CERTIFICATO DI FIRMA DIGITALE

Si certifica che questo documento informatico

phd_unisi_094216_dafirmare.pdf

composto da n°104 pagine

È stato firmato digitalmente in data odierna con Firma Elettronica Qualificata (FEQ), avente l'efficacia e gli effetti giuridici equivalenti a quelli di una firma autografa, ai sensi dell'art. 2702 del Codice Civile e dell'art. 25 del Regolamento UE n. 910/2014 eIDAS (electronic IDentification Authentication and Signature).

PROCESSI INFORMATICI COMPLETATI

- **Apposizione di Firma Elettronica Qualificata Remota** emessa da Intesi Group S.p.A. in qualità di prestatore di servizi fiduciari qualificati autorizzato da AgID, per garantire con certezza l'autenticità, l'integrità, il non ripudio e l'immodificabilità del documento informatico e la sua riconducibilità in maniera manifesta e inequivoca all'autore, ai sensi dell'art. 20 comma 2 del CAD - D.lgs 82/2005.
- **Apposizione di Contrassegno Elettronico**, l'unica soluzione tecnologica che permette di prorogare la validità giuridica di un documento informatico sottoscritto con firma digitale e/o marcato temporalmente, rendendolo inalterabile, certo e non falsificabile, una volta stampato su supporto cartaceo, ai sensi dell'art. 23 del CAD - D.lgs 82/2005.

Per risalire all'originale informatico è necessario scansionare il Contrassegno Elettronico, utilizzando l'applicazione HONOS, disponibile per dispositivi Android e iOS.

UNIVERSITÀ DEGLI STUDI DI SIENA

DIPARTIMENTO DI BIOTECNOLOGIE, CHIMICA E FARMACIA

DOTTORATO DI RICERCA IN

CHEMICAL AND PHARMACEUTICAL SCIENCES

CICLO XXXV

Coordinatore del corso: Prof. Maurizio Taddei

DEVELOPMENT OF AN ANALYTICAL PLATFORM TO DIRECTLY CHARACTERIZE ALUM ADJUVANTED VACCINES

Settore scientifico-disciplinare: CHIM/02

DOTTORANDO
Alessio Corrado

Supervisore
Prof. Alessandro Donati
Università degli studi di Siena

Co-supervisori
Dott. Francesco Berti
GSK Vaccines
Dott.ssa Mila Toppazzini
GSK Vaccines

ANNO ACCADEMICO 2022/2023

Ringrazio il Dipartimento di Biotecnologie, Chimica e Farmacia dell'Università di Siena e GSK Vaccines per avermi dato la possibilità di intraprendere questo percorso di dottorato.

Un ringraziamento sincero ai miei relatori, il Professor Alessandro Donati, il Dottor Francesco Berti e la Dottoressa Mila Toppazzini per avermi guidato e supportato nella realizzazione di questo progetto.

Infine, un ringraziamento speciale a tutte le persone che negli anni sono state coinvolte nel progetto dando il proprio contributo alla realizzazione dello stesso.

Table of Contents

List of Abbreviations

Aluminum hydroxide	Alum; $Al(OH)_3$
Neisseria adhesin A	NadA
Neisserial Heparin Binding Antigen	NHBA
factor H binding protein	fHbp
Capillary electrophoresis	CE
In vitro relative potency	IVRP
Hydroxyl radical foot printing	HRF
Mass spectrometry	MS
Inner diameter	id
High-Performance Liquid Chromatography	HPCL
Capillary zone electrophoresis	CZE
Micellar electrokinetic chromatography	MECK
Capillary gel electrophoresis	CGE
Laser induced fluorescence	LIF
Bare-fused silica	BFS
Background electrolyte	BGE
Electroosmotic flow	EOF
Sodium dodecyl sulphate	SDS
Nanoparticles	NPs
Linear Polyacrilamide (Neutral capillary)	LPA
Tris (hydroxymethyl) aminomethane	TRIS
Hydrochloric acid fuming 37%	HCl
Methanol	MeOH
Isoelectric points	pI
Hexadecyltrimethylammonium bromide	CTAB
Monoclonal antibodies	mAbs
Median Fluorescence Intensity	MFI
Parallel Line assay	PLA
Relative Potency	RP
Ethylendiaminetetraacetate	EDTA
Hydrogen-deuterium exchang	HDX
Dithiothreitol	DTT
Tris(2-carboxyethyl) phosphine	TCEP

Formic acid	FA
Acetonitrile	ACN
Methionine	Met

Abstract

Adjuvants are molecules that boost the potency and the longevity of the immune response to vaccine antigens, with little or no increase of toxicity or reactogenicity.

