV vectors, each vector is based on a modified version of the wild-type AAV. A capsid-modified vector has the same or a similar capsid as the wild-type AAV. The steps taken by a wild-type AAV to enter the cell, to transduce the cell, and express its genome are similar for all engineered vector platforms.

It has been almost 60 years since the discovery of AAV. We know roughly how AAV infects the cells, in that it binds to glycan receptors at the cell surface. This binding is stabilized by proteinaceous co-receptors/entry factors. These proteinaceous entry factors help to internalize the AAV particles. The AAV particles then traffic through different intracellular compartments, including the Golgi. Unlike some other viruses, what is interesting about AAV is that at least some of the intact particles enter the nucleus through the nuclear complexes, where the genome is released and gene expression begins.

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In 2016, the discovery of adeno-associated virus receptor (AAVR) marked a key breakthrough in the field of basic AAV biology. AAVR usage is conserved across different cell types, species, and capsids. Since its discovery, a few follow-up studies have explored AAVR's role as a receptor. AAVR is predominantly localized to the Golgi, which traffics through the cell surface and probably aids the entry of AAV. There is still much to discover regarding the basic biology of AAVR and other entry factors to find out exactly how they impact AAV infection.

Different AAV capsids are used to target different tissues due to differences in tropism. The biggest knowledge gap in this field is the cellular as well as capsid determinants of AAV tropism. Capsid and target cell-specific differences in AAV vector–host interactions, which determine the tropism, are not clearly understood. There are many unknowns, especially within the initial cellular entry step. AAV is of great interest from a basic biology standpoint; it is a fascinating helper-dependent virus that could open up new understanding which might be relevant to other viruses.

NEXT STEPS IN FILLING AAV BASIC BIOLOGY KNOWLEDGE GAPS

As discussed, the cellular entry of AAV vectors is an enigma. Receptor switching is seen, meaning that AAVs are promiscuous in their receptor usage. If we could find receptors that are specific to our target cells, and redesign capsids to target those specific receptors, that would be a huge breakthrough. It would allow us to design capsids targeted to particular disease and prevent off-target effects.

Another recently identified entry factor, GPR108 (PMID: 31784416), is conserved across even the AAVR-independent capsids (although notably, AAV5 – a much more evolutionarily divergent AAV serotype – is independent of GPR108). As a proof of principle, the authors identified the domains within the AAV capsid that engaged with GPR108. They then engineered chimeric capsids to increase the dependence on a particular entry factor – in this case, GPR108. This proof-of-principle study demonstrates that the capsids can be engineered to engage with specific receptors/entry factors. The targeted capsids could then be injected via a particular route to reduce the dosage. For example, for disorders of the brain, we could design capsids specific to a particular neuronal receptor, and inject them in a targeted manner to CNS (e.g., intrathecal, intracranial, etc.) to minimize off-target effects. Technological innovations will allow more targeted and safer vector platforms moving forward.

We work in a vibrant campus comprising the Department of Cell and Molecular Therapies (CMT) at one of Australia's oldest and most respected hospitals and the Gene and Stem Cell Therapy Program Centenary Institute. There are two different streams working with AAV-based gene therapies covering a large portfolio with different branches ranging from clinical trials to basic biology. We are one of only four centers in Australia that conduct AAV-based gene therapy clinical trials for a range of genetic disorders, including hemophilia A and B.

On the basic biology side of things, capsid-specific differences in cellular tropism are a strong focus. I seek to understand capsid and cellular factors that drive the tropism of a particular AAV vector platform. The ultimate goal is to understand the basic biology of both wild-type AAV and AAV vectors to create more targeted, efficient gene therapy platforms.

BIOGRAPHIES

BIJAY DHUNGEL, PhD, is a post-doctoral researcher, Gene and Stem Cell Therapy program Centenary Institute, University of Sydney. He has a PhD from the University of Queensland, an MSc in Molecular Medicine and Biotechnology from the University of Eastern Finland and a B.Tech in Biotechnology from the Acharya Nagarjuna University. He has engineered several AAV-based targeted gene delivery platforms for cancer gene therapy. Dr Dhungel is currently interested

in understanding vector-host interactions of AAV and devises strategies to leverage this knowledge to create safer and more efficient gene therapy platforms.

PROFESSOR JOHN RASKO is an Australian pioneer in the application of adult stem cells and genetic therapy. Since 1999 he has directed the Department of Cell and Molecular Therapies at Royal Prince Alfred Hospital and the Gene and Stem Cell Therapy Program at the Centenary Institute, University of Sydney. John Rasko is a clinical hematologist, pathologist and scientist with an international reputation in gene and stem cell therapy, experimental hematology and molecular biology. In over 170 publications he has contributed to the understanding of stem cells and blood cell development, gene therapy technologies, cancer causation and treatment, human genetic diseases and molecular biology. He serves on Hospital, state and national bodies including Chair of GTTAC, Office of the Gene Technology Regulator – responsible for regulating all genetically-modified organisms in Australia – and immediate past Chair of the Advisory Committee on Biologicals, Therapeutic Goods Administration. Contributions to scientific organizations include co-founding (2000) and past-President (2003-5) of the Australasian Gene and Cell Therapy Society; President (2018–20), President-Elect (2016–18) and Vice President (2008–12) of the International Society for Cell & Gene Therapy; Scientific Advisory Committees and Board member for philanthropic foundations; and several Human Research Ethics Committees. He is a founding Fellow of the Australian Academy of Health and Medical Sciences. In 2018, the Board of the ABC honored him as the sixtieth Boyer Lecturer. He is the recipient of national (RCPA, RACP, ASBMB) and international awards in recognition of his commitment to excellence in medical research, including appointment as an Officer of the Order of Australia.

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CELL & GENE THERAPY INSIGHTS

VECTOR CHARACTERIZATION

INNOVATOR INSIGHT

Simplifying analytical development of viral vector production: robust and sensitive methods for common expression systems

Srinath Kashi Ranganath

The number of viral vector-based gene therapies in clinical trials has recently grown into the thousands due to the tremendous genetic disease-curing potential they harbor. Despite this growth, the comprehensive characterization of critical quality attributes for the safety and efficacy of the material produced for these trials remains a challenge for both manufacturers and regulatory bodies alike. The demands on analytical development teams are oversized compared to legacy biopharmaceuticals and require a unique focus to address issues such as identification, characterization, and enumeration of undesired byproducts. Application of established regulatory guidance, such as limits to residual host cell DNA, requires additional scrutiny due to possible encapsidation and oncogenic potential. This article will focus on the current state of analytical methods in gene therapy workflows, and how leveraging the work Thermo Fisher Scientific has developed can help simplify the burden on analytical development teams.

