Mass spectrometry-based degradomics analysis of toxoid vaccines

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GENERAL INTRODUCTION AND THESIS OUTLINE

Current events in vaccine development

The outbreak of COVID-19 has put vaccine development in the spotlight. The promise of protecting people without the economic downfall caused by lockdowns is appealing. Under enormous pressure with seemingly unlimited funding, the development of the first efficacious COVID-19 vaccines took less than a year [1]. Amongst others, suggestions and improvements are being made to the usually lengthy clinical trials [2]. The fastest vaccines to reach the market are based on existing vaccine concepts that were adapted to SARS-CoV-2. Hundreds of COVID-19 vaccines, spanning every potential vaccine concept, are currently in various stages of development. Every vaccine has its own characteristics and both comprehensive product characterization and the development of suitable quality control assays are fundamental to ensure the safety and efficacy of any vaccine.

Vaccine concepts

Over the past century several vaccine families have been developed. These families can be divided into four categories: live attenuated, subunit, vector and DNA/RNA vaccines, and inactivated vaccines.

Live attenuated viruses can infect the host and induce an immune response (and subsequent protection) but do not cause disease. The first form of vaccination employed this principle by inoculating (vaccinating) people with cowpox virus to protect against (human) smallpox. Although this vaccine was discontinued after the eradication of smallpox, several current vaccines still use live attenuated viruses (*e.g.*, measles, mumps, rubella and oral polio vaccines). The main advantage of live attenuated vaccines is their high and broad immunogenicity and potential for mucosal protection if administered mucosally [3]. There are, however, concerns regarding safety, in particular with respect to potential reversion to a virulent form. Moreover, the rational development of new live attenuated vaccines and evaluating the safety of the vaccine before the first clinical trials is more challenging than with other concepts [4].

For subunit vaccines, specific immunogenic antigens are either purified from a(n) (inactivated) pathogen or produced recombinantly in non-infectious eukaryotic production cells or bacteria, much like the production of recombinant therapeutic proteins, such as insulin and

monoclonal antibodies. The first recombinant vaccine based on this concept was hepatitis B vaccine, for which the surface antigen (HBsAg) was produced in yeast cells (replacing a vaccine based on HBsAg obtained from infected humans) [5]. Other examples of vaccines containing purified antigens include the conjugate vaccines, where an antigen is chemically bound to an immunogenic carrier protein (such as haemophilus B oligosaccharides conjugated to diphtheria toxoid [6]). Vaccines containing purified antigens have clear advantages because the risk of contamination with (other) pathogens or harmful substances such as bacterial endotoxins can be severely reduced. However, some subunit vaccines induce suboptimal immune responses [7,8]. The switch from killed whole cell pertussis vaccines to acellular pertussis vaccines (a subunit vaccine) likely is a contributing factor to the resurgence of pertussis in recent years [9].

In the past decades the concept of RNA- or DNA-based vaccines have gained attention. The main idea is that (messenger) RNA (or DNA) containing the sequence information of antigens can be translated in the cells of the vaccinee. This process mimics an infection and should ensure that the intracellularly produced antigens are displayed to the immune system, similar to the process in an infected cell. The main hurdle with RNA and DNA vaccines is getting the genetic information into the target cells. To facilitate this, viruses deprived of their ability to replicate, or harmless viruses can be used as vectors to deliver the genetic information of the intended antigen. This technology is very new and only recently (2019 and 2020) the first of such vaccines have been approved for use against Ebola [10,11]. Other strategies involve the use of mRNA, either naked or encapsulated in a delivery system such as lipid nanoparticles [12]. Adapting these vaccines to different antigens can be achieved relatively quickly, because different mRNA molecules have very similar physicochemical characteristics, making them suitable for rapid response against new diseases [1].

Inactivated vaccines contain a killed or detoxified pathogen or toxin. Several methods are being employed, but chemical inactivation, usually by treatment with aqueous formaldehyde or β-propiolactone, is the most common one. Licensed vaccines in this category include influenza, polio, diphtheria and tetanus vaccines.