The adjuvant activity of aluminum-containing compounds was first discovered in 1926 when an alum-precipitated diphtheria vaccine showed improved antigenic properties compared to the standard diphtheria vaccine. Since then, aluminum containing compounds have been routinely used in the formulation of vaccines due to their good record of safety, low cost and compatability with various antigenclasses. Despite its extensive use, the immune mechanism of action of aluminum is not yet completely understood . This is in part due to the complexity of developing effective analytical and qualitative tools to characterize the antigen in aluminium adjuvanted vaccines without performing any preliminary treatment to desorb the antigen from the aluminium. Most current methods used to assess the antigen content or purity in formulated vaccines containing aluminum adjuvant complexes, require the desorption of antigens from model aluminum-containing formulations.

The aim of this project was to develop an analytical tool to directly quantify and assess both the content and purity of formulated aluminium-adsorbed antigens, whitout any prelimary sample manipulation (e.g antigen desorption). As an adjuvant model system, we selected three recombinant proteins (Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp)) as antigens, and aluminum hydroxide (AH) as an adjuvant system. The three recombinant proteins were selected due to the substantial quantity of literature regarding their structure and immunological activities, meaning that they are well characterized. In addition, their adhesion rate to aluminium is well known. Capillary electrophoresis techniques showed promising results in detecting and quantifying the antigenic components in a single run. The physical-chemical characteristics, however, of the adjuvanted components impacted the robustness of the separation In addition, other critical issuea related to: (i) aluminium sedimentation, which caused inhomogeneity in the final sample; (ii) poor reproducibility regarding samples with proteins adsorbed to aluminium, presumably due to varying aluminium populations, were found. Although aluminiumsedimentation might be overcome by simple practices such as continuous stirring of the sample, the presence of poor reproducibility remained unsolved.

The study of multivalent (three recombinant proteins) and monovalent (only NHBA

protein) formulations with alum through the In vitro relative potency immunoassay (IVRP) and the hydroxyl radical foot printing (HRF) mass spectrometry showed the peculiar behaviour of NHBA at different alum concentrations, in particular for monovalent formulations. In particular, the IVRP results showed that the relative potency of NHBA increases according to the concentration of alum. As the assay does not discriminate between the percentage of adsorption of the antigen, this was taken to suggest a possible 'rearrangement' of the protein to maximize its adhesion to aluminium when present at low concentrations. This was confirmed by the HRF studies, which monitored the methionine residues that formed part of the known epitope: it was observed that they were less exposed to the solvent (hence less oxidized) at lower concentrations of aluminium. This confirmed that in the presence of low concentrations of aluminium, , the protein tends to change its 'structure' to maximize adhesion, probably leading it to assume a spatial conformation in which epitopes are less accessible to the antibodies used in the IVRP assay. .

Summary

Vaccines, in our time, represent an important tool for preventing and fighting endemic and new diseases, as demonstrated by the recent pandemics of the last two decades. They can be multicomponent products, where the balance between the main principle or active ingredient (antigens) and adjuvants/ excipients is crucial to ensure the efficacy of the final product[1]. Until now, three main types of vaccine have been used in humans: live-attenuated vaccines, consisting of a virus or bacterium that is less pathogenic than the real pathogen; inactivated vaccines that are heat-inactivated or chemically-inactivated particles of the pathogen; or subunit vaccines that are made from components of the pathogen. [2] The great force of vaccination is to generate a strong and persistent immune response against infection (and possibly it sequele), however, boost of adjuvants is critical to enhance the antigen's immunogenicity possibly reducing the amount of active ingredient require, the number of immunizations and improving the overall efficacy of vaccines, in particular for immunocompromised , newborns or the elderly patients.[3]

To evoke effective immune response to vaccine and raise immunogenicity against highly purified components, a variety of adjuvants may be used, becoming more and more crucial elements of modern vaccine formulations.