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Our solutions facilitate one of two highly advanced and well-established core technologies: DNA sequencing, and real time PCR (qPCR). Our MicroSEQ™ microbial identification system utilizes gold standard genotypic DNA sequencing technology to provide accurate identification of potential fungal and bacterial contaminants. This can be used as part of an environmental monitoring plan or a robust microbial risk mitigation strategy. Our solutions for detection of advantageous agents such as mycoplasma, viruses, and quantitation of residual host cell DNA utilize qPCR to achieve fast, accurate, reliable, and actionable results.

SAFETY & EFFICACY IN THE GENE THERAPY FIELD

The gene therapy field is advancing rapidly, with seven currently approved viral-vector gene-therapy-based therapeutics worldwide, and over 3,000 clinical trials in the pipeline. Notable AAV-based therapeutics include Luxturna for treatment of Leber congenital amaurosis-2 (LCA2), and Zolgensma for treatment of spinal muscular atrophy (SMA). Investments in this area are estimated to reach $3.4 billion in 2024 and will more than double to $6.5 billion by 2030. Despite the tremendous success of many viral vector-based gene therapies, recent adverse effects in clinical trials, such as development of liver cancer, have elevated the urgency of addressing safety and efficacy. These issues need to be investigated in preclinical models and patient monitoring in absorption, distribution, metabolism, and excretion (ADME) studies. Some can likely be addressed by analytical teams via monitoring of the critical quality attributes of the drug product itself and through employing novel analytical techniques.

At the 2021 US FDA meeting, the potential vector-mediated integration of non-vector DNA and the risk of oncogenesis in viral vector-based gene therapies were addressed. Assessing the risk requires analysis and elimination of sequences in vectors that increase the risk of oncogenesis.

ANALYTICAL TESTING OPTIONS FOR GENE THERAPY CUSTOMERS

There are three options for analytical testing for gene therapy: developing home-brew methods in-house, using contract testing service labs, or using fully integrated commercial solutions. The general trend in the industry is to use commercial solutions in order to save both time and resources, and as the industry matures more solutions are being developed. This article will address the support provided by Thermo Fisher Scientific toward this goal, and the capabilities of current products to assist in characterization of nucleic acid impurities, removing the challenges of developing these analytical requirements in-house.

ANALYTICAL ASSAYS OFFERED BY THERMO FISHER SCIENTIFIC

In addition to the areas of increased concern and scrutiny discussed above, there are a myriad of analytical assays for characterization and release testing for viral vector products. Applied Biosystems™ resDNASEQ™ residual DNA systems were developed to rapidly and accurately quantify residual host cell DNA and plasma DNA. To address the concern of the presence of mycoplasma, the first regulatory-accepted rapid molecular method was developed: The MycoSEQ™ mycoplasma detection system. To address the requirements for the identification of potentially contaminating microbes, the MicroSEQ™ microbial identification system was developed.

Figure 1 provides a view of the analytical assays Thermo Fisher has developed to simplify in-house analytical development for viral vector manufacturers, which are designed to meet Current Good Manufacturing Practice (cGMP) regulations.

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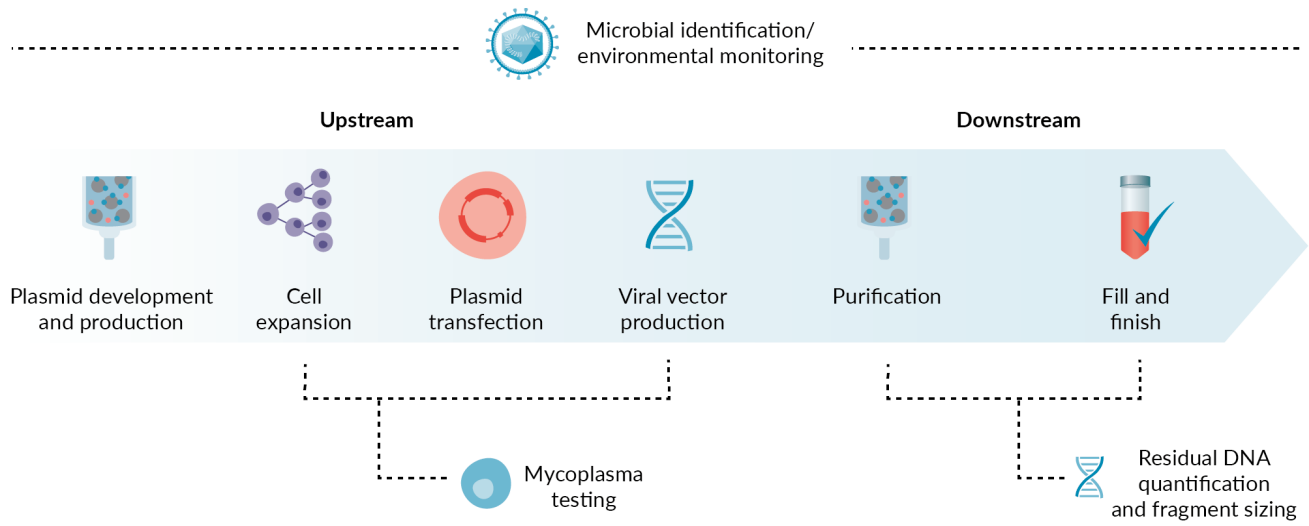
FIGURE 1

Simplifying analytical development to address established regulatory guidance.



RESIDUAL DNA QUANTITATION & FRAGMENT SIZING ANALYSIS

Residual DNA left in a product can impact quality, efficacy, and safety. Regulators worldwide therefore require limitations on the amount of residual DNA in the final dose. The WHO recommends that the amount of residual DNA per dose is kept below 10 ng. It is suggested by the FDA that a method with a sensitivity of 10 pg be used to determine DNA levels. Residual fragment length analysis is expected to demonstrate <200 base pairs (bp). There is increased concern that encapsidation is leading to viral vector products with larger amounts and longer sequences of residual DNA. Oncogenic sequences are of particular concern and must not be present in the final product.

Thermo Fisher offers end-to-end solutions consisting of all-inclusive kits with well-characterized standards and reagents. These assays have been designed to meet regulatory guidance with high sensitivity, reproducibility, and lot-to-lot consistency over several years.

The resDNASEQ™ residual DNA quantitation system (Figure 2) is the first and only fully integrated qPCR system for quantitation of residual host cell and plasmid DNA, including highly characterized DNA reference standards.

There are resDNASEQ™ solutions for both insect (Sf9) and mammalian cell culture-based viral vector manufacturing systems. This article focuses on the solutions for the mammalian expression system.

