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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