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Challenges and opportunities of using liquid chromatography and mass spectrometry methods to develop complex vaccine antigens as pharmaceutical dosage forms

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ABSTRACT

Liquid chromatographic methods, combined with mass spectrometry, offer exciting and important opportunities to better characterize complex vaccine antigens including recombinant proteins, virus-like particles, inactivated viruses, polysaccharides, and protein-polysaccharide conjugates. The current abilities and limitations of these physicochemical methods to complement traditional *in vitro* and *in vivo* vaccine potency assays are explored in this review through the use of illustrative case studies. Various applications of these state-of-the art techniques are illustrated that include the analysis of influenza vaccines (inactivated whole virus and recombinant hemagglutinin), virus-like particle vaccines (human papillomavirus and hepatitis B), and polysaccharide linked to protein carrier vaccines (pneumococcal). Examples of utilizing these analytical methods to characterize vaccine antigens in the presence of adjuvants, which are often included to boost immune responses as part of the final vaccine dosage form, are also presented. Some of the challenges of using chromatographic and LC–MS as physicochemical assays to routinely test complex vaccine antigens are also discussed.

1. Introduction

The development and implementation of vaccines have dramatically improved human health in the past century by controlling, and in some cases, eliminating infectious diseases. For example, smallpox has been eradicated worldwide, and polio has been eliminated in the Americas. The disease burden of measles, mumps, rubella, diphtheria, tetanus and *Haemophilus influenzae* type b has also been greatly reduced [1]. In the past ~15 years (2000s up to the present), several new vaccines have been introduced to protect against additional viral (e.g., human papillomavirus and rotavirus) and bacterial (e.g., pneumococcal and meningococcal) infections. Moreover, research and development of new vaccines remains a very high priority to improve public health for numerous, currently unmet medical needs including therapeutic treatment of various types of cancer, protection against HIV, and responding to new or emerging infectious diseases (e.g., the recent outbreak of Ebola in Western Africa) [2].

The development and regulatory approval of a vaccine candidate not only includes animal testing and human clinical trials for safety and efficacy, but also the CMC (Chemistry, Manufacturing and Control) development of a large-scale manufacturing process to produce a stable, potent vaccine that can be conveniently administered to patients as a medicine (i.e., pharmaceutical dosage form). The first step of the overall CMC vaccine development process includes large scale production of the vaccine antigen which can be one of a diverse set of biological entities. The vaccine antigen is selected to simulate a natural infection, and thus

Abbreviations: LC, liquid–chromatography; MS, mass spectrometry; HA, hemagglutinin; NA, neuraminidase; SRID, single radial immunodiffusion assay; WHO, World Health Organization; RP, reversed-phase chromatography; SEC, size-exclusion chromatography; IEX, ion-exchange chromatography; RSD, relative standard deviation; VLP, virus-like particle; TEM, transmission electron microscopy; HPV, human papillomavirus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HEV, hepatitis E virus; MW, molecular weight; CHIKV, Chikungunya vaccine; FMDV, foot-and-mouth disease virus; rPA, anthrax recombinant protective antigen; HiB, haemophilous influenzae type B; TT, tetanus toxoid; DT, diphtheria toxoid; OMPC, outer-membrane protein complex; MALS, multi-angle light scattering; RT-PCR, real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CBER, Center for Biologics Evaluation and Research; NIBSC, National Institute for Biological Standards and Control; QC, quality control; NMR, nuclear magnetic resonance; Cryo-TEM, cryogenic TEM; AFM, atomic force microscopy.

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trigger a protective immune response in the patient, but without developing the disease itself. Examples include complex natural agents such as live-attenuated viruses (e.g., measles, rotavirus, polio, influenza), inactivated viruses (e.g., influenza, Hepatitis A, polio), live-attenuated bacteria (e.g., typhoid fever, cholera) and inactivated bacteria (e.g., whole cell pertussis, cholera).

As one example, as displayed in the TEM images in Fig. 1, influenza virus (Fig. 1A) [3] particles are typically 80–120 nm in diameter and roughly spherical in shape. This enveloped virus consists of a host cell membrane derived lipid envelope that contains the two major envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The central core of the viral particles contains segmented RNA in association with the viral proteins that package and protect viral nucleic acids. The virus is grown in eggs, harvested, purified and then inactivated by detergent treatments resulting in the inactivated flu vaccine itself which contains micelle-like complexes of the major viral protein (HA) referred to as rosettes (Fig. 1B) [4]. In addition, isolation and purification of key components from bacteria are also effective vaccine antigens including proteins (e.g., acellular pertussis) or polysaccharides (pneumonia, *H. influenzae* type B). More recently, recombinant DNA technology has led to identification and production of recombinantly produced, highly purified vaccine antigens that consist of key component(s) from the infectious agents such as a single proteins (e.g., CRM_{197}, recombinant HA), some of which are viral surface proteins that then spontaneously form a virus-like particle (e.g., Hepatitis B, HPV vaccine antigens).

The second step in the overall CMC vaccine development process includes vaccine formulation or "converting vaccine antigens to medicines" [5]. During vaccine formulation development, an efficacious, long-term storage stable dosage form (which can be conveniently administered to patients) is designed, scaled-up and commercially produced (in some cases requiring tens or even hundreds of millions of doses). One major focus of vaccine formulation development includes evaluating and introducing vaccine adjuvants, substances added to enhance vaccine potency in humans, especially for purified or inactivated antigens that cannot replicate to mimic a natural infection. As shown in the TEM images in Fig. 1C [6], the HPV bulk vaccine, consisting of the recombinantly expressed L1 viral surface protein in yeast, spontaneously forms 60 nm virus-like particles or VLPs (which require *in vitro* disassembly and reassembly for proper particle formation) [7]. The VLP is then adsorbed to an aluminum salt adjuvant, as shown in the TEM image in Fig. 1D [6], as the final drug product.

Vaccine pharmaceutical dosage forms containing inactivated or purified antigens (often with adjuvants) are formulated as liquid preparations that are filled into glass vials or prefilled syringes. Inactivated and recombinant vaccines are generally administered by injection [8], typically IM (intramuscular), but also by subcutaneous and intradermal delivery. In contrast, live-attenuated viruses and bacteria based vaccines are less thermally stable and tend to be formulated as lyophilized products for long term storage (with some exceptions such as the more inherently stable, non-enveloped polio virus and rotavirus). Live vaccines can be administered not only by injection, but also orally (e.g., rotavirus, adenovirus, typhoid) or nasally (e.g., live influenza vaccine) to better mimic the natural route of infection. Both liquid and lyophilized vaccine formulations require identification of pharmaceutical excipients used as stabilizers, buffers and tonicifying agents [9]. The design and development of stable vaccine formulations is beyond the scope of this work but several recent reviews are available [5,10,11].

The third and final step in the overall CMC vaccine development process is vaccine analysis. Method development and validation of quality control assays are critical and key steps in vaccine development since the results from analytical testing ensure a link between the production process and the safety and efficacy of the vaccine (as established in clinical trials). Moreover, the purity, potency and stability of the vaccine as it is produced and during long-term storage are monitored by analytical assays to ensure the quality of the vaccine throughout its shelf-life. Vaccines are labile and usually require cold storage in refrigerators, and in some cases freezers, for long term storage and distribution [12]. This often is referred to as the vaccine cold chain and its maintenance is a very important aspect of successful vaccine distribution and administration. Careful monitoring of vaccine potency by an appropriate analytic test is a cornerstone of a successful testing plan to monitor and ensure the quality of vaccines [13]. Since most vaccine antigens have no easily measurable inherent biological activity other than to stimulate an immune response upon administration, vaccine potency assays have historically consisted of *in vivo* animal tests where the ability of the vaccine to generate an antibody response in animals is measured, for example, by serum ELISA assays. Regulatory agencies emphasize the importance of the development of alternative potency assays to limit the use of laboratory animals during testing [14],and *in vitro* potency assays can be used when the identity of a key epitope within the vaccine antigen is known, and its integrity is monitored, using immunological reagents (e.g., a monoclonal antibody against the epitope). In the case of live-attenuated viruses and bacteria, cell based assays can be used to monitor the number of infectious particles (e.g., viral plaque assays). Both *in vivo* and *in vitro* vaccine potency assays have many practical challenges including inherent variability in their accuracy and precision as well as being technically challenging, labor intensive and time consuming to routinely run in an analytical laboratory.

Thus, there is ongoing interest in assessing the ability of more robust and quantitative physicochemical assays to better characterize complex vaccine antigens. This is especially true for recombinant protein antigens as well as highly purified isolated protein and polysaccharide vaccine antigens. There are several challenges (and opportunities) in using physicochemical assays to monitor the quality and stability of vaccine antigens. First, due to the complex nature of vaccine antigens (e.g., protein, polysaccharide or virus-like particle), a combination of assays are required to monitor different aspects of vaccine structure and composition. Second, the physical analysis requires not only developing methods to monitor the overall structural integrity and stability of the antigen, but also assays to monitor loss of local structure of key epitopes within the antigen. Similarly, for chemical analysis, both the overall chemical composition as well as chemical alterations within key epitopes (e.g., Asn deamidation or Met oxidation) must be considered. Finally, the low dose of vaccine antigens (e.g., micrograms) and the presence of adjuvants (e.g., aluminum salts, emulsions, immune activating compounds, etc.) can result in numerous analytical challenges in terms of matrix interferences, sample handling and assay sensitivity.

