Meeting analytical challenges in a brave new world of next generation biotherapeutics

The need for innovative advanced analytical tools to accelerate the development of immunotherapies and other complex biologics that may one day cure genetic diseases and cancer has never been greater. Advances in mass spectrometry and capillary electrophoresis combined with new smart computing solutions is bringing that dream closer.

By Mani Krishnan and Susan Darling

Imagine, after months of hospital visits, finally getting a diagnosis for your nine-month-old son. "Take him home; love him; take a lot of pictures. There's nothing you can do," the doctor says[1]. Jake* has just been diagnosed with spinal muscular atrophy (SMA) type I, a rare genetic degenerative neuromuscular disease[2]. Most (historically, eight out of 10) babies with SMA type I do not survive beyond two years of age[1]. Less severe forms of SMA, types II-IV still bear a high mortality and morbidity rate. A 29-year-old woman with SMA type II described at a Cure SMA meeting in 2018 that there were many routine tasks she missed being able to do. "I can only imagine now being able to feed myself (or as I joke, binge eat in secrecy), scratch an itch, defend myself from insects, change a tampon, cook meals, nurture the people that I care about, clean my house, dress myself, do my own hair and make-up; and I want to hug people. I want to reach out and cuddle with my fiancé[1]."

But now there is hope, as two novel treatment options for SMA were approved within the last four years, the latest only hitting the market last year[2]. The first, Spinraza (nusinersen), approved in 2016 is indicated for the treatment of children and adults with SMA[3]. It is an antisense oligonucleotide (ASO) drug that targets the dysfunctional gene, *SMN2*, that causes SMA to create more functional protein[2]. The second is Zolgensma (onasemnogene abeparvovec), also a gene therapy, which is indicated for the treatment of SMA in babies up to two years old. It uses adeno-associated viruses (AAVs) to deliver functional copies of the SMA-causing gene, *SMN1*, to supplement the defective gene[2]. These biotherapeutics exemplify the industry trend towards focusing on biopharmaceutical therapeutics.

Trending towards new biotherapeutic modalities

With opportunities rife, the biologics drug development landscape is evolving faster than ever. Not only are we now treating previously untreatable diseases such as SMA, but advancements in drug discovery are allowing us to better understand the root causes of diseases, thereby paving the way for more profound interventions. New modalities like oligonucleotide drugs and next-generation therapeutics such as 'Frankenbodies' – novel immunotherapies the like of which we have never seen before – will transform medicine, providing one-shot cures and potentially curing certain genetic diseases. Over the next decade, these new, targeted, safer and more efficacious drugs are going to change the way we treat disease. To keep pace with these developments, analytical capabilities need to advance to provide the higher precision, sensitivity and resolution needed to develop these new medicinal products and quality control (QC) their biomanufacture. Innovations in technologies, including mass spectrometry (MS) and capillary electrophoresis (CE), are under way to address these needs, ensuring that these new medicines get to clinic and market as quickly and efficiently as possible.