Toxoid vaccines

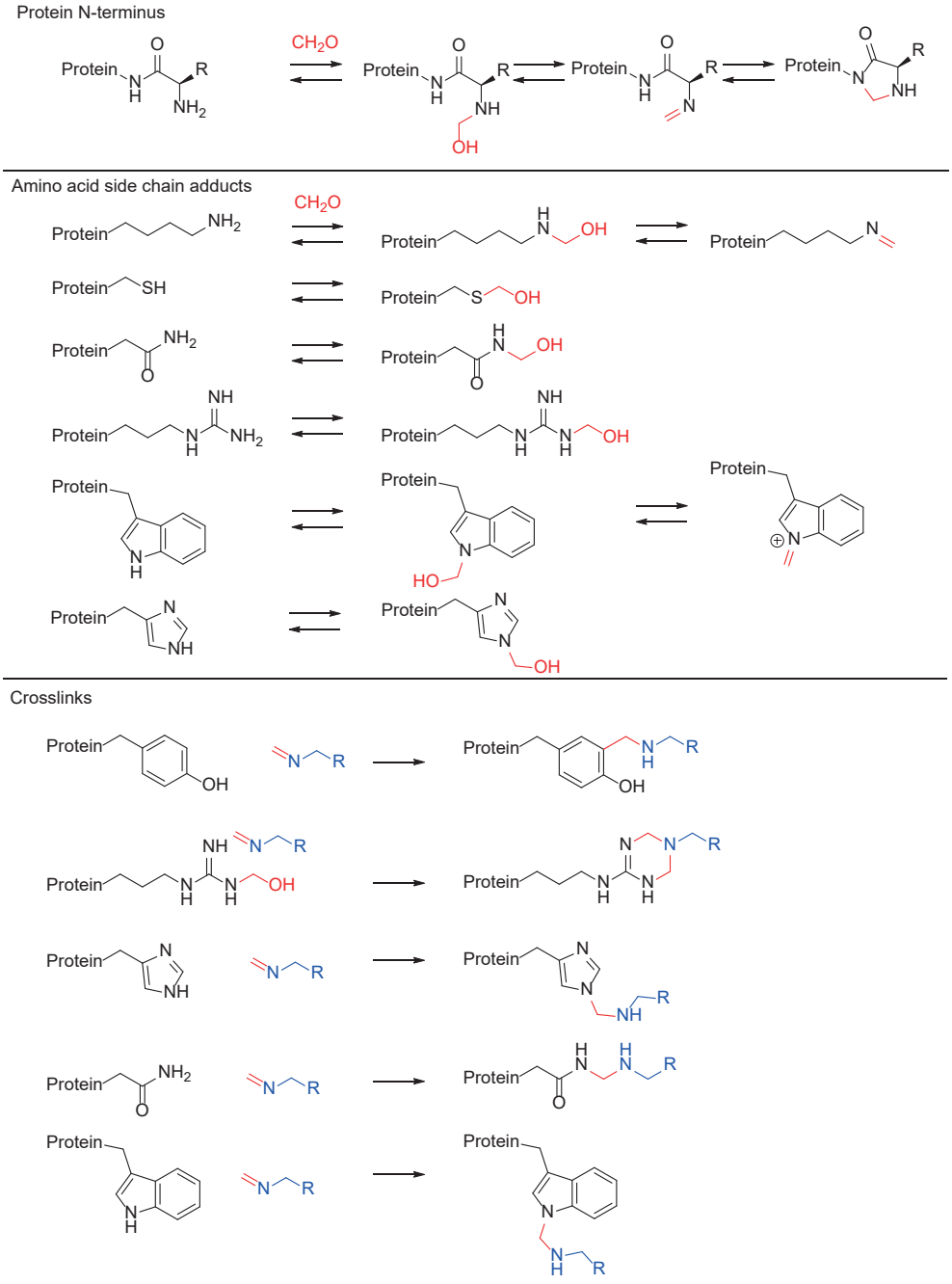
In this thesis, some of the oldest effective human vaccines –those prepared by formaldehyde-inactivation– are studied and characterized. Toxoid vaccines were discovered by serendipity. In 1891, antibodies obtained from the serum of animals that were injected with small amounts of diphtheria toxin were first used to successfully treat a patient suffering from diphtheria [13]. Following this success, more diphtheria toxin was needed for both research and for the production of more antibodies for the treatment of other patients. During such a diphtheria toxin production, a cultivation vessel was cleaned with formaldehyde but not all the residual formaldehyde was removed before adding new toxin to the vessel [14]. This toxin was then found to have a much lower toxicity in animals without detrimental effect on the antibody production. Further optimization based on formaldehyde-detoxification of the so-called *toxoid* (*i.e.,* toxin-like) by Gaston Ramon in 1923 eventually resulted in vaccines that were suitable for human use [15]. Nowadays, diphtheria and tetanus vaccines are still produced by formaldehyde treatment of the respective toxins.