Adjuvants can be classified in two groups:

1. Substances that increase the immune response to the antigen,

2. Immunogenic proteins that modify T-cell activities[4]

The adjuvant activity of Aluminum-containing compounds were first discovered to have adjuvant activity in 1926 when an Alum-precipitated diphtheria vaccine showed better antigenic properties than the standard diphtheria vaccine. Since then, aluminum containing compounds have been commonly used in the formulation of vaccines due to their good record of safety, low cost and good adjuvanticity with various types of antigens. Despite its extensive and continuous use, the immune mechanism of action of aluminum is not yet completely understood [5]

Physico-chemical and functional attributes of all components and of the whole vaccine formulation are crucial and they must been controlled. One of the main analytical challenges is to determine purity and quantity of the antigen in the final product of adjuvanted vaccines. The commonly used methods for characterizing adjuvanted

vaccines require the desorption of antigens from model Aluminum-containing formulations, due to the Alum interference. Desorption, generally, involves a high salts concentration inducing a changing in the physico-chemical proprieties of the compounds. For this reason, there is a big need for tools able to directly quantify adsorbed antigens. This project was divided in two main parts, the first part was aimed to develop analytical platforms able to directly quantify antigens and determine purity in the final container of adjuvanted vaccines, without any further manipulation, like desorption. In this context, capillary electrophoresis techniques were explored to achieve the goal. However, several challenges linked to adjuvants interference have been experienced with the explored electrophoretic techniques.

The second part was focused on establishing whether antigen adsorption on adjuvants could affect the structure of the antigen or its immunogenicity. The aim was to understand the antigen-antibody interaction by exploiting the potential of immunobased techniques, such as in vivo relative potency (IVRP), and Hydroxyl radical footprinting (HRF) mass spectrometry techniques as an orthogonal assay. The choice of these two assays lies in their ability to be directly applied to our vaccine model without antigen desorption and to combine accurate physiochemical characterization with immunological studies to characterize the antigen-adjuvant interaction.

As an adjuvant system model, we selected three recombinant proteins: Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp) as antigens and Aluminum Hydroxide (AH) as an adjuvant system. The three recombinant proteins were selected due to the large mole of literature in which their structure and immunological actives were well described. In addition, their alum adhesion rate is well known and documented.[6-8]

Regarding the direct analysis of adjuvanted vaccine without desorbing process, CE techniques showed that it is feasible to detect and quantify the antigenic components in a single run but unfortunately the chemical physical characteristics of the adjuvanted components affect the robustness of the separation. One important obstacle is related to Alum sedimentation, that causes inhomogeneity in the final sample. Another issue of the analysis was that Alum with the adsorbed proteins appears as multiple peaks presumably due to different Alum populations: the peaks distribution differs from run to run, causing not reproducible quantifications. We investigated these two-principal issues and, even if sedimentation was solved, the presence of not reproducible multi

peaks remained an open point.

The study of multivalent (three recombinant proteins) and monovalent (only NHBA protein) formulations with Alum through the IVRP and HRF assay showed a peculiar behaviour of NHBA at different Alum concentrations. In particular, the IVRP results showed that the relative potency of NHBA increases as the Alum concentration grows. Since the assay is not dependent on the percentage of antigen adsorbed, this suggested a possible 'rearrangement' of the protein to maximize its adhesion to Alum when present at lower concentrations. This was confirmed by HRF studies, monitoring the methionine residues forming part of the known epitope: it was seen that they were less exposed to the solvent (hence less oxidized) at lower Alum concentrations. This confirmed that in the presence of less Alum available, the protein tends to change its 'structure' to maximize its adhesion on it, probably leading it to conformation in the space less accessible to the epitope.

Disclosure

Disclosure
Alessio Corrado is a PhD student at the University of Siena and participates in a post graduate studentship program at GSK. This work is sponsored and funded by GlaxoSmithKline Biologicals SA. Alessio Corrado is an employee of the GSK group of companies.