The purpose of this review is to examine the ability of liquid chromatographicand mass spectrometric analysis to characterize complex vaccine antigens. The physicochemical analysis and characterization of complex vaccine antigens is presented through the use of informative case studies. We first explore the utility of these methods as applied to the analysis of flu vaccines including inactivated whole virus and recombinant hemagglutinin. Second, examples of the analytical characterization of currently available recombinant virus-like particle (VLP) vaccines (e.g., HPV, HBsAg) and VLP based vaccine candidates (e.g., HEV, CHIKV) are presented. Third, the characterization of vaccine antigens in the presence of adjuvants is discussed. Finally, the analytical testing of polysaccharide vaccines linked to protein carriers by these techniques is reviewed. Some of the analytical challenges in characterizing complex vaccine antigens by chromatographic and LC–MS, including sample preparation and data analysis with samples containing

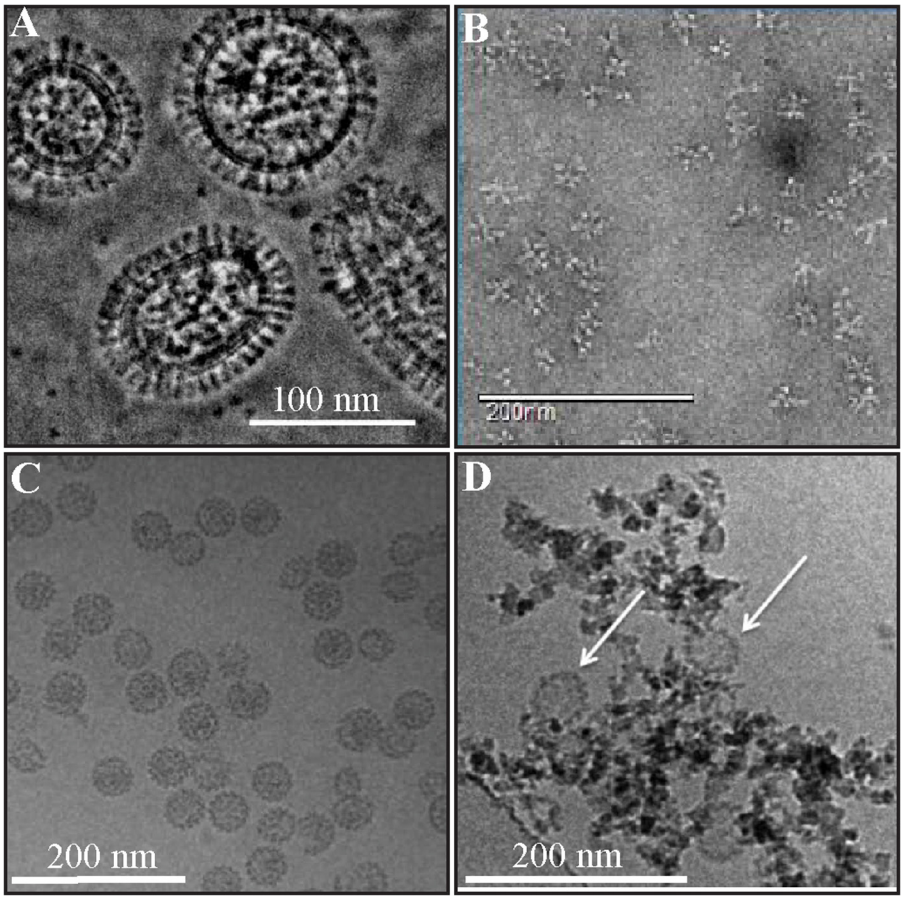

Fig. 1. Transmission electron microscopy images of (A) influenza A viruses, (B) purified rHA forming rosettes in solution, (C) HPV VLPs, and (D) HPV VLPs (indicated by white arrows) absorbed onto aluminum salt adjuvant. Images were adapted from Refs. [3,4,6] with permission.

complex mixtures and low doses, are also discussed as part of these case studies.

2. Influenza vaccines

Influenza is an 80–120 nm diameter single-stranded RNA *Orthomyxoviridae* virus. The viral RNA encodes eleven proteins, each of which is essential for infection, replication, and subsequent release of progeny virions [15]. Primarily two genera (Influenza A and B) from this family infect humans. Influenza A is further classified into subtypes based on the antigenicity of the two major surface glycoproteins on the virion, hemagglutinin (HA) and neuraminidase (NA). Both proteins elicit an antibody response upon infection and antibodies against HA confer protective immunity [16].

Approximately 5–10% of adults and 20–30% of children are infected with influenza annually, which results in the hospitalization of 3–5 million people [17]. The best strategy for preventing seasonal influenza epidemics is vaccination, although one major challenge is that a new vaccine needs to be developed, produced, and distributed annually to combat antigenic drift in the virus. In the U.S. alone, approximately 150 million influenza vaccines were distributed in 2014 [18]. Current FDA-approved vaccines are composed of either trivalent or quadrivalent mixtures of the most globally-prevalent influenza subtypes (A/H1, A/H3 and B). Many of these vaccines are produced using methods that date to the 1940s, in which the virus is grown in embryonated chicken eggs, harvested and processed into either live attenuated, or more commonly, inactivated viral vaccine preparations [19]. Since these production methods take approximately six months to make and release test a vaccine, and often contain unwanted impurities such as residual egg proteins in addition to their viral components, numerous alternative influenza vaccine antigen platforms (e.g. DNA- or recombinant protein subunit-based vaccines, virus-like particles, and cell-culture grown virus, etc.) are in development. For example, a recombinant hemagglutinin (rHA) vaccine has been produced in insect cells (Flublok®, Protein Sciences Corporation) and has recently received regulatory approval for use in the U.S. [20,21].

Despite the emergence of newer technologies to manufacture influenza vaccines, analytical methods for monitoring the purity, potency and stability of these vaccines are still often dictated by the use of well-established but low-resolution methods. The two archetypal assays to characterize influenza antigens for *in vitro* testing are a single radial immunodiffusion assay (SRID) and SDS-PAGE. The SRID assay is used to determine the potency of the vaccine. The assay was implemented by the World Health Organization (WHO) in the late 1970s [22,23]. The method not only has high assay variability, it is costly and time-consuming in that strain-specific reagents (antigens and anti-sera) need to be generated, standardized, and distributed by regulatory laboratories (e.g., CBER, NIBSC, etc.) prior to analysis [24]. SRID consists of adding detergent-treated virus to an agar plate embedded with strain-specific antibodies. The antibodies recognize the precursor conformation of the HA protein (HA0) in which the proteins two subunits (HA1 and HA2) are associated through a disulfide bond. The protein–protein interaction causes a precipitin ring to form. The diameter of this ring is then measured and the relative potency of the vaccine sample is determined using a reference antigen of known concentration that has been determined by regulatory laboratories. The accuracy and precision of the assay are affected by a

variety of experimental factors (e.g., effectiveness of detergent solubilization, variability between agar plates, variability in measuring the precipitous ring between analysts, etc.). Therefore, the acceptable relative standard deviation (RSD) for the assay is 20%. Given this relatively high assay variability, regulatory authorities have recommended developing and evaluating modern, high-resolution technologies and analytical methods to more accurately and precisely assess flu vaccines to improve their quality, efficacy, and safety [25].

In addition to the SRID assay, electrophoretic assays are routinely performed as part of development of influenza vaccines. Capillary isoelectric focusing has been used to assess the purity of the virus during production and RT-PCR methods are used to identify clinical isolates [26,27]. SDS-PAGE, in particular, has been integral to the standardization of influenza vaccines since the implementation of the SRID assay. The assay is used to measure the amount of HA within a sample to generate reference reagents for the SRID assay, quantitate the HA content in candidate vaccines, and can be used to help identify mechanisms of potency loss when observed by the SRID assay [28,29]. HA is currently quantified in the SDS-PAGE assay using densitometry, which can be potentially inaccurate due to strain-dependent co-migration of influenza proteins [28]. The amount of HA within different influenza subtypes can also vary (typically 20–50% of total protein), which further complicates quantitation [24,30,31]. Over the past few decades, chromatography and mass spectrometry based methods have been developed as higher-resolution techniques to isolate and quantify HA, and serve as orthogonal assays to SRID and SDS-PAGE [29,30,32–35]. The effectiveness of these newer, more modern analytical approaches in characterizing influenza vaccines, including their associated protein components, will be discussed in the following sections.

2.1. Chromatography

Chromatography has emerged as a major analytical method for better characterizing and monitoring the quality of influenza vaccines. Reports of using chromatographic techniques to purify influenza components began in the 1980s, in which reversed-phase chromatography (RP), size-exclusion chromatography (SEC), and ion-exchange chromatography (IEX) methods were developed to separate influenza proteins based on their different sizes and physicochemical properties [36,37]. The utility of these techniques as analytical tools to characterize the virus, however, was relatively absent in the literature. Over the past decade, the number of publications focused on developing chromatographic methods for characterization and QC testing of influenza vaccines has grown dramatically. The emerging interest in these techniques has been driven by a variety of factors, including the need for more robust analytical technologies to quickly and accurately characterize pandemic viral outbreaks, the development of new non-egg derived influenza vaccines, and the improved resolution and automation of chromatographic systems.