*Not patient's real name

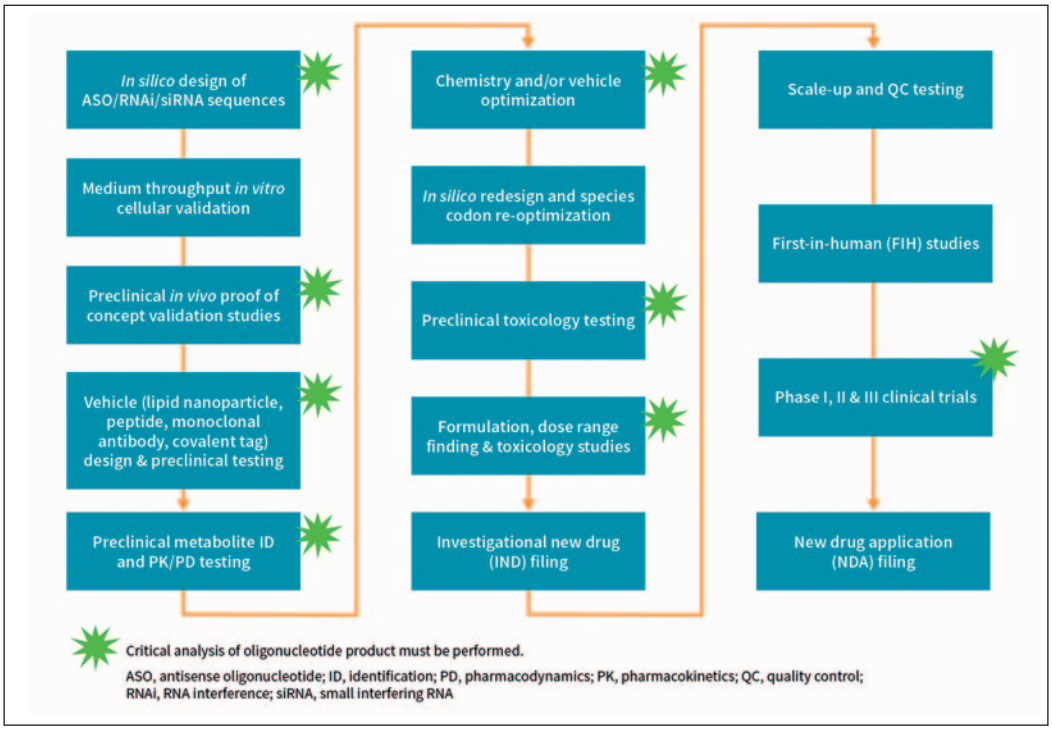


Figure 1
Analytical checkpoints in the development and manufacture of oligonucleotide-based therapies

Key areas of improvement that biopharma scientists are looking for in the development of new analytical assays include higher throughput, easier and more streamlined sample preparation, higher levels of specificity due to the complexity of new molecules, the ability to process data and interpret results without expert intervention, and the need for high-quality service and support from manufacturers of analytical instruments. Meeting these needs can substantially hasten the development and manufacture of new therapeutics, which would, in turn, impact the bottom line of drug manufacturers and, more importantly, bring relief to the patients that are waiting for new treatment options to manage their disease. That is why instrument companies need to collaborate closely with scientists in biopharma and academia to identify real-world issues and develop tried and tested solutions that improve on existing technologies and increase their laboratories' analytical capability and capacity.

Not your grandparent's oligonucleotide medicines

The new modalities in biologics include oligonucleotide-based medicines, gene (and cell-based) therapies and next-generation antibodies. While oligonucleotide drugs are technically a form of gene therapy, we will be discussing them as a category in their own right due to the particular challenges and opportunities that they pose, which do not necessarily arise with other forms of gene therapy. Oligonucleotide-based medicines come in many guises, including antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), messenger RNAs (mRNAs) and aptamers, which are delivered using common vehicles such as liposomes and small molecule or antibody-based conjugates. While ASOs tend to dominate in the pipelines of most biopharma developers, siRNAs and miRNAs are also being investigated. They are being targeted to treat diseases such as cancer, cystic fibrosis, Alzheimer's disease, hepatitis B, HPV, Duchenne muscular dystrophy (DMD), asthma and inflammatory arthritis. A relatively recent success story is that of Spinraza, which was the first disease-modifying treatment option available for people with SMA. Other examples include Exondys 51 (eteplirsen) and Defitelio (defibrotide sodium), which treat two previously undruggable diseases[4,5].

The road to these successes, however, was not easy. It has taken around 30 years to get to where we are today[6]. This modality has had a rocky beginning, with first-generation therapeutic oligonucleotides exhibiting potency and safety issues, and advancements occurring in fits and starts. But after decades of disappointment, the recent convergence of several developments has spurred a renaissance in oligonucleotide medicines. Better understanding of the basic oligonucleotide biology, improved

chemistries, more sophisticated delivery systems, and increasing clinical success has led to the regulatory approval of next-generation oligonucleotide therapies. This generation of oligonucleotide drugs varies greatly in molecular size, structure, ion charge and nucleotide number, accounting for their diverse mechanisms of action (MoAs)[7].

New modalities bring new challenges and new solutions

The complexity of oligonucleotides raises challenges for manufacturing and regulation. While chemically synthesised in a similar manner to that of traditional small-molecule drugs, their diverse MoAs at the cellular level more closely resemble those of biologic therapies. This lack of a ready categorisation as a small or large molecule means that regulatory authorities have had to focus special attention on this new therapeutic modality, for marketing approval and manufacturing quality control. Moreover, although scaling up manufacturing processes for Good Manufacturing Practice (GMP)-compliant commercial production is more feasible than with many cell-based therapies and other types of biologics, achieving high enough productivity and yield continues to be a challenge.