CHAPTER I.
GENERAL INTRODUCTION AND OBJECTIVE OF THE THESIS

1.1 *Vaccines*

"Vaccines are a substance used to stimulate the production of antibodies and provide immunity against one or several diseases, prepared from the causative agent of a disease, its products, or a synthetic substitute, treated to act as an antigen without inducing the disease".[9]

In the last century, vaccination has been a crucial role to reduce death and morbidity caused by infectious diseases. It is believed that vaccines save at least 2-3 million lives per year worldwide. [10]

The history of vaccines has its roots in antiquity: the earliest evidence of vaccination was recorded in 16th century China, where smallpox was most likely prevented by inoculation; some studies hypothesize the presence of smallpox-like rashes on Egyptian mummies, suggesting that the phenomenon could date back at least 3,000 years[11].

Only in 1700s, a young Doctor, Edward Jenner, paid his attention to the unsullied complexions of milkmaids and inferred that cowpox protected them from the ravages of smallpox. Jenner decided to attempt, what is known today as a "clinical study", inoculating vaccine smallpox pustules fluid from on milkmaiders infected with the disease, noting that the subjects expose to human smallpox, did not get sick, thus acquiring immunity to human smallpox. This experiment marked the basis for modern vaccines and vaccinations. Indeed, at the end or nineteenth century, Luis Pasteur identified microorganisms as the agents responsible for infectious diseases. Moreover, by accidentally leaving of a bacteria culture without nutrients, he observed that it was possible to artificially obtain micro-organisms showed attenuated virulence and were capable of conferring immunity against disease. Just in honor of Edward Jenner’s discovery, Pasteur named these attenuated bacteria “vaccines”.

Even in our time, more than a hundred years after Pasteur's observation, vaccines are considered a fundamental milestone for healthcare providing life-long protection against several infections. At the begging, the guiding principle was that proposed by the French microbiologist, which involved identification of the microorganism, inactivation by heat or drying, and subsequent injection. The greatest innovation in the field of vaccines occurred in the 1920s, when Gaston Leon Ramon developed a new technology to inactivate diphtheria and tetanus toxins. Gaston's great insight was to identify the two toxins as the causes of the respective diseases and the possibility of inactivating them with formaldehyde, a procedure like that used in modern vaccine production. This made it possible to have a vaccine that did not consist of the entire bacterium, but only part of it. In this way, Gaston developed two new vaccines against Corincebaterium dipheteriae and Clostridium tetanii, with their respective toxins. [12] Findings over the time allow to categorize vaccines in three main groups: live-attenuated whole microorganism, heat or chemically inactivated microorganisms and microorganisms' subunits. The new technology improvements and the continuous attentions of safety concerns drive vaccine development to limit the use of whole pathogens in favor to the identification and isolation of well-characterized components responsible of conferring immunity to the disease. Indeed, the most challenges when working with whole inactivated microorganism is the reversion of toxicity and high levels of reactogenicity, that may be caused adverse effect.

The continuous development of vaccines (fig 1) over the years has allowed constant progress toward newer and more sophisticated technologies. One of the main examples in this context, was the combination for protection against multiple diseases, that stated over sixty years ago with the combination of diphtheria toxoid (DT), tetanus toxoid (TT) and whole-inactivated pertussis bacteria (wP) into a single DTwP vaccine that has become the cornerstone of pediatric and adult immunization programs.[13]