ANALYTICS FOR THE HEK293 PRODUCTION SYSTEM

Thermo Fisher Scientific offers multiple assays that apply to the HEK293 production system. These include solutions to quantify residual host cell and plasmid DNA and a residual adenovirus early region 1A (E1A) fragment sizing assay. The resDNASEQ™ quantitative E1A DNA fragment length kit is the newest assay developed specifically for HEK293 processes to address two additional aspects of regulatory guidance, in addition to quantitation of the residual DNA. This kit can simultaneously detect and quantify E1A DNA of different fragment sizes. All of these assays can be used throughout the downstream process to support the characterization and optimization of your process and for routine quality control (QC).

FIGURE 2

ResDNASEQ residual DNA quantitation system.

Ultrahigh sensitivity
0.3 pg/reaction LOQ for HEK293 DNA
30 copies/reaction LOQ for Kanamycin-Plasmid Resistant DNA
0.3 pg/reaction LOQ for Sf9 and Baculovirus DNA

Rapid testing and streamlined workflow
Time to results <5 hours with optimized sample prep

Reliable performance
Consistent performance kit to kit, lot to lot, year to year

ADENOVIRUS EARLY REGION 1A (E1A)

E1A is an oncogene integrated in chromosome 19 of HEK293 cells, providing essential genetic regulatory modulation for viral vector manufacture. This gene allows HEK293 and various related cell lines to be used to produce recombinant adenovirus, recombinant adeno-associated virus (AAV) and recombinant lentivirus. One current challenge in viral vector manufacturing is co-packaging of the host cell DNA within recombinant viral vector capsules. As E1A is both part of the HEK293 host cell genome and a known oncogene, any potential residual E1A requires detection and quantification as a harmful process-related impurity. Regulatory guidance requires the method used to demonstrate the effectiveness of the DNA reduction process to <200 bp fragments.

As shown in Figure 3, the E1A assay design involves three single-plex assays targeting known overlapping fragment sizes of short, medium, and long fragments. The assay requires three standard curves, one for each fragment size, to quantitate E1A fragments of unknown samples.

The E1A kit comes with all reagents and standards for all three single-plex assays. Each assay shows high linearity and efficiency and enables accurate qualitative results across a broad range of DNA concentrations. The kit has been validated in various matrices used in gene therapy to reflect typical application situations, including inhibitors and at varied concentrations. The kit has shown excellent performance under these conditions. The standard curve performance of the kit, as shown in Figure 4, demonstrates high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations.

RESDNASEQ QUANTITATIVE PLASMID DNA KITS

Since its launch in late 2020, the Applied Biosystems™ resDNASEQ™ quantitative HEK293 DNA kit has seen tremendous

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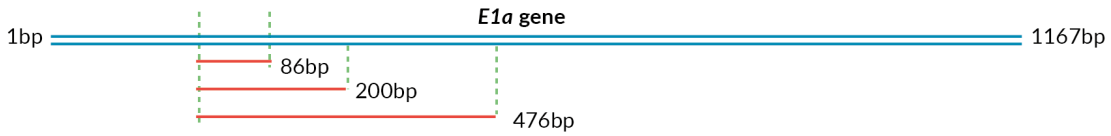
FIGURE 1

E1A fragment length assay concept.



Assay design

- Three single-plex assays for known fragment sizes: short, medium and long
- Three standard curves for quantitation of each fragment size
- Sample Prep with PrepSEQ Chemistry, KingFisher Flex, qPCR with 7500 Fast or QuantStudio 5
- Readout directly in AccuSEQ software in copy number for unknown fragments

Example

Quantification

Short Frag assay (86bp)	110+100+90 = 300 copies
Med Frag assay (200bp)	100+90 = 190 copies
Long Frag assay (476bp)	90 copies

Ratio

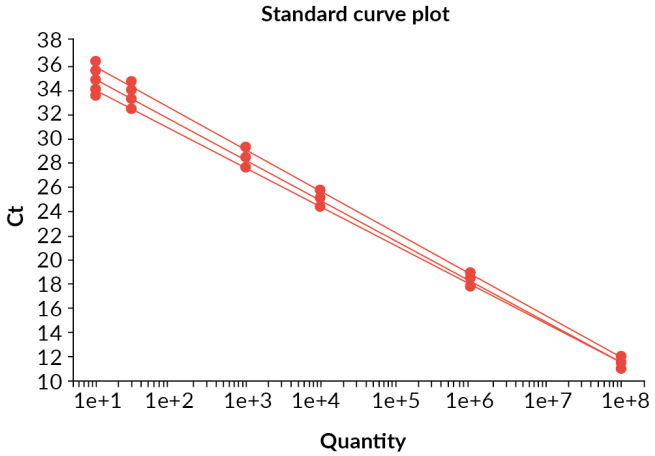
<200bp	110/300
200–476bp	100/300
>476bp	90/300

success in the gene therapy field. The resDNASEQ™ kanamycin resistance gene kit was launched in 2021 and has been implemented in several gene therapy production processes. Both kits follow the same general workflow, including a DNA extraction procedure using PrepSEQ™ solutions and a qPCR assay for quantitation of the target DNA fragments.

The resDNASEQ™ quantitative plasmid DNA and kanamycin resistance gene kits, and the HEK293 residual DNA kit assays provide high sensitivity and broad dynamic range allowing testing of a wide range of samples. The method linearity within the dynamic range easily meets specifications, as demonstrated by R2 values of >0.99, as shown in

FIGURE 4

Standard curve performance.



	Short	Medium	Long
Fragment size	86–200 bp	200–476 bp	>476bp
Slope	3.284	3.363	3.433
R^2	0.999	0.999	0.999
Eff:	101%	98%	95%

Table 1. In addition, the PCR efficiency has been calculated to 100% ± 10%.

Rigorous internal validation of the resDNASEQ™ assays has been completed. Internal validation studies are executed using our total workflow solution to verify our assays perform to specifications designed to meet regulatory guidance and validation criteria.

TOTAL WORKFLOW SOLUTION

The streamlined workflow begins with a manual or automated sample preparation. A semi-automated version utilizing the KingFisher™ Flex allows for up to 96 extractions at a time. Regardless of the level of automation chosen, the sample prep features well-established PrepSEQ™ chemistry based on magnetic particle-based suppression. The second and third steps in the workflow are setting up and running the resDNASEQ assay on one of our recommended Applied Biosciences real-time quantitative PCR (qPCR) instruments. The results are reported on the AccuSEQ™ software.

AccuSEQ™ real-time detection software supports you with the setup, running, and analysis of your qPCR experiments. The software has Security, Audit, and e-Signature (SAE) functionality that helps enable 21CFR part 11 compliance and full traceability of all actions within the software.

OPTIMIZED SAMPLE PREPARATION

Gene therapy sample matrices are typically associated with PCR-inhibiting components such as benzonase, detergents, media components, and potentially high levels of other non-target nucleic acid material. Various sample matrices were tested, and spiked DNA was successfully recovered and quantitated with expected limits across all sample types. Results show that PrepSEQ chemistry allowed to successfully prepared samples from a variety of matrices common to gene therapy bioproduction workflows.