There are several points in the development and manufacture of oligonucleotide therapeutics when analysis is critical (see **Figure 1**). Various techniques are used to analyse these oligonucleotide medicinal products, including electrospray ionisation (ESI)-MS, enzyme-linked immunosorbent assays (ELISAs), CE and high-performance liquid chromatography (HPLC). However, scientists we have spoken to often complain about the lack of adequate analytical sensitivity and the requirement for resolution down to the base pair level. Extracting oligonucleotides out of complex biological matrices, such as serum and tissue samples, can also be very challenging and time-consuming. Moreover, data analysis is often slow and laborious because multiple software packages need to be used to accomplish a single task. Therefore, we have worked on finding a better solution, with a novel microflow liquid chromatography (LC)-ESI-MS workflow[8].

Qualitative and quantitative oligonucleotide analysis generally utilises ion-pairing reversed phase (IP-RP) LC-MS at analytical flow rates. A fundamental challenge with this methodology is the limit of detection and quantification, which can be compromised by electrospray ion suppression resulting from charge competition. Moreover, the routine use of ion-pairing reagents leads to accumulation within the mass spectrometer, which contributes to contamination that accelerates the need for front-end cleaning and maintenance intervals. The new microflow LC-ESI-MS strategy resolves both these issues[8]. It incorporates the well-documented sensitivity advantage phenomenon observed with low flow rates in LC-MS along with the associated enhancement in ionisation efficiency[8,9]. Improved sensitivity was observed with the new microflow LC-ESI-MS assay compared with a conventional

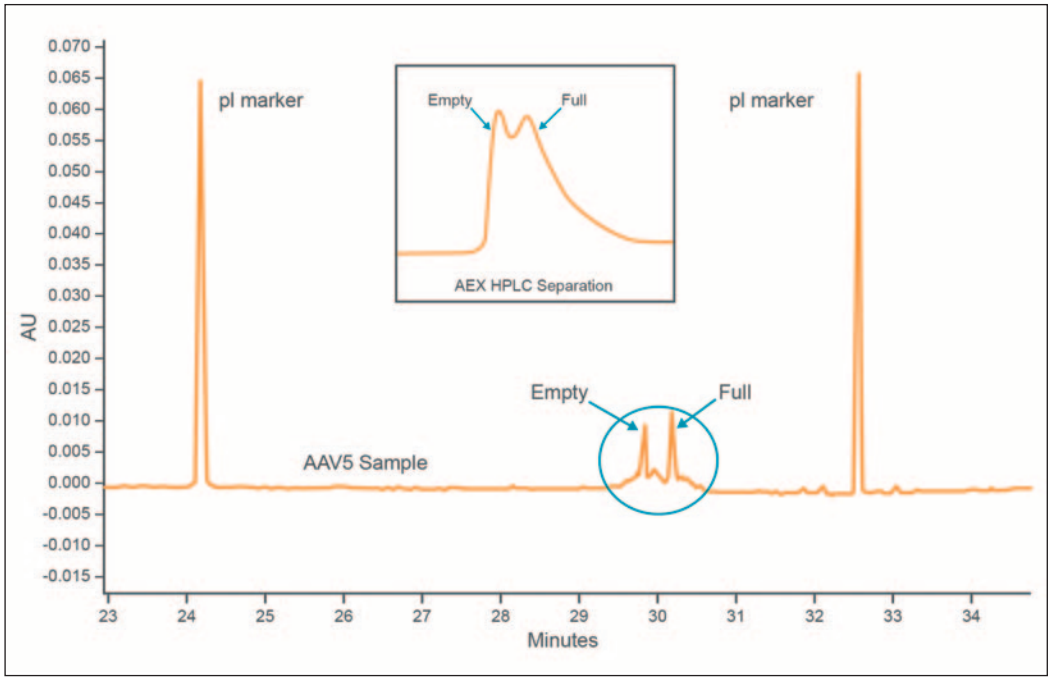
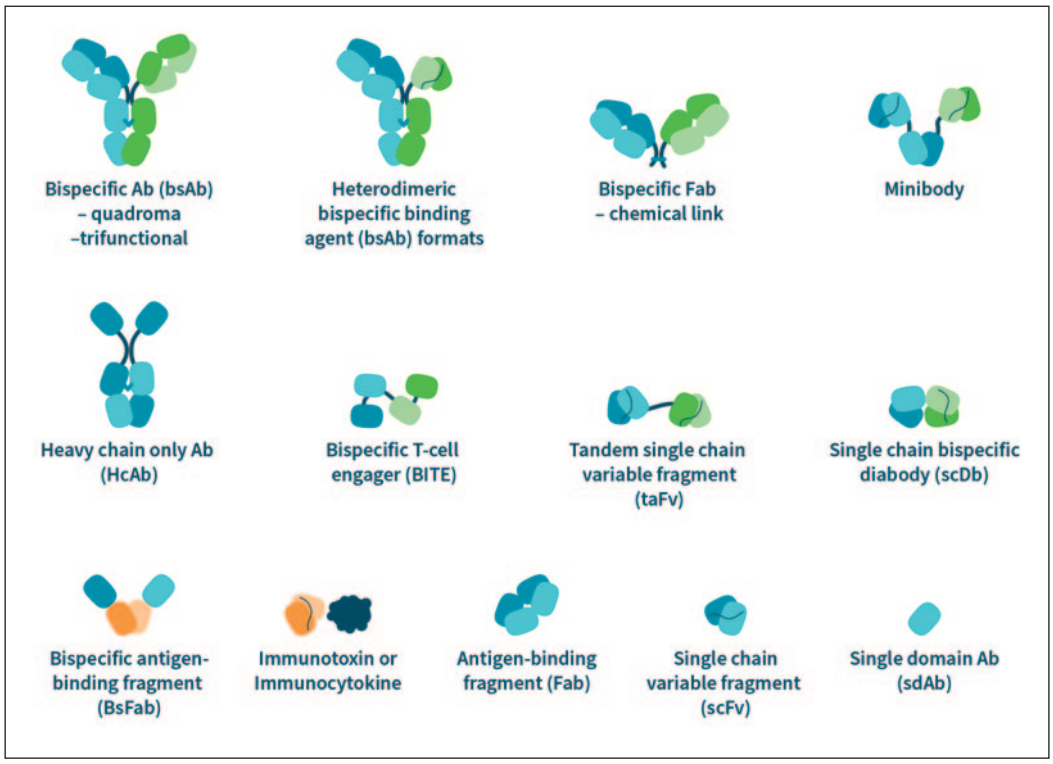