Current analytical chromatographic methods of studying influenza components/vaccines typically require unique sample preparation and/or unconventional running methods, given the inherent complexity of the virus/vaccine. For example, SEC is commonly used to separate oligomeric species within a solution primarily based on their respective molecular sizes but some influenza proteins have relatively similar masses (e.g., HA and NA are ~77 kDa and ~60 kDa, respectively) [38]. Furthermore, these two glycoproteins natively form higher-order oligomeric species (i.e., rosettes), which further complicate chromatographic separation. In 2010, Garcia-Cañas et al. reported the development of a robust and reproducible SEC method capable of separating viral influenza component proteins and even differentiating various influenza stains based on their elution profile [38]. The authors assessed a variety of mobile phase additives and found that 0.1% sodium dodecyl sulfate (SDS) substantially improved peak separation in comparison to denaturants and non-ionic detergents. The optimized method resulted in six highly reproducible (< 1.2% RSD) peaks. The protein components of each peak were subsequently identified using electrophoresis and mass spectrometry. This study demonstrated the effectiveness of SEC chromatography as a quick and reliable analytical tool to characterize the components of commercial inactivated influenza vaccines.

2.1.1. Reversed phase HPLC as an orthogonal technique to the SRID assay

In 2006, a novel RP-HPLC method was reported in which influenza viral components were separated and characterized [39]. A limitation of this method was that the viral proteins needed to be enriched prior to RP-HPLC analysis to be detected. Lorbetskie et al. refined this initial method and developed a highly sensitive and reproducible chromatographic technique to characterize influenza vaccines without the need to concentrate the sample prior to analysis [34]. As shown in Fig. 2, a peak corresponding to the major antigenic subunit of HA (HA1) was well resolved in the RP-HPLC chromatograms of three influenza vaccine stains and within the equivalent trivalent vaccine [34,40]. The authors then proceeded to validate this analytical method by determining the limit of detection and quantification, repeatability, precision, etc. A recent publication by Wen et al. further optimized a RP-HPLC method to accurately measure the HA protein content in multiple influenza vaccines and correlate the amount of the antigen to its potency as measured in a SRID assay (Fig. 2) [33]. A critical aspect of this method was the treatment of the influenza sample with trypsin prior to RP-HPLC analysis. Proteolysis degraded the "active" conformation of HA (i.e., the low-pH induced HA conformation observed within an infected host cell). The presence of the active form of HA would have reduced the correlation between the two assays because this protein conformation is not reactive in the SRID assay yet would contribute to the peak area in the RP-HPLC chromatogram. This technique can be used as an orthogonal assay to SRID to better understand the mechanism of potency loss in an influenza vaccine, given its high sensitivity and potential to differentiate proteins with post-translational modifications. Another application for this RP-HPLC technique would be its use to approximate the potency of rapidly produced pandemic influenza vaccine candidates while the immunological reagents for the SRID assay are generated, which generally takes 6–8 weeks [41].

2.2. Mass spectrometry

Mass spectrometry (MS) has been used in numerous stages during the development of influenza vaccines and to identify newly emerging influenza strains. The use of MS to characterize whole viruses and their individual components has increased dramatically over the past few decades, which is attributed to revolutionary advancements in ion source, analyzer, and detector technologies [42–44]. Initial MS experiments with influenza using modern techniques were focused on elucidating antigenic sites on HA from whole virus digests [45,46]. Recent applications of MS during the development of influenza vaccines include: glycosylation profiling of recombinant HA protein [47], rapid genotyping and serotyping of clinical influenza isolates [48], identification of conserved phosphorylation sites that appear critical for viral entry and replication [49,50], and using hydrogen-deuterium exchange to observe dynamic changes in the structure of HA upon exposure to a low pH environment [51]. These few examples demonstrate the diversity and high-resolution capabilities MS methods offer to characterize influenza.

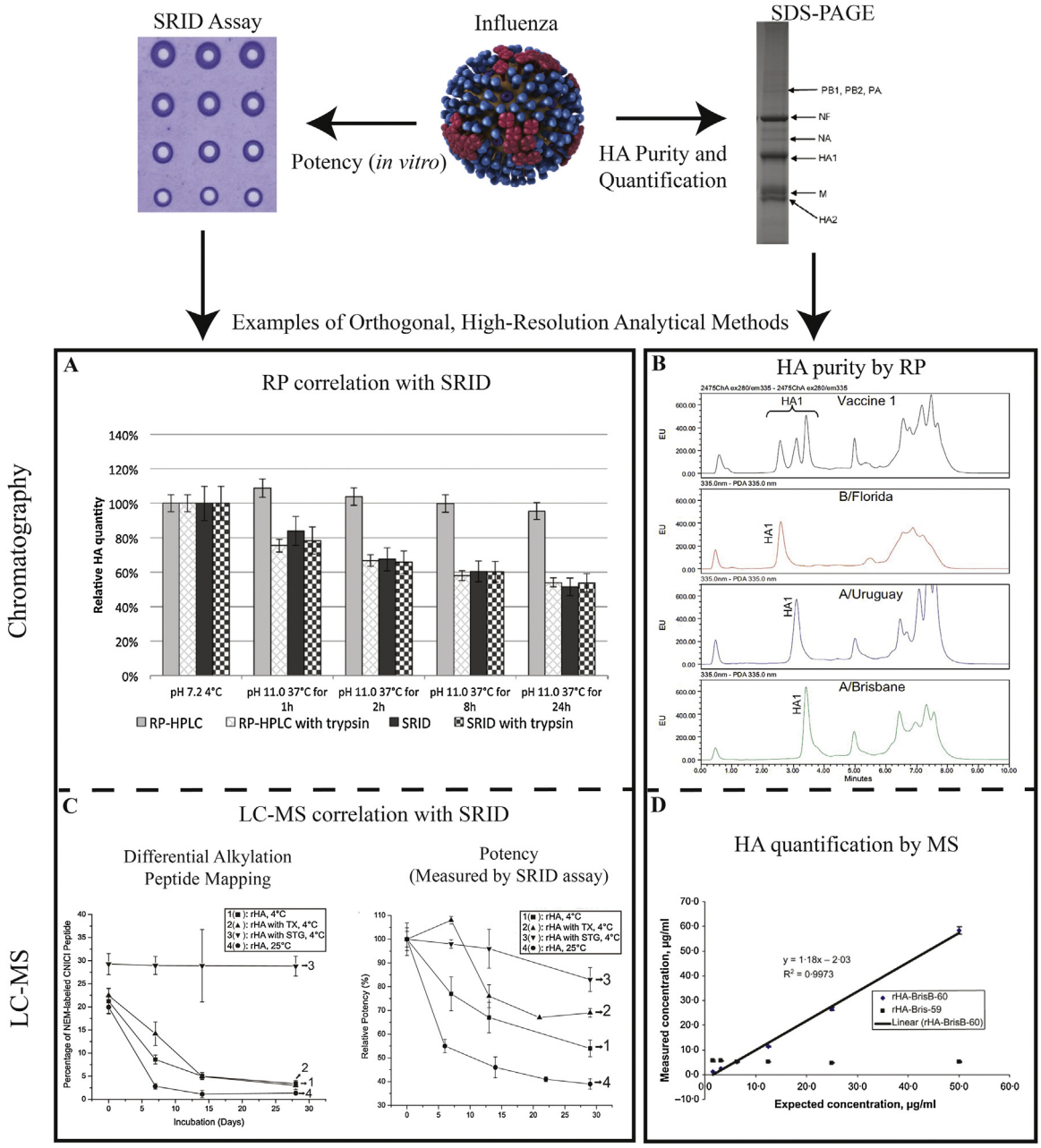

Fig. 2. Examples of RP-HPLC and LC–MS analytical assays to characterize influenza vaccines. Top Panel: RP and LC–MS methods have been developed as orthogonal techniques to the SRID and SDS-PAGE assays, which measure the *in vitro* potency and HA purity/concentration, respectively. Bottom Panel: A) SRID correlation with HA quantitation using RP-HPLC, B) HA purity by RP-HPLC in three influenza vaccine strains, C) SRID correlation with LC–MS, D) expected vs. MS-measured HA concentrations. Images were adapted from Refs. [29,30,32–35] with permission. The influenza virion was adapted from http://www.cdc.gov/media/subtopic/library/diseases.htm (#11823, Created by Dan Higgins).

Mass spectrometry analysis of influenza is generally coupled with chromatography given the inherent complexity of the virus. Technological advances in chromatography and MS instrumentation have made modern LC–MS techniques of characterizing influenza vaccines rapid, sensitive, and accurate. For example, multiple LC–MS methods have been recently described for absolute quantification of influenza proteins (Fig. 2) [35,52,53]. The authors of these studies propose the use of LC–MS as an initial HA quantitation method for seasonal and pandemic influenza vaccines until SRID reagents are generated. While these approaches appear promising, HA quantification is still reliant on the accuracy and consistency of an internal reference standard(s). Furthermore, LC–MS is a multistep process wherein systematic and random error can influence the integrity of the data if not properly mitigated. Given these technical challenges, the high cost of instrumentation, and required technical expertise, MS quantitation of HA protein levels will probably not replace the more standard SDS-PAGE assays in a quality control (QC) setting anytime soon, but these studies demonstrate the use of MS-based techniques as orthogonal methods to the current repertoire of analytical techniques to better characterize influenza vaccines.