PrepSEQ™ kits involve a combination of alcohol precipitation and magnetic bead-based extraction of nucleic acids from a plethora of sample types. This is a universal sample prep for extracting double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and RNA. The PrepSEQ kit has shown excellent performance in obtaining quantitative recovery, high precision, and consistent performance with complex matrices including low pH, high salt, and high protein.

The PrepSEQ chemistry can be used in both manual and automated workflows. Depending on the type of automation and experimental design chosen, 1–96 samples can be processed in one run. The manual method is low throughput and allows 1–16 extractions per day. The KingFisher™ Flex system is

TABLE 1

Residual DNA kit specifications.

Specification	Kanamycin-resistance gene plasmid DNA kit	HEK293 DNA kit	E1a Fragment Length Kit
Linearity	$R^2 > 0.99$	$R^2 > 0.99$	$R^2 > 0.99$
PCR efficiency	100% ±10%	100% ±10%	100% ±10%
Precision	≤10% CV	≤10% CV	≤10% CV
Limit of detection (LOD)	15 copies	30fg	10 copies
Limit of quantitation (LOQ)	30 copies	300fg	30 copies
Assay range	300,000 to 30 copies	300fg to 3ng	10^8 to 30 copies
qPCR instruments tested			
Applied Biosystems™ 7500 Fast Real-Time PCR QST System			✓
QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1 mL			✓
Gene Therapy Matrices tested			
Sample derived from a bioreactor at harvest			✓
Sample after chromatography			✓
Sample after final purification			✓

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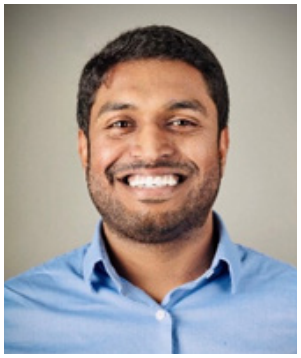
high throughput, allowing for up to 192 extractions per day. The KingFisher Flex offers many advantages such as easy setup using graphical interface, high speed purification of nucleic acids, control of cross-contamination, and high-quality nucleic acid recovery from a wide variety of sample types.

INSIGHT

In summary, the resDNASEQ™ system is a robust residual DNA quantitation solution for therapeutic grade AAV production. This all-inclusive system includes highly characterized DNA standards and reagents necessary for residual DNA quantitation. It provides an optimized sample preparation for quantitative DNA recovery and ultra-high sensitivity and specificity with no cross-reactivity to unrelated DNA. The streamlined workflow provides reliable data within five hours. Our quality assurance team provides consistency so that customers will receive the same high-quality performance from kit to kit, and our worldwide technical support network will assist you throughout all phases of the implementation process from early qualification and validation, all the way to lot-release and routine testing.

Q&A with Srinath Kashi Ranganath

Charlotte Barker, Editor, *BioInsights*, talks to **Srinath Kashi Ranganath**, Field Applications, Pharma Analytics Group, Thermo Fisher Scientific



SRINATH KASHI RANGANATH is a Staff Scientist – Field Applications with the Pharma Analytics group at Thermo Fisher Scientific, supporting customers in implementing, optimizing and validating the Pharma Analytics workflows for biomanufacturing processes across various therapeutic modalities. Prior, Srinath served as a Bioassay Scientist and an SME for the development and optimization of assays for residual DNA and other process impurities for 6 years. Srinath has an MS in Pharmaceutical Sciences from Campbell University, NC. His thesis is focused on understanding the expression profile of certain intracellular signaling molecules and how altering their function will affect the downstream cell signaling.

Q Can I use the resDNASEQ quantitative DNA fragment length kit instead of the resDNASEQ HEK293 DNA kit?

SKR: The resDNASEQ™ HEK293 kit is specifically designed to yield highly sensitive quantitation of residual HEK293 DNA samples. The E1A DNA fragment length

kit will provide additional information about the fragment sizes in the sample. There are significant differences in the kit design; the HEK293 kit targets a conserved repeat region of the genome giving the confidence in sensitivity requirements designed to meet regulatory guidance, while the E1A kit targets just one gene. There is preliminary data to show that there is some correlation between the E1A quantitation and the total residual host cell DNA for HEK293. It is not linear, but when you look at clearance studies in the downstream purification process, similar orders of clearance are observed between the two types of DNA. At this point, I do not think you will be able to use the E1A kit to replace HEK293 testing.

Q What is the regulatory track record for the HEK293 residual DNA kit?

SKR: That information comes from feedback from the field, but because many of customers purchase via the website and do not require a lot of support during implementation, qualification, and validation, it can be hard to collect. It has been purchased by more than 100 different customers and, to date there are no known issues with the acceptance of this method by regulatory agencies.

Q Do you have any available solutions for a droplet digital PCR (ddPCR) platform?

SKR: These kits are designed to work very well on qPCR, but customer feedback indicates that the E1A, kanamycin, and Sf9 baculovirus strips also perform well on digital PCR (dPCR) platforms. A ddPCR platform will probably not work well with an assay design that targets a highly repetitive element.

Q There are two genes encoding kanamycin resistance that are commonly used in plasma. Does your kit detect both?

SKR: The kanamycin resistance gene kit was developed to pick up the vast majority of antibiotic-resistant plasmids used in biopharmaceutical manufacturing by targeting sequences common to three gene families of kanamycin resistance. So it is very likely that it will work with most plasmids containing the kanamycin gene. The address specific questions about whether or not the kanamycin resistance gene kit is able to quantitate a specific plasmid, please contact a Thermo Fisher Scientific representative, and bioinformatic information may be available.

Q Is there a cross-reactivity between this kit and other non-HEK293 cells?

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SKR: It is important for a residual DNA kit to be specific. Extensive exclusion primer testing is performed so that these kits meet specificity requirements. Additionally, resDNASEQ kits are species-specific, but not specific to a certain cell line. For instance, the HEK293 kit will pick up and actively quantify human DNA. In addition, the only low level off-target reactivity is that with Vero (African Green Monkey) DNA, which is unsurprising given the close relationship between humans and monkeys. For unrelated species, no cross-reactivity was observed.

Q Does the residual human DNA kit over or underestimate the DNA concentration of HEK293 DNA? If so, what is the magnitude of the difference?

SKR: There is data to show that the HEK293 and human DNA standard curves line up on top of each other. There should not be any major differences between measures with both kits. The HEK293 kit is specifically designed for gene therapy customers who use HEK293 cell lines, and includes a HEK293 DNA standard, although both kits are shown to quantitate the HEK293 or human DNA the same way.

Q Do you see any interference from the presence of digested envelopes in DNA quantitation assays?