Formaldehyde-inactivation has been very successful to obtain vaccines against bacterial toxins such as diphtheria, tetanus and pertussis toxins and viruses such as polio and influenza. Moreover, it has shown potential for new vaccines, such as those targeting enterovirus 71 [16], coxsackieviruses [17] and coronaviruses (*i.e.*, SARS-CoV-1[18]). Besides some unfounded criticism towards formaldehyde in vaccines [19] (the concentration limits in vaccines are lower than the endogenous formaldehyde concentrations found in the human body [20]), several failures have decreased its popularity for new vaccine concepts. The infamous Cutter incident with inactivated polio vaccine was related to the formaldehyde not reaching every polio virion, leading to improper inactivation, which resulted in 260 cases of paralytic polio in vaccinees [21]. These types of incidents can be avoided by proper quality control and designing a robust production process. Other serious incidents involve the clinical trials of formaldehyde-inactivated viruses which caused enhanced disease upon contracting the wildtype disease in recipients instead of offering protection. This was the case with formaldehyde-inactivated measles vaccine and a formaldehyde-inactivated RSV vaccine [22]. Understanding the biochemical and immunological fundamentals is essential for the development of new vaccines.

Because formaldehyde-inactivation of toxoid vaccines was invented by chance and not by design, the inactivation chemistry at the foundation of these vaccines was not immediately clear. Over the years, several groups have worked on understanding the interactions between formaldehyde and proteins [23-30]. An overview of common reactions and reaction products is depicted in **Scheme 1**. The reactions start with the formation of an hydroxymethyl on an amine, amide, guanidino group or thiol. For amines this product is in equilibrium with the corresponding imine. These imines can then act as electrophiles in subsequent (crosslinking) reactions. Crosslinks can be formed either between molecules in the matrix (such as amino acids) and the protein of interest, between various protein molecules, or within the same protein molecule. Even though amides (*e.g.*, the amide in the backbone of an N-terminus, glutamine residues or asparagine residues) are usually considered poor nucleophiles, reaction products with imines are commonly observed. The most commonly encountered crosslinks are between lysine residues and arginine or tyrosine residues [23]. Both K-R and K-Y crosslinks also occur as double crosslinks, in which two different lysine residues are linked to the same arginine or tyrosine residue. Overall, the conversion rates are low and the potential reaction products for a given amino acid are numerous. This results in very heterogeneous mixtures containing many different molecules when treating large proteins (such as diphtheria or tetanus toxin) with formaldehyde. This heterogeneity drastically complicates the analysis and characterization of toxoid-based vaccines.

Toxoid (and polio and hepatitis A and B) vaccines are often adsorbed to aluminum salts to enhance the immune response. The exact immunological mechanism remains poorly understood. The three most commonly suggested hypotheses are the so-called depot-effect (slow release of the antigen from the injection site over time), enabling phagocytosis of the adsorbed antigen by increasing the particle size and triggering local inflammation [31,32]. While after years of research no definitive answer has been given, the increase in efficacy of the vaccines that benefit from aluminum-based adjuvants is clear [33].

Scheme 1. Overview of formaldehyde-induced modifications described in the literature.