2.2.1. *LC–MS as an orthogonal technique to the SRID assay*

Modern MS techniques offer robust capabilities to monitor the potency/stability of influenza vaccines. As described above, the SRID assay is the standard technique for determining the potency

of an influenza vaccine *in vitro*. This immuno-assay is a relatively low-resolution technique and if potency loss is observed, additional techniques are required to determine the mechanism(s) of potency loss. Recently, the utility of MS analysis of a HA protein to better understand loss of potency in the SRID assay was described [29]. In this study, potency loss (determined by the SRID assay) was observed during storage of a rHA influenza vaccine (Fig. 2) [29]. Potency loss was investigated using diverse biophysical techniques. The loss of potency however was not correlated with a measurable change in the antigens overall secondary structure, conformational stability, or hydrodynamic size. SDS-PAGE suggested non-native disulfide crosslinking correlated with potency loss in the SRID assay; therefore a differential alkylation LC–MS peptide map method was used to monitor free and disulfide bonded Cys residues within HA as a function of time when stored at two temperatures and under different formulation conditions. This LC–MS peptide map strategy identified and measured the relative percentages of a free Cys residue in the C-terminus of the protein in the different samples, the results of which correlated with the observed potency loss determined by the SRID assay. In a subsequent study, the C-terminal Cys residues in HA were genetically altered and resulted in an influenza vaccine that did not lose potency during storage for 12 months at 25° C [54]. These studies demonstrate the utility of LC–MS to complement the SRID assay and to provide further insight into the molecular mechanisms of HA potency loss.

3. Virus-like particle vaccines

The majority of commercial viral vaccines are based on either inactivated or live attenuated viruses. While these types of vaccines have been successfully used to prevent diseases for more than half a century, inactivated or live attenuated viral vaccines can have limited efficacy, can raise safety concerns due to reversion or incomplete inactivation, can be difficult to manufacture, and can be unstable during storage [55]. Recombinant DNA technology has led to the production of virus-like particles (VLPs), which has subsequently led to the development of VLP-based vaccines. These VLPs consist of viral capsid proteins expressed in host systems and then self-assembled into a particle that present antigens on their surface to mimic a virion, but lack infectious genetic material and therefore is inherently non-virulent [56,57]. VLPs-based vaccines have immunologic advantages over other recombinant soluble protein antigens since they can induce better immune responses by presenting multiple copies of antigens [58]. The size of VLPs (typically ranging from 20 to 200 nm) also permits their efficient uptake by dendritic cells [59]. In addition, VLPs can elicit immune response at low doses (typically in the μg of protein antigen range), which can reduce the effective cost of the vaccine [60,61].

Currently, four VLP-based vaccines have been approved for human use and consist of vaccines against human papillomavirus (HPV) and hepatitis B virus (HBV), which are available in many countries, a hepatitis E virus (HEV) vaccine that is currently only available in China, and a malaria vaccine using hepatitis B surface antigen VLP as a carrier. Quality control and analytical characterization testing are both crucial aspects of vaccine development due to an increased emphasis and stricter requirements for the safety, efficacy and quality of vaccine candidates in clinical development [62]. For the successful commercial development of a VLP-based vaccine, ensuring manufacturing consistency and final product stability are paramount, and are generally ensured by characterizing potency, purity, size, stability, and aggregation state [10,63,64]. Modern analytical methods such as SEC and LC–MS have been developed for such purposes with several VLP-based vaccines as discussed below.

3.1. Characterization of licensed and candidate VLP-based vaccines

3.1.1. Human papillomavirus (HPV) vaccine

Human papillomavirus (HPV) infection can cause the development of cervical cancer, which is the second most common cause of death in women worldwide from cancer [65]. HPV belongs to the *papillomaviridae* family of viruses and are present in the skin and mucosa of various animal species. Multiple types of HPVs can lead to cervical cancer in humans, with HPV types 16 and 18 responsible for ~70% of cases [61]. The first licensed VLP-based vaccine against HPV was Gardasil® (Merck) introduced in 2006 and was shown to effectively reduce HPV infection among teenagers [66]. Gardasil® is a quadrivalent VLP vaccine composed of recombinant L1 viral capsid protein expressed in yeast (containing HPV types 6, 11, 16, and 18) and adsorbed onto aluminum hydroxide. In 2009, a second VLP-based HPV vaccine was licensed (Cervarix®, GlaxoSmithKline). This vaccine contains both HPV type 16 and 18, which are adsorbed onto aluminum hydroxide, and a second adjuvant (MPL, Monophosphoryl Lipid A) [67]. A third VLP-based HPV vaccine that includes nine different VLPs against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 [56] has recently received FDA approval.

Multiple expression systems such as yeast, *Escherichia coli* and insect cells have been used to produce HPV VLPs [56]. The VLPs produced from these recombinant expression systems can differ in particle morphology and size. For example, the HPV L1 protein when expressed in yeast cells, produces VLP particles that differ in their size and shape depending on the type of the L1 protein [7]. The L1 protein assembles into a pentameric form, which in turn spontaneously assembles into a viral capsid-like VLP containing 72 pentamers. By reducing and reoxidizing disulfide bonds under certain specific solution conditions that maintain protein solubility and stability (i.e., by altering the solution pH, NaCl concentration, and adding/removing reducing agents and detergents), these VLPs expressed in yeast can be reversibly disassembled and reassembled *in vitro* [7]. This dis/reassembly method facilitated the production of HPV VLPs at large scale with uniform morphology and resulted in VLPs that were more immunogenic, stable, and less prone to aggregation in solution. Interestingly, the HPV VLP type 18, as opposed to types 6, 11, and 16, formed correct particles in yeast and thus did not require the dis/reassembly treatment, demonstrating that this behavior is dictated by the specific L1 protein being expressed and produced.

SEC has been used to characterize recombinant HPV VLP types 6, 11, and 16 expressed in yeast before and after this treatment using a 4000 Å pore column (Fig. 3A) [7]. A single but tailing peak was observed with dis/re-assembled HPV VLPs compared to more heterogeneous distributions with the untreated HPV VLPs. Such tailing behavior implies some non-specific interaction of the VLP with the SEC column resin. Interestingly, this non-specific interaction was greatly enhanced with VLPs prior to the dis/reassembly treatment in a type specific manner where the largest effect on sample recovery was with HPV VLP types 6 and 11. Using transmission electron microscopy (TEM), the dis/reassembled VLPs were more uniform in size and morphology in comparison to untreated VLPs, which were much smaller in size and more heterogeneous in morphology (HPV VLP type 6 is shown as one example in Fig. 3B) [7].

In addition to VLP morphology and size, sequence fidelity of the recombinant proteins that compose the VLPs is also a critical control aspect of producing these vaccines for human use. Peptide mapping LC–MS can be used as a powerful tool to study the composition profile of the VLPs and can identify sequence modifications that could affect the biological properties of vaccine products. For example, peptide mapping LC–MS was used to confirm the primary sequences of the L1 protein from HPV types 16 and 18

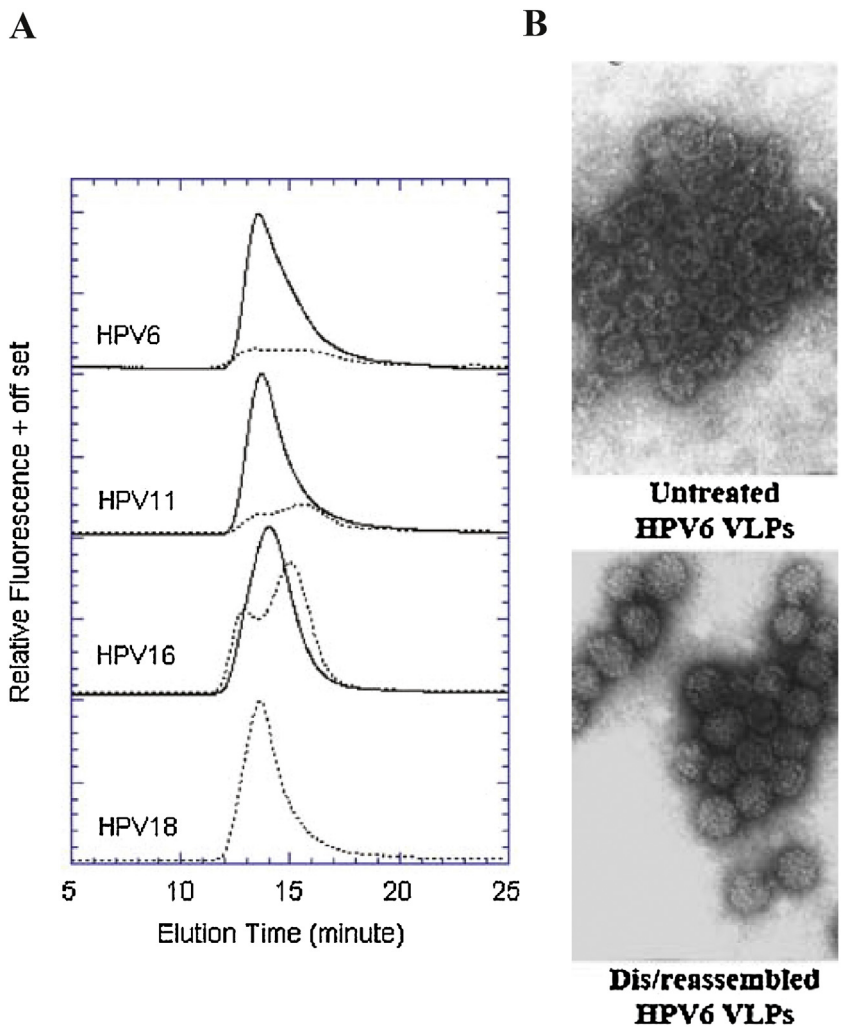

Fig. 3. Size and morphology analysis of HPV VLPs produced in yeast before and after *in vitro* dis/re-assembly treatment. (A) SEC traces of four different types of HPV VLPs where the solid line represents the treated VLPs while dashed lines depict untreated VLPs. (B) Representative TEM image of untreated and treated HPV type 6 VLPs. Figure was adapted from Ref. [7] with permission.

that were expressed in a baculovirus expression system [65]. Three separate batches of each L1 protein were digested with trypsin, chymotrypsin or endoproteinase Asp-N and the resulting peptides were identified by LC–MS[65]. Peptide sequence coverage of ~97% was observed for both HPV 16 and 18 VLPs. These data also showed that there was only one post translational modification, N-terminal acetylation, in the LI viral proteins within the VLPs. This example demonstrates the use of peptide mapping LC–MS to help verify batch to batch consistency and identify post-translational modifications that can occur during protein expression and purification.