SKR: In development, Thermo Fisher Scientific tested a representative sample that might not correlate with the results that you see in your sample because your process might be unique. There are a few known customers who are working to answer the same question: is detection of any incorporated residual DNA in the capsules possible without protein digestion and without lysis

Q Are the qPCR assays compatible with the QuantStudio 6 and 7?

SKR: It is highly recommended that the kits be used on the complete system that have tested and validated during in-house testing: the 7500 Fast and the QuantStudio 5 instrument with the AccuSEQ software. This is particularly because 21 Code of Federal Regulation (CRF) Part 11 compliance is only possible with AccuSEQ. It is not possible with the software on the QuantStudio instruments such as the design and analysis such as the QuantStudio rtPCR software. If this is used simply for research and development testing and 21 CRF Part 11 compliance is not needed, then the kit works just as well as any qPCR instrument that fulfils the filter and sample block requirements.

Q Where in my workflow do I need to test for residual fragment length?

SKR: Given the rapid turnaround, fragment size testing can be performed at any point during the process. However, the clear utility of this kit is to measure the efficiency of the DNA size-reduction steps such as benzonase, further upstream, or right at the harvest stage when you are performing the DNA size-reduction step and the lot-release testing stage. That being said, the kit may also be used in process development to test different elusions for the presence of the E1A gene to demonstrate clearance.

Q Are there any plans to add other types of cell line to the resDNASEQ family?

SKR: The resDNASEQ™ portfolio has evolved over time. The Chinese hamster ovary (CHO) assay came first, but now there are many more targets. As emerging needs become clear, additional targets will be added. If a particular residual DNA assay is needed for your process, please get reach out to Thermo Fisher Scientific. More feedback received by Thermo Fisher Scientific helps enable quicker development of solutions to customer problems.

AFFILIATION

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applied biosystems

Characterization of advanced therapies: leveraging advanced analytics to avoid FDA holds

Dana Cipriano & Alexei Saveliev, Center for Breakthrough Medicines

The accurate characterization of cell and gene therapies is currently mission-critical to the industry. The past two years have seen a swathe of rejections, delays, and suspensions from the FDA due to CMC issues, many driven by a lack of product characterization and insufficient understanding of mechanisms of action (MoA).

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The MoA of advanced therapies is often poorly understood and consequently, assay design has not been robust. Developers are finding it hard to provide the proof that regulators need to approve products.

To bridge this gap, developers need access to services and tools that allow for sensitive and specific assays, with the flexibility to test across the five key domains required throughout the manufacturing process by the FDA: identity, stability, safety, purity, and potency (Figure 1).

ORTHOGONAL METHODS OF PRODUCT CHARACTERIZATION

Taking an orthogonal approach to early product characterization is key to the testing strategy of the Center for Breakthrough Medicine (CBM), as detailed in Figure 2. Potency testing, next-generation sequencing (NGS), and mass spectrometry should be leveraged early in the drug development cycle to avoid later delays and address CMC challenges.

The use of advanced analytical capabilities, which are specific to a client's product, allows for early testing that can support demonstration of comparability later in development, including when changes occur to improve the manufacturing process. Using stability programs to evaluate product and reference standard stability throughout the product lifecycle, as well as having a CMC regulatory team to support clients during the regulatory filing process, enables swifter patient access to approved medicines.

Figure 1. The five key domains required by the FDA for regulatory approval.

IDENTITY

Final cell product should undergo an **identity test** (e.g., an assay to measure the presence of vector material) If it is ex vivo genetically modified.

Sample assays to be tested include:
- Cell surface marker assays
- Expression assays
- Vector genome
- Sequencing
- Restriction digest testing

STABILITY

FDA recommends a **minimum viability of 70%** for ex vivo genetically modified cells.

If this level cannot be achieved, manufacturers can submit data to support and demonstrate the **effectiveness of a product** with a lower viability specification.

The protocol should describe the **storage container, formulation, storage conditions, testing frequency, and specifications** (i.e., test methodologies and acceptance criteria).

SAFETY

Information assessing **risk of potential contamination** with adventitious agents should be provided.

Sterility testing should be performed before a product is frozen, purified, or cultured and a **rapid microbial detection test**, such as a Gram stain, along with sterility test on the final formulated product.

Testing methods include:
- Mycoplasma testing
- Adventitious agent testing
- Antibody production tests

PURITY

Freedom from extraneous matter in the finished product.

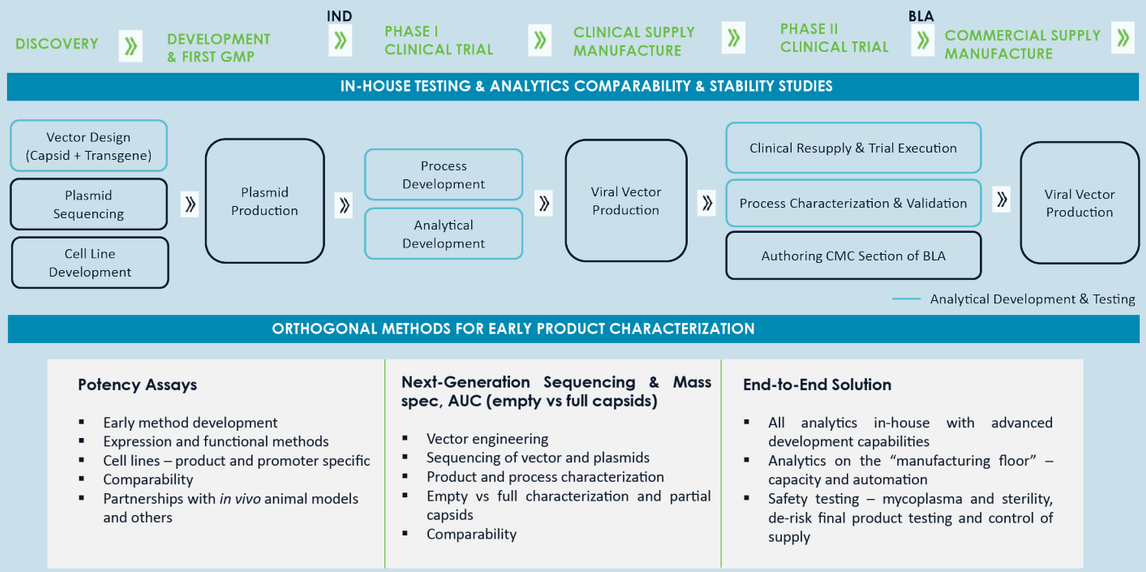
- Purity testing includes assays for **pyrogenicity or endotoxin** and **residual manufacturing impurities**
- **Rabbit pyrogen test** method is the current required method for testing certain licensed biological products for pyrogenic substances,
- FDA also accepts alternative test methods, such as the **Limulus Amebocyte Lysate (LAL)**

POTENCY

Potency assay is not required to initiate early phase clinical studies, but FDA recommends well-qualified assay to determine dose

- Assay should **represent the mode of action (MOA)** of the drug and be quantitative in nature
- Measure the **expressed product** and not the vector
- Prior to Phase III, assays that are not well defined may be used, including **quantifying gene therapy vector expression**

Figure 2. CBM testing strategy for advanced therapies including orthogonal methods for early product characterization.