Figure 2
Results from cIEF method: full and empty capsids profile of AAV (serotype 5) sample analysed by cIEF is depicted by the red trace, compared with the anion exchange high performance liquid chromatography (AEX- HPLC) profile denoted in the inset[21]

Figure 3
Schematic of different types of next-generation antibodies



LC-MS system in the analysis of an ASO, with significant improvement in peak area, peak intensity and signal to noise ratio. In addition to the improvement in sensitivity and overall quantitative performance, it is also notable that the microflow LC-MS assay dramatically reduced the required mobile phase additives that inherently contribute to front-end contamination of the mass spectrometer[8]. The superior utility of this new assay is aimed at supporting and accelerating the development of investigational oligonucleotide drugs and speeding their arrival to market and clinic.

Gene therapies have finally arrived

Gene therapy is another hot area of development in the biotech industry right now. Similar to the development of oligonucleotide drugs, gene therapies have taken several decades to come to fruition. Although the basic concept of gene therapies – a DNA-based medicine that inserts a healthy gene into cells to replace a mutated, disease-causing variant – is simple, the delivery of this new biologic modality has been far from straightforward[10]. One important hindrance was delivering the therapeutic genetic material. The use of early viral vectors resulted in detrimental clinical outcomes and set back the development and acceptance of gene therapies by many years[11-14].

Today, however, both viral and non-viral vectors have seen a renaissance in innovative modifications and applications in both preclinical and clinical settings. Modern viral vectors include non-replicating lentiviruses, adenoviruses and adeno-associated viruses (AAVs), whereas non-viral vectors refer to nanoparticles, lipid vesicles and other similar constructs. Along with Zolgensma, regulatory authorities have approved several AAV-delivered gene therapies, such as Luxturna (voretigene neparvovec-rzyl), Imlygic (talimogene laherparepvec), and Glybera (alipogene tipavovec)[10,15-17]. More than 50 candidate AAV-delivered gene therapies are in development, with several being evaluated in clinical trials[11,18]. AAVs are also being investigated for their potential as vectors for vaccination against infectious agents[19]. This includes vaccines being developed based on plasmid DNA, mRNA, protein subunit, or viral vector platforms, against 2019-nCov, which causes coronavirus disease 2019 (COVID-19)[20].