3.1.2. *Hepatitis B virus (HBV) vaccine*

According to the WHO, approximately 1 million deaths occur every year from Hepatitis B-related infections [61]. HBV belongs to a family of *hepadna* viruses [68] and is encapsulated with an outer envelope made of lipid and three envelope proteins known as small (S), middle (M) and large (L). These envelope proteins self-assemble to form a HBsAg (Hepatitis B surface antigen) particle, which elicits protective immunity upon vaccination [69]. The Hepatitis B vaccine is the first example of utilizing recombinant DNA technology to produce a vaccine[70]. The vaccine consists of recombinant Hepatitis B Surface Antigen (S-protein) that are expressed in yeast, which spontaneously forms ~22 nm particles that also contain lipid from the host cell. Several recombinant HBV vaccines have been approved for human use including Engerix-B® (USA, GlaxoSmithKline) and Recombivax HB® (USA, Merck). Newer versions of the vaccine, containing the S protein and additional "preS" regions include GenHevac B (France, Pasteur-Merieux Aventis), and Sci-B-Vac (Israel, Scigen) [5,56,71].

Ensuring proper assembly of the HBsAg VLP and presentation of the key conformational epitopes are critical factors affecting the efficacy of this vaccine [72]. For instance, intact HBsAg-VLPs have been reported to be 1000-fold more immunogenic than the disassembled HBsAg protein [73]. Conversely, HBsAg-VLP aggregates (i.e., >40 nm diameter particles) have been shown to elicit weaker antigenicity than the ~22 nm particles [74,75]. Therefore, monitoring VLP particle integrity during manufacturing and subsequent storage is a critical attribute to ensure vaccine efficacy. SEC is a fast and sensitive analytical method that can be effectively used to monitor HBsAg VLP assembly and stability over time. Fig. 4 shows the SEC profile of HBsAg VLPs that were stored at 4 °C for up to 12 months [62]. An earlier elution of the main peak and the emergence of additional high MW species were also observed during storage and were attributed to aggregation of the VLPs. The increase in peak area at 17 min resulted from the dissociation or disassembly of the VLPs over time. This example demonstrates the capability of SEC as an analytical method to monitor VLP vaccine integrity and stability during storage.

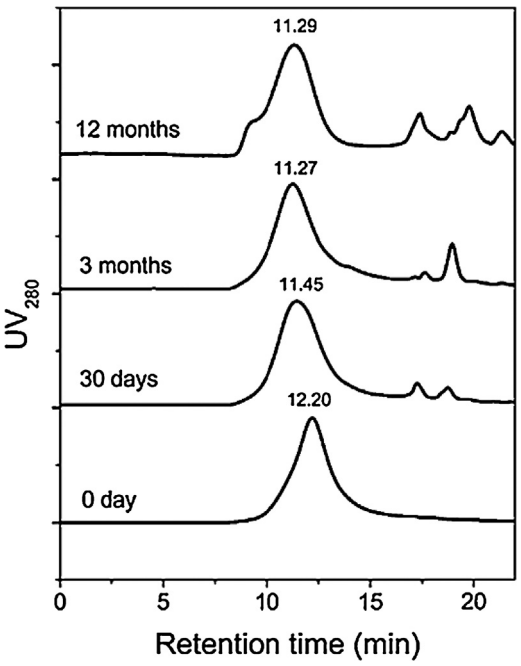

Fig. 4. Comparison of SEC chromatograms of bulk HBsAg VLPs stored at 4 °C over a period of 12 months. Figure was adapted from Ref. [62] with permission.

3.1.3. Hepatitis E virus (HEV) vaccine

HEV infects up to one third of the world's population and is the leading cause of acute hepatitis with jaundice [76,77]. HEV infection also poses serious problems in pregnant women with an estimated maternal and fetal mortality rate of ~20% [78,79]. The first licensed HEV VLP vaccine (Hecolin®, Innovax) was approved in 2011 in China and provides almost 100% protection [80]. This VLP vaccine consists of a truncated, recombinant HEV capsid protein (p239) expressed in *E. coli* that spontaneously forms a VLP particle of ~ 20–30 nm in diameter [79].

Several analytical methods have been employed to examine the quality, size and stability of the p239 protein, which comprises this VLP vaccine. This includes LC–MS based peptide mapping, SEC, dynamic light scattering, and differential scanning calorimetry [79]. For example, to ensure consistency in terms of size and quality, multiple batches of commercial scale p239 containing VLPs were characterized by SEC and LC–MS [79]. Fig. 5 shows the molecular weight (MW) determination and peptide sequence coverage of p239 protein within the VLPs from three different lots as measured by MALDI-TOF MS and LC–MS, respectively. The observed MW was consistent with the theoretical MW of p239 (Fig. 5A) and 100% sequence coverage (Fig. 5B) was obtained across all three batches [79]. These results demonstrate the utility of these techniques to ensure lot-to-lot consistency of this VLP vaccine produced at commercial scale (i.e. 50L).

3.1.4. Chikungunya virus (CHIKV) vaccine candidate

CHIKV is a mosquito-borne alphavirus that infects humans, causing fever, rash, nausea, and joint pain [81–83]. A licensed CHIKV vaccine is currently not available but several are in development [84]. For example, a recent animal study in rhesus macaques showed protection against the virus when vaccinated with a candidate CHIKV VLP-based vaccine [85]. This vaccine consists of several structural proteins (i.e., the envelope (E1, E2, E3, 6 K) and capsid (C) proteins) from the virus that self-assemble into VLPs when expressed as recombinant proteins in cell culture. To develop a CHIKV VLP based vaccine, it is important to quantitate CHIKV VLP components to determine the antigen-specific vaccine mass. RP chromatography has been used to separate, quantitate, and identify protein degradation/chemical modifications in the viral capsid and envelope proteins of the CHIKV VLP [86]. Fig. 6 compares the RP-HPLC profile of control and stressed CHIKV VLPs where the stressed VLPs were stored at 25 °C for 1 week at pH 9.0 [86]. The trace shows changes in the elution profile for the stressed VLP sample with an E2 protein degradant peak observed. This result suggested that the E2 protein underwent Asn deamidation (a known chemical degradation pathway for Asn residues under high pH and temperature conditions). Thus, this method can be used as a fast and quantitative (precision of 12% RSD) method to monitor the chemical stability of these VLPs [86].

4. Vaccines with adjuvant

The clinical success of many vaccine candidates in protection against disease is highly correlated with their ability to generate neutralizing antibodies in animals and humans. A common approach of increasing the immunogenicity of inactivated viral/bacterial preparations, as well as recombinant protein vaccine antigens, is to include an adjuvant in the formulation. For such adjuvanted vaccines, multiple injections are also often required to generate protective immunity. In contrast, live attenuated vaccines, which can replicate and thus better mimic a natural infection, typically do not include adjuvants and often only require a single dose.

The first historic example in the literature of the use of an adjuvant to increase vaccine immunogenicity was diphtheria toxin, where it was found that when bound to an aluminum salt, the toxin generated much greater immunogenicity than controls without an adjuvant [87]. Although still an active area of research, it is generally accepted that aluminum salt adjuvants generate increased immunogenicity by certain immunological mechanisms including recruiting immune cells to the administration site and enhancing antigen uptake [88–90]. Currently, two types of aluminum salt adjuvants are present in many commercial vaccines including aluminum hydrophosphate (Adju-Phos®) and aluminum hydroxide (Alhydrogel®), which differ in terms of their composition, morphology and surface charge [91]. Some vaccine manufacturers produce their own aluminum salt adjuvants with varying physical properties between Alhydrogel® and Adju-Phos® depending on the manufacturing process used to produce the adjuvant [5,92–94]. Other classes of adjuvants include emulsions (e.g., MF59), and various types of immune stimulating compounds (e.g., MPL) that activate various toll-like receptors in the innate immune system [95].