NGS TECHNIQUES FOR PRODUCT CHARACTERIZATION

CBM offers comprehensive analysis for plasmids, viral genomes, and cell and gene therapies by Illumina NGS. Clients have further options for confirmation of transgene and antibiotic resistance genes, detection of substitutions, insertions and deletions in viral genome encoding and plasmid backbone sequences, detection of viral integration sites, RNA sequencing, and whole-exome sequencing.

The Sanger platform can also be used to confirm viral identity. In addition to short-read technologies, long-read platforms, such as PacBio Sequel and Oxford Nanopore, allows for cutting-edge genetic characterization.

In partnership with:
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VIEWPOINT

INTERVIEW

CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHT: MAY 2022

VIEWPOINT

A new wave of allogeneic CAR-T cell therapies in oncology treatment

Silvan Tuerkcan, PhD
JMP Securities, a Citizens Company

"The two big goals of allogeneic CAR-Ts are to get into earlier lines of therapy, and to succeed in solid tumors."

VIEWPOINT

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On April 25 2022 David McCall, Editor *Cell and Gene Therapy Insights* spoke to Silvan Tuerkcan about CAR-T cell therapies in oncology treatment. This article has been written based on that interview.

WHAT DO INITIAL OUTCOMES OF ALLOGENEIC CAR-T CLINICAL TRIALS TELL US ABOUT A DIFFERENCE IN MODALITY, THERAPY & FUTURE USE?

In general, the clinical efficacy data obtained to date for allogeneic CAR-T therapies is of the same standard as autologous CAR-Ts in terms of response rates, although the durability of response and duration of transplant is as yet not fully understood. We believe this could position allogeneic CAR-Ts as a different product altogether compared to autologous products.

For example, the overall response rate (ORR) of approved autologous CD19-directed should be: CAR-Ts – Breyanzi, Kymriah, and Yescarta – ranges from 50 to 73%, and in terms of complete responses, these numbers range from 32 to 54%. In the ALPHA trials of Allogene Therapeutics' anti-CD19 cell therapy, ALLO-501, data showed a response rate of 81% in follicular lymphoma (FL) and 64% in large B-cell lymphoma (LBCL) patients, when ALLO-501 was dosed in combination with a CD52 lympho-depleting antibody (ALLO-647). Importantly, complete response (CR) rates were also impressive, at 58% in FL and 43% in LBLC. In the initial trial of Allogene's ALLO-501A, we saw improved responses (especially with consolidation dosing, which is re-dosing shortly after the first dose) with initial response rates of 60% reached, although only a 10% CR rate was achieved. CRISPR Therapeutics' CTX110 – another CD19-directed CAR-T – provides a further example, recently showing initial response rates of 58% in the highest dose cohort (DL2+) in LBCL patients in the CARBON trial, without both enhanced or antibody-aided lympho-depletion and consolidation dosing.

With this data, we are approaching the lower limit of the commercial autologous CAR-T products in terms of overall response (ORR) and CR, especially if one considers the Intention to Treat (ITT) population. However, duration of response is an important dimension in which we do see some differences. For example, CTX110 showed a 21% (4/19) 6-month CR rate, and for ALLO-501, the 6-month CR rate was 43% (3/7) in LBCL patients. We have seen long CRs of 15+ months, which is good, but these numbers are low in comparison with other (autologous) CAR-Ts such as Kymriah, for which 75% of responders are still in remission at 6 months, and 64% at both 9 and 12 months.

For now, it appears as if autologous and allogeneic modalities differ considerably, with much longer persistence and greater expansion peaks for autologous CAR-Ts. Indeed, the durability of allogeneic CAR-Ts may never reach that of the autologous products. Many approaches are being explored to reduce rejection by host CD8+, CD4+, and NK cells and to improve the cell's metabolism, but they have not yet progressed into the clinic. That may seem disappointing to many, but in fact, allogeneic CAR-T products may not need to reach this sort of durability to be effective and adopted. Allogeneic CAR-Ts could be considered an entirely different modality of drug – one that could require re-dosing as needed in order to fight remission due to low CAR-T levels, or antigen-mediated escape of the tumor. If a patient has received a particular CAR-T and then their tumor mutates to present a different antigen, allogeneic CAR-Ts can allow a simple swap to the next target required. Re-dosing of allogeneic CAR-Ts may well be a necessity, but it may also be an advantage.

WHY PURSUE SO MANY CD19-TARGETED & BCMA-TARGETED THERAPIES?

Investors often point out that the CAR-T arena is very crowded. However, in my opinion, these indications are vital stepping-stones for the field. It is important to show proof of concept of a cell therapy platform in an established tumor type prior to making the move into solid tumors.

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Preclinical optimization of CAR-Ts has not always translated well into the clinic, and optimization of therapies in patients will be key for future iterations. It will become important, as CAR-Ts with additional edits arrive, to establish these novel platforms in an existing setting. Early experience in established liquid tumors with well-known benchmarks helps companies to evaluate and refine their manufacturing process, their analytics, and their transplant and dosing regimen.

WHAT MIGHT CONTINUOUS INCREMENTAL PRODUCT ITERATIONS OF ALLOGENEIC CAR-Ts LOOK LIKE?

The two big goals of allogeneic CAR-Ts are to get into earlier lines of therapy, and to succeed in solid tumors. Therapies that are entering the clinic in a first-generation configuration help to establish a tidemark against existing options. Subsequent programs in clinical development could leapfrog their predecessors, and subsequent optimization of the process, analytics, and transplant procedures will all help to improve the therapy itself. For example, Cellectis currently has UCART22 in dose optimization – the Phase 1 portion of Phase 1/2 trial. Preclinical UCART20×22, which is a dual-targeted CAR-T, is coming up behind UCART22. The early clinical experience with UCART22 will inform dosing and other parameters for UCART20×22. In addition, Allogene Therapeutics firstly established ALLO-501 in the clinic, and is now utilizing the clinical learnings to make improvements (ALLO-501A).

Continuous cell therapy development is creating the opportunity to build a pipeline on a backbone that is continuously improved. As discussed above, on the product and clinical development side, companies can build and reference previous data and progress. On the operational side, there may also be benefits that could help to establish strong ties with practitioners. Currently, each cell therapy and indication requires its own workflow and systems. Eventually, since these workflows and systems are not standardized between these different therapies, this will begin to create a large burden on transplant centers as offered numbers of therapies and patients increase, because of the different paperwork, modalities, and workflows for each product. Developers of cell therapies could lock locked-in customers, continuously supplying new products based on the same existing system.