New assays for quality control of toxoid vaccines

The combination of product heterogeneity caused by the formaldehyde-inactivation process and the highly turbid nature of aluminum salt-containing vaccines makes characterization of the final product challenging. Historically, the two most important parameters of vaccines: safety and efficacy, have been studied by using animal tests, which are not affected by these challenges [34,35]. Efforts to reduce, refine or replace animal tests (3R concept) are being made [36]. To date, an important part of these efforts is to use a so-called consistency approach; if *in vitro* assays can show batch-to-batch consistency of vaccines or their intermediates, then comparison to a gold standard with confirmed potency and safety can circumvent additional animal tests [37]. *In vitro* assays have several advantages beside the obvious ethical considerations: the assay variability is generally smaller and they are considerably cheaper than *in vivo* assays. This results in the unique situation where every stakeholder (government, regulatory agencies and vaccine manufacturers) supports the development of alternatives to animal tests for the batch release of vaccines [38,39]. Several assays have been developed over the years, including antibody binding assays such as the flocculation test [40], ELISAs and biosensor assays [41,42] (non-adsorbed antigen fraction) or direct Alhydrogel formulation immunoassays (DAFIA, analysis of the adsorbed antigen fraction) [43]. These serological tests are aimed at the refinement of animal tests. Moreover, physicochemical techniques, such as SDS-PAGE, primary amino group determination, fluorescence spectroscopy and circular dichroism analysis, have been developed for the characterization of toxoids in a consistency approach [42]. The assays employed in a consistency approach are usually intended for the actual replacement of animal tests. Additionally, complementary assays, preferably those capable of analyzing the final product, are required to expand the panel of tests capable of testing various product characteristics to ensure that the product quality meets safety and efficacy requirements.

In search of a new animal-free assay to confirm the quality of toxoid vaccines, we looked into the adaptive immune system for inspiration. Globally the adaptive immune system can protect the body against pathogens with (a combination of) two types of responses: a humoral response with the production of antibodies, and a T-cell response. T-cells can be divided into cytotoxic T-cells expressing the CD8 receptor and T-helper cells expressing the CD4 receptor. To become effector T-cells, an antigen presenting cell has to present a

fragment of an antigen (a T-cell epitope) to the right T-cell. Almost all cells in the body present fragments of proteins (peptides, predominantly originating from within that particular cell) to naïve CD8 T-cells through Major Histocompatibility Complex I (MHC-I) molecules. Peptides displayed to the immune system in this way can show either endogenous self-proteins not resulting in a T-cell response, or unknown proteins which could be indicative of, for instance, a viral infection. The cytotoxic T-cell can then kill an infected or otherwise aberrant cell and, for instance, prevent the production of more virions. Contrarily, CD-4 T-cells are activated by peptides presented on MHC-II molecules, which are expressed by specific immune cells, with dendritic cells being the most important cell type. Dendritic cells take up potential antigens and process them in their endo-lysosomal vesicles to eventually present the peptides derived from these antigens on their MHC-II molecules. Activation of CD-4 T-cells is pivotal for the other arm of the adaptive immune response: humoral immunity [44]. Therefore, the antigen processing by dendritic cells is a key step in achieving a good immune response for formaldehyde-inactivated toxoid vaccines. Several groups have identified a correlation between the intracellular antigen degradation speed and the immunogenicity of the antigen. Antigens that were found to be more resistant to proteolytic degradation by dendritic cells were found to be more immunogenic [45-51]. We hypothesized that by mimicking this part of the immune system in a simplified way, we should be able to identify potential variations in the protein antigens used in vaccines. By studying the enzymatic proteolysis of antigens in such a *degradomics* approach, mimicking a small component of the immune system could be part of a panel consisting of several *in vitro* assays, replacing the need for *in vivo* studies.

Thesis outline

The chemical and structural heterogeneity of toxoid vaccines makes their analysis challenging. However, detailed insights on a molecular level can be obtained by mass spectrometry. Our initial focus was the identification of formaldehyde-induced modifications in diphtheria toxin, which is described in **Chapter 2**. Subsequently, the methods described in **Chapter 2** were applied to study what effects formaldehyde-induced modifications on model proteins have on their susceptibility to enzymatic proteolysis (**Chapter 3**). During the analysis of these model proteins, unknown formaldehyde-induced modifications were observed. The structural elucidation of these modifications, the discovery of a new type of crosslinks and various other subsequent reaction products are described in **Chapter 4**. The degradomics analysis

described in **Chapter 3** was applied to tetanus toxoids to distinguish heat-denaturated toxoids from their original state (**Chapter 5**). In order to reduce the analysis time and further improve the degradomics approach, an optimized strategy using Tandem Mass Tag multiplexing for the relative quantification of peptides was developed for the analysis of diphtheria toxoids (**Chapter 6**). Finally, **Chapter 7** provides a brief discussion on the results presented in this thesis and offers some perspectives on implementation of the findings for toxoid vaccine development, quality control and further research.

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