Although aluminum adjuvants are routinely incorporated in commercial vaccines, the number of high resolution analytical techniques available to study the physicochemical properties of either component of the vaccine (i.e., protein antigen(s) or adjuvant) is very limited. Although beyond the scope of this review, some traditional biophysical methods such as front face intrinsic tryptophan fluorescence, FTIR spectroscopy, Raman spectroscopy, and differential scanning calorimetry, which are commonly used to characterize bulk protein antigens, can also be used to characterize the structural integrity and conformational stability of proteins adsorbed to aluminum adjuvants [10,11]. These techniques however, present limitations in characterizing protein antigens in the presence of adjuvants including (1) lack of information about the primary structure, and (2) limited sensitivity for detecting structural alterations at low doses of vaccine antigen. The potential use of LC–MS to examine vaccine antigens under these conditions offers several potential opportunities to address these limitations, but examples of applying LC–MS methods to characterize adjuvanted vaccine antigens are limited. Some of the available studies can

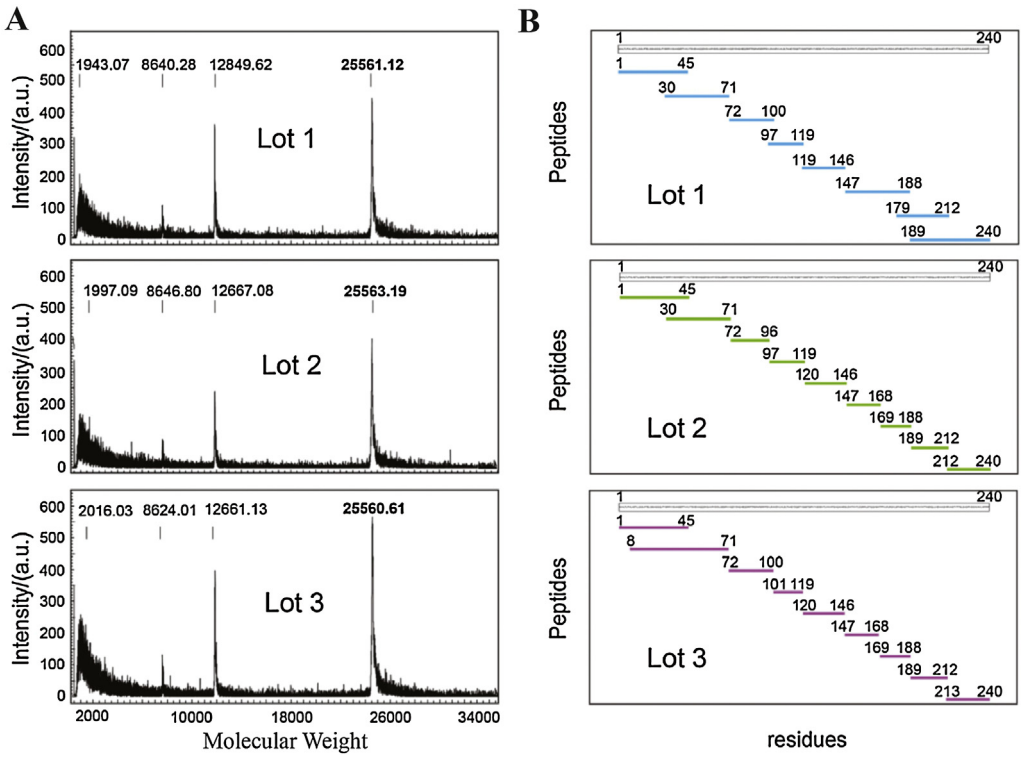

Fig. 5. Characterization of the p239 protein within HEV VLPs from different manufacturing lots. (A) MW determination of the p239 protein in HEV VLPs from different lots by intact mass analysis. (B) Peptide sequence coverage of different lots of HEV VLPs determined by LC–MS. Figure was adapted from Ref. [79] with permission.

be broadly categorized as follows: LC–MS use to identify protein antigen(s), characterization of the protein antigen and adjuvant components of the vaccine, and the use of LC–MS to help evaluate the *in vivo* behavior of an adjuvanted vaccine. From the relatively limited number of publications, LC–MS appears to be a very promising technique to study these types of vaccines; however, additional studies in this area are needed to better understand the physicochemical integrity and interactions between the components of these vaccines and the subsequent impact on vaccine efficacy.

4.1. Identification of protein antigen(s)

The most common use of LC–MS with regard to adjuvanted vaccines is the identification of the protein antigen component [96–103]. For example, Harmsen et al., recently described a novel technique involving the immobilization of antibodies against Foot-and-mouth Disease Virus (FMDV) on a microchip to capture and identify viral antigens [96]. FMDV is a positive-stranded RNA virus that affects livestock. A vaccine against FMDV is economically important because it can disseminate quickly within an animal population. Recombinant single-chain anti-FMDV antibody fragments were produced in yeast and bound to ProteinChip arrays (Bio-Rad). The arrays were then incubated with FMDV to capture antigens, which were subsequently identified using surface-enhanced laser desorption TOF-MS. Major findings of this study include: (1) the identification of several FMDV antigens, (2) an observation of mass heterogeneity within the antigens that had not been observed previously, which the authors attribute to the presence of chemical modifications, and (3) the application of this method to identify antigens in a water-in-oil-in-water double emulsion formulation, where the authors found that FMDV antigens do not disassociate from FMDV upon emulsification. This method has several advantages compared to a more conventional technique such as Western blotting which includes higher sensitivity, lower sample volume, and a faster and more reproducible analytical method [96].

4.2. Characterization of protein antigen bound to adjuvant

Characterization of the protein antigen component of an adjuvanted vaccine is another use of LC–MS, with several examples reported in the literature [53,104–107]. For example, Estey et al. employed unique sample handling and LysC peptide mapping methods prior to MALDI-TOF to study a complex trivalent botulism vaccine, which was composed of three protein antigens (rBoNTA(H_C), rBoNTB(H_C), and rBoNTE(H_C)) bound to aluminum hydroxide [108]. The chemical stability of each protein antigen either bound or unbound to adjuvant was evaluated during storage at 4° C or 30° C for 9 wks. During incubation, the authors observed a decrease in peptide sequence coverage in rBoNTE(H_C) bound to adjuvant (78% → 10% sequence coverage over 9 weeks), which they attributed to increasingly poorer desorption of the protein antigen from the adjuvant. After investigating several desorption methods, incubating the protein-adjuvant mixture in 4 M urea at room-temperature for 24 h increased sequence coverage to >70%, although some Asn deamidation was observed in these samples, which may have been an artifact of the desorption conditions.

Sequence coverage was also consistently lower (by 15–30%) in trivalent samples compared to monovalent forms. During desalting and concentrating of the peptides (from protein desorbed from aluminum by urea and then digested using LysC) using C18 ZipTips® (EMD Millipore), the authors developed a fractionation elution method in which the ACN concentration was gradually increased and subsequent analysis of the eluted peptides resulted in improved sequence coverages of 88–96%. Using the peptide mapping procedure combined with the described desorption and fractionation elution methods, the authors identified several chemical modifications that appeared earlier or were unique to the adjuvanted antigen samples compared to unbound control

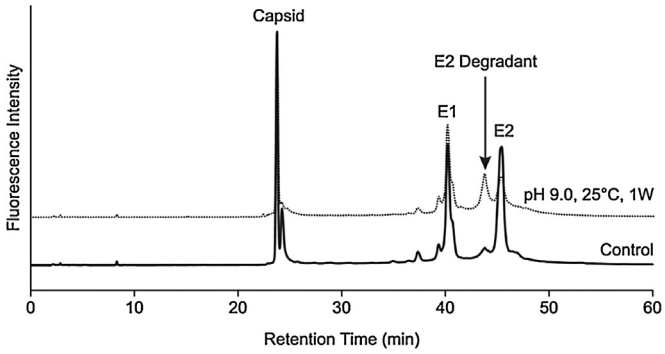

Fig. 6. RP-HPLC profile of control and stressed CHIKV VLP samples that are composed of three structural proteins (Capsid, E1, and E2). The indicated E2 degradant is potentially due to Asn deamidation. Figure was adapted from Ref. [86] with permission.

antigens. For example, at +16 Da mass increase (likely Met375 oxidation) was observed in a rBoNTA(H_C) peptide (residues 375–398) in week 2 when bound to adjuvant, but a similar mass increase was not observed in the protein antigen in solution (no adjuvant) until 9 weeks. Overall, the authors identified ~1–5 chemical modifications in each protein antigen during storage, which generally appeared earlier when the protein was bound to adjuvant. The authors speculate that binding of proteins to adjuvant destabilizes their conformation and changes in local flexibility make the proteins more susceptible to chemical modifications.

In another example, D'souza et al. used LC–MS to detect and monitor Asn deamidation during storage of a recombinant protective antigen (rPA) from a *Bacillus anthacis* vaccine when absorbed to aluminum hydroxide gel and compared the results to in-solution controls (see Fig. 7 below) [109]. The authors first removed buffer components by passing the adjuvanted rPA mixture through a 30 kDa molecular weight cutoff membrane. Protein was desorbed using 100 mM Tris, 7.5 M guanidine, 1 mM EDTA, pH 7.4. The resuspended protein was then reduced, alkylated, trypsin-digested and the resulting rPA peptides were analyzed by LC–MS. As shown in Fig. 7, authors observed that when rPA was bound to adjuvant, deamidation increased in two specific Asn residues (713 and 719) during storage at 4° C over 90 days [109]. Furthermore, deamidation in these two residues correlated with a decrease in toxin neutralizing antibodies, but at the same time, the rPA-specific antibody titers remained unchanged. Similar to the Estey et al. example above, this study demonstrates the ability of LC–MS to monitor the chemical stability of protein antigens when adsorbed to adjuvant (via methodology that desorbs the antigen from the adjuvant).

4.3. *Characterization of vaccine adjuvant*

The use of LC–MS to characterize the adjuvant component of an adjuvanted vaccine has also been described. For example, Hamdy et al. used LC–MS to quantify two lipids (7-acyl lipid A and PET lipid A) in a solution and to develop the mixture as an adjuvant [110]. Cotte et al. used LC–MS to characterize two surfactants (ceteareth-12 and sorbitan oleate) for use in an emulsified oil-in-water adjuvant and state that their method could be used to quantify and characterize the surfactants directly in the final formulation. Thus, this method could potentially be used as a quality control assay for the final bulk and drug products [111].