WHICH KEY ATTRIBUTES OF ALLOGENEIC CAR-Ts ARE BEING TWEAKED?

Internal edits and modifications of the CAR-T biology are being performed, with the goal of improving the durability and fitness of the cells. Key hurdles are minimizing graft versus host disease (GvHD) and addressing rejection from host T cells, including CD8 (HLA-I) and CD4 (HLA-II), and NK cells (low HLA-I). Regarding immunogenicity, we have seen genetic ablation of the TRAC locus to reduce the effect of the immune system. Many alterations have been explored with a view to improving activity, including PD-1 and CTLA-4 knockouts, metabolic reprogramming, and CAR-Ts that secrete pro-inflammatory cytokines to modulate the tumor microenvironment. There are also decoys being added to cells to moderate the tumor microenvironment and remove other cytokines.

In terms of novel antigens the list is long. Cells targeting two antigens, simultaneously, could reduce tumor escape, when toxicity is controlled. An example is Cellectis planning to move UCART20×22 into the clinic this year. There are also universal modular CARs, which could be retargeted as needed throughout a patient's lifetime.

The conditioning regimen and transplant procedure is a very important aspect of CAR-T therapies, and this will be even more the case with allogeneic CAR-Ts, especially if they need to be re-dosed. There are CAR-T cells that have been made resistant to

depletion via anti-52 monoclonal antibodies – alemtuzumab, for example, which can be added to the pre-conditioning regimen to create a better niche for successful engraftment and expansion. The aim is to achieve a long-lasting cell expansion curve. There is also significant data to support consolidation dosing from clinical trials, boosting ORR. The effector-to-tumor cell ratio, or the total number of administered CAR-Ts, has also been found to be important. This highlights the importance of careful dose optimization during early clinical development, which has more dimensions compared to small molecules or antibodies. Lastly, companies such as Jasper Therapeutics and Magenta Therapeutics are making advances in chemo-free pre-conditioning.

Another key area is in manufacturing and the gene engineering modalities used. There are many different ways of engineering or editing cells, including using viruses, zinc finger nuclease (ZFN), TALEN, CRISPR, base editing, PiggyBac transposon, and many more. Each of these platforms has its own advantages and disadvantages, especially as the edits get more numerous and complex.

The delivery system used with the gene editing platform is also key. Intellia Therapeutics is using lipid nanoparticles (LNPs) in the manufacturing of its cell therapies, which could have certain advantages over electroporation, including being gentler on the cells. On the other hand, Cellectis has doubled down on electroporation by investing heavily in the hardware and development of the systems.

The final, but by no means. most insignificant consideration is the safety of these CAR-T therapies. This involves the conditioning regimen and the cells themselves. Cytokine release syndrome (CRS) is produced by super-physiological responses due to activation of the CAR-Ts, leading to release of a cascade of pro-inflammatory cytokines. We want to disrupt the subsequent recruitment of other immune cells to reduce the burden on the patient – for example, by using adaptive expression systems for cells such as transient CAR expression. We have also seen inducible controls using inducible suicide system, as well as autonomous control via logic-gated CAR-Ts.

All of these dimensions can be optimized; there is endless work that can be done. Again, this is why we believe continuous clinical development will be critical, building on prior results.

HOW WILL REGULATORS LOOK AT INCREMENTAL CHANGES IN ALLOGENEIC CAR-Ts?

There is no clear-cut regulatory solution. For now, each therapy stands on its own, and each subsequent improvement in CAR-T cell therapy requires a separate development program and sign-off from regulators.

Most time and resource savings will come from the clinical development side. The range of parameters that need to be explored may be inferred from knowing how the predecessor CAR-T works. Continuous refinement of assays already in place from prior products will be important, as this allows interface with regulators and the prevention of roadblocks.

A recent trend is the tightening of the accelerated approval pathways that we have seen in oncology and more specifically, in targeted therapies. For cell therapies to gain approval in the future, incremental changes that are introduced will need to translate into significant benefit for the patient.

BIOGRAPHY

SILVAN TUERKCAN is a Director and equity research analyst covering the biotechnology and biopharmaceutical industries at JMP Securities, a Citizens Company. Prior to joining JMP Securities, Silvan was an Executive Director and senior research analyst at both UBS and Oppenheimer & Co. with coverage of small- and mid-cap biotechnology companies. He previously worked in equity research at Goldman Sachs, where he concentrated on large-cap pharmaceuticals. Silvan began his career as a postdoctoral fellow both at Institut Pasteur & Ecole Polytechnique in France and later at the Stanford University School of Medicine. Silvan holds a PhD in biophysics

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CELL & GENE THERAPY INSIGHTS

BUSINESS INSIGHT: MAY 2022

INTERVIEW

David McCall, Editor, *Cell and Gene Therapy Insights* speaks to Ewan Townsend, Partner, Arnold & Porter

Trends and considerations in cell and gene therapy M&A, partnering and IP

EWAN TOWNSEND's practice at Arnold & Porter focusses on the commercial transactions involved in the development, exploitation and commercialization of medicinal products, representing many of the world's most sophisticated biotechnology and pharmaceutical companies. His experience includes structuring, drafting and negotiating licence and collaboration agreements, manufacturing, distribution and supply agreements, material transfer agreements, clinical trial agreements, services agreements, co-promotion/co-development alliances, pharmacovigilance and quality agreements. Ewan also advises on the regulatory issues that frequently arise in the context of those transactions and throughout the medicinal product life cycle, particularly in relation to marketing authorisations, manufacturing, distribution, advertising, pricing and reimbursement.

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Q What trends do you see in the cell and gene therapy (CGT) space relating to deals around technology platforms?

"...we are seeing platform companies becoming more interested in commercializing their own product candidates, and seeking rights to co-commercialize with their larger pharmaceutical company partners."

ET: We continue to see an ongoing focus on collaboration and alternative structuring approaches, moving away from the traditional mergers and acquisitions (M&A) model, where a platform company develops an asset before a large pharmaceutical company acquires or licenses that asset.

In the CGT space, pharmaceutical companies have started looking at assets at an earlier stage, working out ways to partner with platform companies in developing early-stage assets or even to generate assets that do not yet exist. A clear advantage is that it's not necessary to buy the whole platform company if you can pay a relatively small upfront fee and then pay more in royalties and milestones at a later date if the collaboration is a success. This allows risk to be pushed to a later stage of development. However, striking an earlier-stage deal and the focus on partnership and collaboration from the outset requires expertise in and an increased investment of management time to develop successful commercial relationships.