Analytical checkpoints from bench to bedside

As with oligonucleotides, there are several key stages in the development and manufacture of gene therapies, including the plasmid DNA and vectors, where bioanalysis is critical for go/no-go decisions. These include determining whether the plasmid is comprised of the right sequence and has the right

structure, whether there is any contamination from residual genomic DNA or RNA during production, whether the viral vectors have incorporated the transgene payload, whether there is contamination from host cell proteins or other impurities in the final medicinal product, and whether the gene therapy is eliciting the correct therapeutic effect. Numerous analytical methods need to be developed to answer these questions.

To support the advancement of gene therapies, we have developed a new method for determining whether viral capsids are empty or full[21]. This capillary isoelectric focusing (cIEF) method has sufficient resolution to quantify the proportion of capsids that are empty, full, or partially full (see **Figure 2**). This is a critical determination because confirmation that the capsid contains the correct transgene is needed. Empty capsids may cause increased immunogenicity, and capsids with malformed transgenes (DNA with truncations, insertions, etc) may fail to have the desired therapeutic effect due to lack of effective transduction. Along with its high resolving power, the cIEF-based solution offers further advantages over other current methods[22]. Taking less than one hour to analyse a sample, it is markedly faster than analytical ultracentrifugation (AUC) and electron microscopy (EM), which can take days. It can also be optimised to work with AAV samples across multiple serotypes. This method enables process developers of these products to optimise their manufacturing processes such that a consistent specific activity (ratio of empty to full viral particles) can be administered to patients and predictable production yield obtained[23].

Going beyond monoclonal antibodies to the next generation

Traditional monoclonal antibodies (mAbs) dominate the pharmaceutical and biopharmaceutical market. They continue to be particularly successful in oncology, with blockbuster drugs such as Herceptin (trastuzumab) for breast cancer, and immunology, with drugs like Humira (adalimumab), which is used to treat several systemic immune disorders, such as rheumatoid arthritis[24-26]. Humira remains the top-selling drug in the US for the third year running[27,28]. But while they still represent the most significant piece of the biologics pie, the biopharma industry is quickly embracing the development of new, radically different antibodies (see **Figure 3**). These next-generation antibodies are expected to deliver much greater clinical utility and may well outperform the success of traditional mAbs. With six of the top 10 bestselling biologics on the market being mAbs, this would be no mean feat[29].

Despite the huge success in treating cancer and immune disorders, mAbs have their limitations. Their mono-specificity limits their application. It can hinder access to tissues and thus prevent successful penetration of solid tumours or the crossing of the blood-brain barrier. The pharmacokinetics (PK) of mAbs can also be constrained to long half-lives, which in some cases is beneficial, but in others not.

> "For gene therapy developers, time to market is critical. Unlike other classes of drugs, where a disease can be treated by multiple medications, gene therapeutics cure diseases by targeting specific genes. The one who gets there first wins. There is no second place. That is why scientists in biopharma are driven to develop a robust manufacturing process. But there is a lack of reliable and reproducible methods to consistently produce AAV-based gene therapies, which means there is a lot of risk to get these therapies to market. This new method offers a key to improving and streamlining the development and production process for AAV-based therapeutics. When you have the right analytics for the entire in-process samples as well as release testing, you can navigate the upstream and downstream process to develop better quality and safer products – helping to reduce the cost of the manufacture."
>
> *Dr Rachel Legmann, Director, Technical Consultancy – Gene Therapy and Viral Vectors at Pall Biotech and SCIEX research partner*[22]

Several types of next-gen antibodies

The next-gen antibodies can be divided into immunoglobulin G (IgG)-like antibodies and non-IgG-like antibodies. IgG-like antibodies include bi- and tri-specific antibodies and antibody-drug conjugates (ADCs). Non-IgG-like antibodies – or proteins – include fusion proteins and peptides, with Fc-peptide fusion proteins and single-domain antibodies sometimes being referred to as 'Frankenbodies' because, like Frankenstein's monster, they are made up of different component parts. Nplate (romiplostim) is one such antibody; it is an Fc-peptide fusion protein or 'peptibody' that is a thrombopoietin receptor agonist indicated for the treatment of thrombocytopenia in patients with chronic immune thrombocytopenia (ITP)[30,31].