Schlegl et al., used LC–MS to detect and quantify elemental impurities in adjuvanted vaccine products [112]. Aluminum adjuvants are manufactured by exposing aluminum salts to alkaline environments and collecting the precipitated hydroxides. Heavy metal contaminants can co-precipitate with hydroxides and since different manufacturers may use different processes and raw materials, the amount of heavy metal impurities may vary. Among the adjuvanted vaccines tested, the authors found differences in the identities and amounts of heavy metal contamination between manufacturers, and even between lots from the same manufacturer. The study also suggested that the Japanese encephalitis vaccine IXIARO® (Intercell Biomedical), when adsorbed to aluminum adjuvants with elevated heavy metal impurities, degraded more quickly during accelerated stability testing, which the authors attributed to heavy-metal catalyzed oxidation [112].

4.4. *Adjuvanted vaccine* In vivo *behavior*

The use of LC–MS to study the *in vivo* behavior of either the protein antigen or adjuvant component of a vaccine is very limited. Vaarala et al., serves as an example of LC–MS use for evaluation of the protein antigen component of commercially available adjuvanted vaccines [113]. The conclusions of this study indicated an increase in the attributable risk of narcolepsy with Pandemrix when compared to Arepanrix, both of which are H1N1 vaccinations adjuvanted with AS03, an adjuvant consisting of squalene, DL-α-tocopherol and polysorbate 80 (manufactured by Glaxo-SmithKline). The authors used high resolution gel electrophoresis and MS to identify higher levels of altered viral nucleoprotein in Pandemrix. The increased altered nucleoprotein appears to be linked with higher levels of serum IgG-antibody in children with Pandermix-related narcolepsy compared to healthy children, although the precise mechanism of the Pandermix-related narcolepsy has not been fully elucidated.

There are also limited published examples of MS use for the detection of aluminum in mice following injection to evaluate clearance rates with time [114,115]. In Flarend et al., adjuvants labelled with an aluminum isotope were injected into mice, and blood and urine samples were collected for 28 days. The samples were digested twice in nitric acid and the resulting solution was allowed to evaporate completely. The resulting solids were exposed to 800 °C producing AL_2O_3 ash. The ash was mixed with silver powder and analyzed by accelerator mass spectrometry. While differences were noted in the adsorption levels between aluminum phosphate and aluminum hydroxide adjuvants, the authors reported that both adjuvants were cleared by the mice after administration.

5. Vaccine Carriers/Conjugates

Since the initial success of the polysaccharide-conjugated *Haemophilous infulenzae* type B (Hib) vaccine (PedvaxHIB®, Merck) in the late 1980's in reducing the occurrence of invasive Hib in infants, the development and use of conjugate vaccines for immunization against encapsulated bacterial pathogens has greatly increased [116,117]. Polysaccharides are relatively poorly antigens individually, especially in children, and therefore are typically conjugated to more immunogenic carrier proteins to elicit a robust T cell-dependent immune response [118–120]. Examples of carrier proteins used in commercial vaccines include: tetanus toxoid (TT), diphtheria toxoid (DT), *H. influenza* protein D, the outer membrane protein complex (OMPC) of *Nisseria meningitis*, and cross-reacting material 197 (CRM_{197}) (Table 1) [121,122]. CRM_{197} is a non-toxic variant of diphtheria toxin isolated from *Corynebacterium diptheriae* [123–125]. The different chemistries involved in conjugation of polysaccharides to carrier proteins and their corresponding vaccine immunology are increasingly understood but are a complex topic which has been reviewed elsewhere [125,126]. The subsequent sections will focus on the use of two multi-component high-resolution analytical methods (SEC-MALS and LC–MS) to better characterize

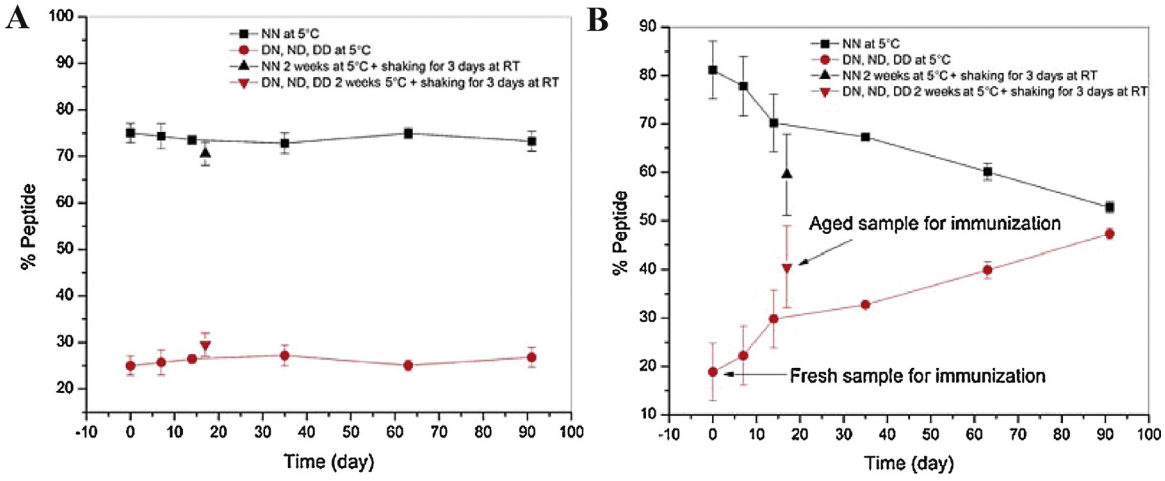

Fig. 7. LC–MS peptide mapping was used to monitor deamidation of specific Asn residues (Asn 713 and 719) in recombinant protective antigen (rPA) over time under different conditions: (A) rPA in-solution, and (B) rPA adsorbed to an aluminum adjuvant. This figure was adapted from Ref. [109] with permission. NN, ND, DN, and DD denote the residue (either Asn or Asp) at positions 713 and 719 in rPA.

Table 1
Source of polysaccharides and nature of carrier protein used in commercially available polysaccharide conjugated vaccines [122].

Vaccine	Polysaccharide Source (Organism)	Manufacturer	Carrier Protein
Menveo®	*Neisseria meningitidis*	Novartis	CRM_{197}
Prevnar®	*Streptococcus pneumoniae*	Pfizer	CRM_{197}
Prevnar-13®	*Streptococcus pneumoniae*	Pfizer	CRM_{197}
Hiberix®	*Haemophilous influenzae*	GlaxoSmithKline	Tetanus Toxoid
ActHIB ®	*Haemophilous influenzae*	Sanofi Pasteur	Tetanus Toxoid
Menhibrix®	*Neisseria meningitidis* *Haemophilous influenzae*	GlaxoSmithKline	Tetanus Toxoid
Menactra®	*Neisseria meningitidis*	Sanofi Pasteur	Diphtheria Toxoid
PedvaxHIB®	*Haemophilous influenzae*	Merck	OMPC

polysaccharide conjugate vaccines during development as vaccine candidates.

5.1. Characterization of vaccine carriers/conjugates using SEC-MALS

SEC coupled with multi-angle light scattering (SEC-MALS) has become an increasingly popular analytical method for molar mass determination in a wide variety of biological samples. Since the calculated molar mass is an absolute measurement (i.e., calculation of molar mass is directly proportional to the molar mass of the sample of interest and not dependent on a SEC calibration curve that is often imprecise due to elution volumes that are not completely size-based), SEC-MALS has become an attractive choice for molar mass determination and soluble aggregate characterization not only for polysaccharide conjugate vaccines, but for a wide variety of macromolecules as well [127,128]. The molar mass of a given macromolecule is calculated by the following equations:

$$I(\theta) \propto R(\theta) = K^* McP(\theta)\left[1 - 2A_2 McP(\theta)\right] \quad [127]$$

$$K^* = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \times \left(\frac{dn}{dc}\right)^2 \quad [127]$$

where I(θ) represents the intensity of scattered light at a given angle, R(θ) represents the excess Rayleigh ratio at a given angle, n_0 represents solvent refractive index, N_A represents Avogadro's number, λ_0 represents vacuum wavelength of incident light, dn/dc represents the refractive index increment, M represents molar mass, c represents protein concentration, and P(θ) represents the form factor which is related to the angular dependence of the scattered light, and A_2 represents the second virial coefficient [127]. The differential refractive index (dn/dc) is an important parameter that is often assumed to be 0.185 mL/g for protein or is estimated from the primary sequence [129]. When analyzing polysaccharide-protein conjugate vaccines, however, it is important to determine the dn/dc empirically since this parameter can vary even more widely than for proteins alone [130–132]. Since the dn/dc is squared, any error in dn/dc could be amplified by as much as two-fold in the calculated molar mass. Measurement of the dn/dc is relatively straightforward and can be performed with a differential refractometer by measuring various sample concentrations that cover an order of magnitude and fitting the differential refractive index values at these concentrations to a linear function [129].