The nature of CGT means that there are a relatively higher proportion of licensing and collaboration deals compared to M&A, but we are also seeing companies enter acquisitions of CGT platforms – Roche's acquisition of Spark Therapeutics, Gilead's acquisition of Kite, and Bayer's acquisition of AskBio are just a few examples. Competition is fierce for CGT assets and companies are making bold bets on platform technologies (both in licensing and M&A), often on the basis of relatively limited data.

While traditionally a platform provider would be focused on operating its platform, we are seeing platform companies becoming more interested in commercializing their own product candidates, and seeking rights to co-commercialize with their larger pharmaceutical company partners. That sets the stage for some interesting negotiations, with the platform companies on the one hand looking to leverage the collaboration to upgrade their own commercialization capabilities, while on the other hand, pharmaceutical companies instinctively look to limit the platform companies' ability to do so to a small number of territories or programs.

Q What are the challenges or considerations for companies in the sector relating to these trends, from the lawyer's perspective?

ET: Platform deals are complex transactions. The nature of a platform technology means there can be multiple collaborators using the same platform in the same or different therapeutic areas. A platform company may be managing multiple relationships in parallel with different collaborators, many of whom may be providing funding, and many of whom may be competitors amongst themselves. The platform provider may be balancing competing

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interests, which means that clarity around who carries which aspect of research and development, timings and how critical decisions are taken is key.

Another tricky question is determining what success looks like. Setting objective criteria for success in a potentially brand new field can be a challenge, which in turn can lead to difficulties in establishing when milestones have been met, when the next phase of work should commence and whether the collaboration is proceeding as it should. Establishing clear and objective criteria requires creativity and detailed consideration by the lawyers, scientists, and the commercial team.

Intellectual property (IP) is a notoriously difficult area in platform deals. Clearly, who owns the IP generated in the collaboration is a key question. The platform provider will typically retain the rights in its background IP in the platform, but ownership of IP generated using the platform as part of the collaboration requires a very careful approach, based on the platform and therapies in question. Similarly, establishing who should control the prosecution, enforcement, and defense of the IP is often hotly debated.

Determining which rights the parties should retain at the end of the collaboration can also be challenging. If the collaboration terminates but the collaborator is still interested in one of the targets, should it receive a grant-back license? What happens if the collaborator starts challenging the platform owner's IP? These questions can lead to negotiations which may seem abstract at the time but can become extremely valuable at the end of the collaboration.

Non-compete clauses are another heavily negotiated topic. If the platform company can generate multiple products in the same therapeutic area, and grant sequential licenses to different parties, then licensees will want to ensure their competitors are not able to access the same IP to develop therapies for the same therapeutic indication. This often conflicts with the platform owner's interest in commercializing and exploiting its platform in a broad space with as many players as possible.

Q Much has been said about the increasingly cluttered IP landscape for some platform technologies, most notably CRISPR. What is the likely future direction in terms of its evolution in IP terms?

ET: Most people think of CRISPR as a single technology, but there are actually a huge number of potentially patentable molecules and processes within the CRISPR field. We have seen battles over Cas9 (specifically, the patent battle between University of Berkeley and Massachusetts Institute of Technology), but there are whole families of CRISPR complexes around it, such as Cas12, Cas4, CasX and CasY, that have a variety of applications. Even Cas9 is not just one molecule, as there are different aspects depending on whether it is wild-type or engineered for particular activities.

As a result, the IP landscape is indeed cluttered, and navigating it is a challenge. And this is not only a concern for biotechnological applications - CRISPR has applications in a range of other industries, such as agriculture and making cell-cultured meat products. This means that patent rights are held by a number of different proprietors across a variety of fields, which makes navigating the landscape and avoiding inadvertent infringement a challenge. There has

been some debate over whether the various parties active in this space should establish a patent pool arrangement. Clearly, that is only likely to work if the royalties that a patent owner can achieve from joining the pool are better than that in a direct licensing arrangement, and I'm not sure we are at that stage yet.

Other possible outcomes could be a major consolidation with one or a few companies emerging as the key players in the field, or an alternative technology arising which outcompetes CRISPR simply on the basis of being easier to license. It's difficult to predict what may happen at this stage.

Q With patent landscape analysis and due diligence becoming increasingly important for a maturing industry, what are the best practices in this regard, particularly for small biotech companies?

ET: Small biotech companies need to ensure that their IP assets form a key part of their business plan. The strength and scope of a company's IP in the CGT space is often hard for the investors to grasp, and so demystifying the picture is an important way to attract interest in the company.

Investors will want to, not only understand the basics of the small biotech's position, but also see that the organization has the right structures and processes in place. Small biotechs need to ensure that they have robust processes for capturing IP as it is being developed. That means having the right templates, policies, and procedures to ensure that (for example) confidential disclosure agreements are put in place at the right time; that agreements with employees and contractors contain appropriate assignment provisions; that development activities are documented properly; and that the IT systems are secure.

Doing the basics right (and being able to show that they have been done right) will help to avoid any headaches when it comes to due diligence. However, it is also important to recognize that this is a fast-moving field in which cross fertilization and sharing of ideas is common. Companies need to take appropriate steps to stop that from happening, and to try to protect themselves when it does, but there will inevitably be some issues, with third parties infringing or challenging IP or perhaps ownership disputes with employees. Biotechs need to be prepared to explain these issues and their potential impact on value in the course of diligence discussions, and what steps they are taking to resolve them.

"Moving from orphan products into more mainstream indications will require a number of hurdles to be overcome."

Q What are your expectations for ongoing and future trends in both M&A and licensing and partnering in CGT – and what do they mean for the field as a whole?

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ET: We have discussed the focus on early-stage collaborations and partnerships in CGT. However, the sector is evolving, so as these early-stage collaborations start to bear fruit, companies will start to exercise their options and realize the value in these products. As the products mature, so will the market and we will see more M&A activity.

A number of CGT products to date have been very niche, treating ultra-rare genetic disorders in small numbers of patients. Moving from orphan products into more mainstream indications will require a number of hurdles to be overcome.

First, supply chains will need to become cheaper and more efficient. The supply chain for CGT products is significantly more complex than for more traditional small molecule products, often involving the shipment of cells and tissues around the world and the manufacture of bespoke products for individual patients. Moving into mainstream indications will increase pricing pressures, leading to enhanced focus on the costs of manufacture.

Second, both companies and payers will need to develop novel approaches to pricing and reimbursement. In rare diseases, payers have found ways to deal with CGT products with million-dollar price tags, but such high prices will be difficult to justify for more mainstream indications. Even so, prices will inevitably be higher than traditional products, requiring innovative approaches to pricing and reimbursement. There are plenty of ideas around how prices can be amortized over time or be linked to successful outcomes, but these often run up against payers' budgetary and procurement mechanics, which tend to have been designed for more traditional products and operate over a relatively short term.

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AUTHORSHIP & CONFLICT OF INTEREST

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