Another type of Frankenbody is the nanobody, also known as a single-domain antibody (sdAb). This type of antibody was discovered by chance, as with many great discoveries, when the serum from a dromedary was analysed by a couple of biology students during a practical course at the Free University of Brussels in the late 1980s[32]. In addition to the usual distribution of immunoglobins, the students also discovered a group of smaller antibodies that

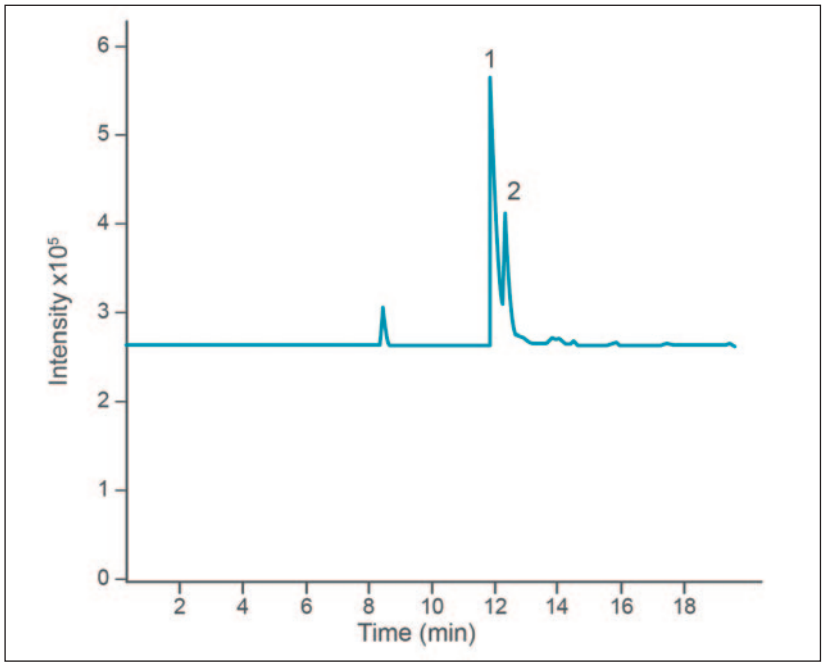


Figure 4
Base peak electropherograms showing the separation of a nanobody (Peak 1) from its deamidated form (Peak 2) using CESI-MS[31]

did not correspond to anything known to science. After careful characterisation, it became clear that they had discovered a new class of antibodies. These antibodies were devoid of light chains and had a single antigen recognising domain. This class of antibodies was also later found to exist in other camelid species, including llamas, alpacas and sharks.

As the name suggests, the nanobody is a relatively small protein, comprised of a single monomeric variable antibody domain with a molecular mass of about 12-15kDa. It can bind selectively to a specific antigen, in the same way as an IgG, but is thought to better infiltrate solid tumours, bind to a larger number of regions on a target molecule and be excreted from the body without much trace, minimising allergic responses. They also exhibit higher chemical stability and can be tailored to have a half-life *in vivo* ranging from only a few hours to a few weeks, depending on whether it is required to treat an acute or a chronic condition.

Separating out charge variants of nanobodies

In the development and manufacture of nanobodies, intact protein analysis is beneficial as it offers a general picture of the heterogeneity of the protein population while minimising artefacts arising from sample treatment. For classical mAbs, traditional LC-MS methods are usually employed for this kind of analysis. However, these LC-MS methods struggle to separate proteins that differ in mass by less than 10Da. Moreover, when proteins larger than 10kDa of nearly identical mass co-elute, their spectra overlap making it nearly impossible to deconvolute the data and identify the individual species. Therefore, the identification of charge variants, such as deamidation that causes a mass charge of just 1Da, is very difficult using traditional LC-MS approaches[32].

In response to these analytical limitations, we developed a CE-ESI-MS (CESI-MS) workflow for the assessment of intact nanobodies that is sensitive enough to detect nanobody charge variants from sample volumes as low as 5µL without the need for sample digestion. The inherent separation efficiency of CE provided sharp peaks for better separation and identification, while the direct combination of CE with MS technologies allowed for the very low flow rates for the charge-based separation of the proteins, including the separation and challenging detection of deamidated nanobody proteins (see **Figure 4**). With this CESI-MS method, it was possible to separate charge variants of nanobodies and nanobody fusion proteins, including deamidated forms, as well as products of truncation of the antibody that arose during the preparation of the protein samples[32].

Looking to the future

As more and more solutions are developed to meet the needs of discovering and producing these new biotherapeutics, more and more new biotherapeutics should hit the clinic and market. Not only is the industry supported by advancing CE and MS technologies, but it is also garnering a whole new world of knowledge from smart computing solutions incorporating machine learning or deeper forms of artificial intelligence (AI), which are applied to analyse big data and map systems biology. After almost half a decade since the idea of gene therapies was published, it seems that we are fortunate enough to finally be witnessing the era of gene, cell and immuno-therapy. DDW

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