Lockyer et al. used SEC-MALS to characterize glycoconjugates for a variety of polysaccharides from Hib, *Neisseria*, and *Streptococcus* that were conjugated to tetanus toxoid (TT) [131]. In this study, the authors used SEC-MALS to monitor the size of the carrier protein before and after conjugation, calculate the molar mass, determine the presence of aggregates, and to obtain a measure of polydispersity. They found that each conjugate had a unique MW, which would have been miscalculated if based on SEC column elution behavior alone [131]. The elution of glycoconjugates is often non-Gaussian, so it is crucial to use MALS for accurate MW determination. Several other studies have used similar approaches to determine the molar mass, aggregate content, and polydispersity of polysaccharide conjugates [130,132]. In fact, Jumel et al. noted that by employing UV absorbance detection alone, they found 5–10% of aggregate present in a Men-C-CRM_{197} conjugate, but based on MALS, this peak made up a negligible amount of the total peak area [130]. If a MALS detector is not available, conjugation can be

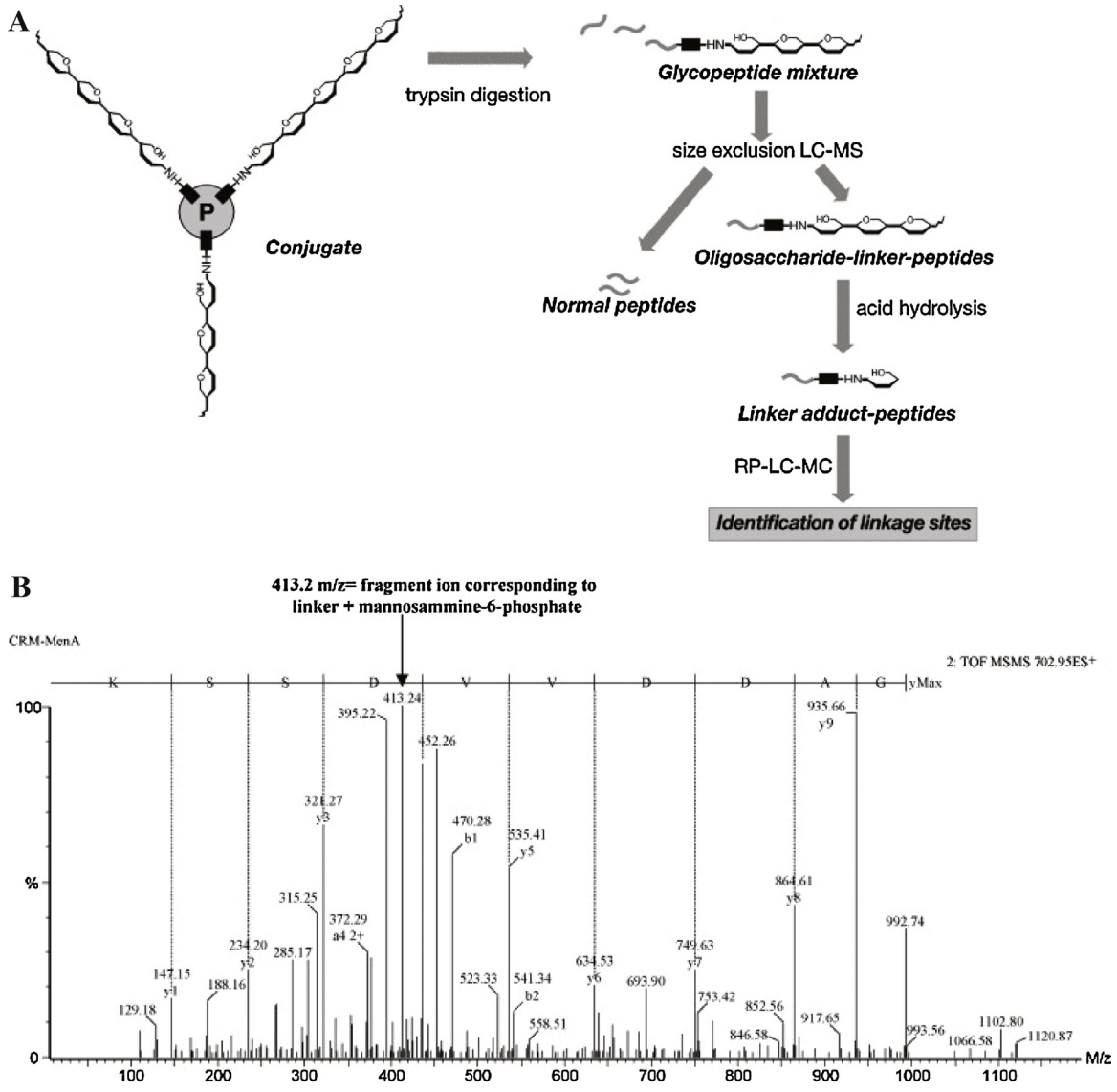

Fig. 8. LC–MS analysis of *N. meningitis* polysaccharides conjugated to CRM_{197}. A) LC–MS workflow to identify Lys residues of the carrier protein of a polysaccharide conjugate vaccine. B) Tandem MS analysis of the N-terminal peptide of a *N. meningitis* oligosaccharide to CRM_{197}. The arrow indicates a molecule resulting from conjugation. Figure was adapted from Ref. [132] with permission.

monitored by SEC and SDS-PAGE although a precise molar mass determination would not be possible [133,134].

5.2. Characterization of vaccine carriers/conjugates using LC–MS

LC–MS is a powerful tool for analyzing polysaccharide conjugate vaccines since it provides detailed information on both conjugation sites and glycosylation. Bardotti et al. outlined a strategy to characterize glycosylated lysine residues in *Neisseria meningitides* serotypes, A, C, W135, and Y polysaccharides that were conjugated to CRM_{197} (Fig. 8A) [132]. After trypsin digestion of the polysaccharide-conjugate, peptides were analyzed by SEC-MS since conjugated peptides generally eluted earlier then their non-conjugated counterparts. The samples were then subjected to mild acid hydrolysis in 0.1 N HCl to remove all but the final polysaccharide repeating unit, which is covalently linked to the carrier and not susceptible to hydrolysis. The resulting CRM_{197} peptides that contained conjugated lysine residues were then identified by LC–MS. A representative mass spectrum is shown in Fig. 8B of the N-terminal peptide of CRM_{197}-MenA that showed a peak with a 413.2 m/z (indicated by the arrow) which represents a molecule formed by conjugation with the respective oligosaccharide [132].

Another study performed by Crotti et al. used LC–MS to determine the relative reactivity of lysine residues in CRM_{197} to determine which of these amino acids were more susceptible to conjugation [135]. After labelling the lysine residues with linker molecules, semi-quantitative LC–MS was performed after a combination of trypsin, GluC, and AspN/GluC digestion steps. Of the 39 lysine residues in CRM_{197}, 31 were found to be modified with the linker to varying degrees (95% sequence coverage). After a thorough analysis of the crystal structure of CRM_{197}, the authors found a strong correlation between solvent accessibility and lysine residue reactivity, in which the most reactive lysine residues were the most solvent accessible. Using this information, the authors devised a two-step conjugation process to conjugate various glycans to CRM_{197} using only the surface exposed lysine residues in a controlled fashion. Presumably, the resulting conjugates would be better physicochemically defined and possess greater and more consistent biological activity.

6. Conclusions

Quantitative and robust analytical methods to monitor and ensure structural integrity, stability and efficacy of vaccine antigens are critical during vaccine development. In this review, we highlighted illustrative recent case studies describing recent applications of liquid chromatographic and mass spectrometric methods to better characterize a variety of diverse and complex macromolecular vaccine antigens and demonstrate the effectiveness of these higher-resolution techniques. These analytical methods offer extensive insight into the structural and chemical integrity of a diverse set of vaccine antigens in solution, and even when bound to an adjuvant. This is not possible with many traditional biophysical methods and can be an important supplement to the use of traditional vaccine potency assays.

Improved, higher resolution analytical technologies for physicochemical characterization of vaccine components (antigens and adjuvants) can facilitate vaccine formulation development (to identify a stable preparation for long term storage and convenient administration), comparability studies (to assess the effect of process changes and scale-up on the quality of the vaccine), and finally during commercial vaccine manufacturing (to ensure reproducible product is made from a validated process). Furthermore, recent developments suggest that it may be possible to use MS to analyze large bioparticles (e.g., intact viral particles) without the need to break down the complex into constituent components (e.g., proteins, nucleic acids, lipids) [42,44,136]. Although the presence of some components (e.g., nucleic acids) may complicate this possibility, successful analysis of something as complex as a ribosome suggests MS analysis of large bioparticles may be possible in the future. In addition to MS, other high-resolution techniques (e.g., NMR, Cryo-TEM, AFM) have been successfully applied to characterize and evaluate the stability of vaccines [63,137]. These types of high-resolution techniques complement lower resolution biophysical methods more commonly used during vaccine development (e.g., dynamic light scattering, analytical ultracentrifugation, SDS-PAGE) and can offer greater insight into the composition and structural integrity of a vaccine antigen.

The application of these analytical technologies to characterize a variety of vaccine antigens/adjuvants has not only facilitated their development as vaccine candidates, but has expanded our general knowledge of the correlative relationship between physicochemical properties and vaccine potency which can play a pivotal role in developing stabilized vaccine formulations. Despite these achievements, numerous challenges still remain. Chromatographic and especially mass spectrometric analyses generally have to be customized to each macromolecular antigen and/or modified to account for physicochemically diverse vaccine components, especially the presence of adjuvants. Furthermore, method parameters (e.g., sample handling, proteolysis conditions, data analysis methods, etc.) can potentially introduce artifacts and/or influence data interpretation in these high-resolution, sensitive techniques. The required investment in developing and implementing such analytical technologies pays off handsomely during vaccine development and enables critical scientific insights into the structural integrity and stability of complex vaccine antigens when manufactured, stored and administered as pharmaceutical dosage forms for human use.

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