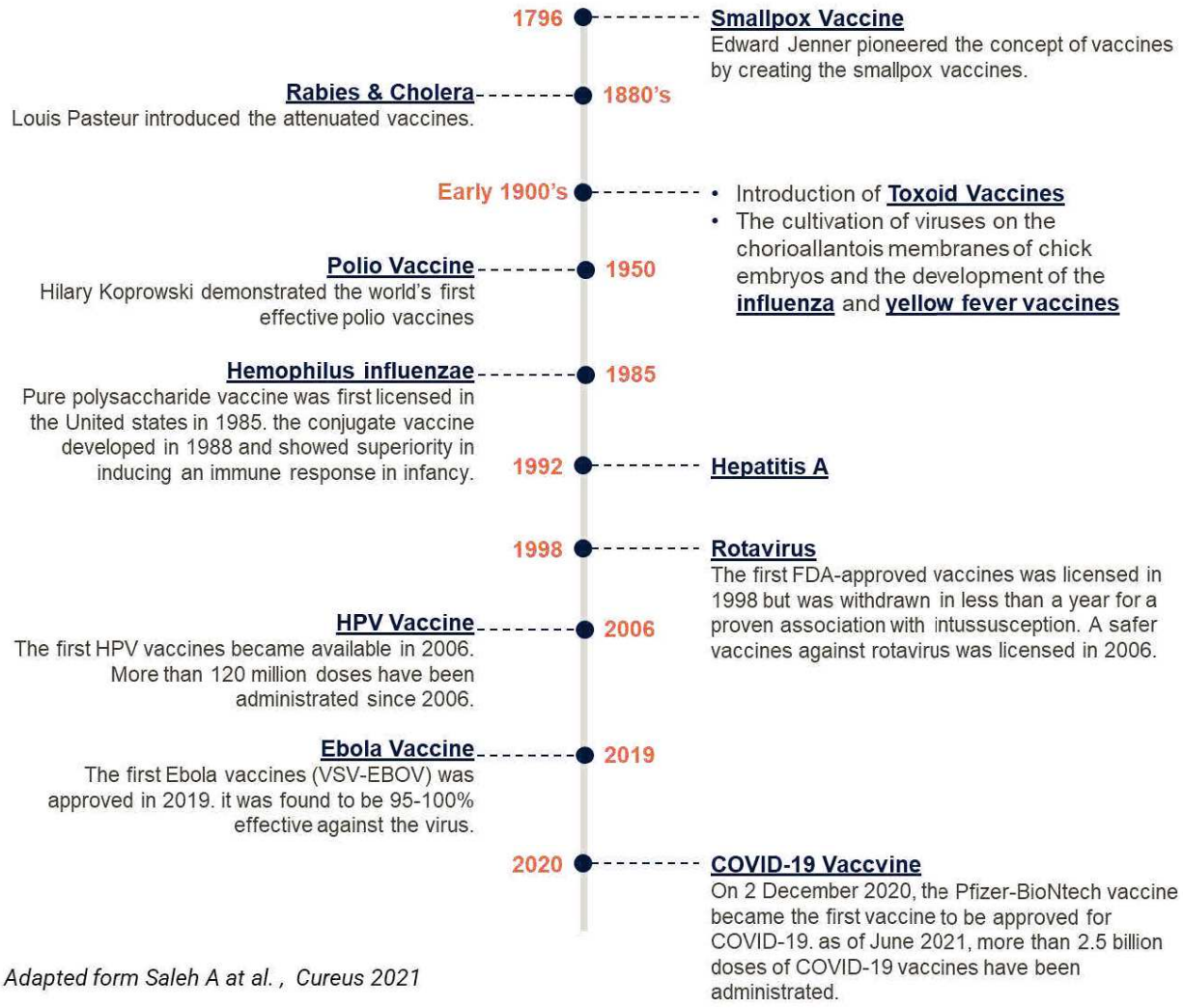


***Figure 1**:Vaccine History timeline*

One of the milestones in the field of vaccines was the *Reverse Vaccinology (fig 2)*[14, 15]: this particular technique has been changing the way to things at the vaccines. The main components of vaccines (as proteins or sugars) are those usually found on the bacteria and virus surfaces. Commonly used procedures required to: isolate the antigen, discover its functions and biologic sequence, introduce it into a biological "fermenter" (formerly brewer's yeast) to produce it in large quantities [12]. Reverse vaccinology avoids this longer procedure starting by the entire genome. Through bioinformatics techniques screening the entire genomes of pathogens to identify the potential genes that could lead at good epitopes, the peptides involved in the antibodies binding and proteins in surface. Once the proteins/peptides have been identified, these are produced and tested in animal model and finally formulated in the final vaccine. This allowing to identify a great magnitude of antigens compared to the traditional method. Group B

meningococcus (MenB) represents the first example of a successful application of reverse vaccinology. [16-18]

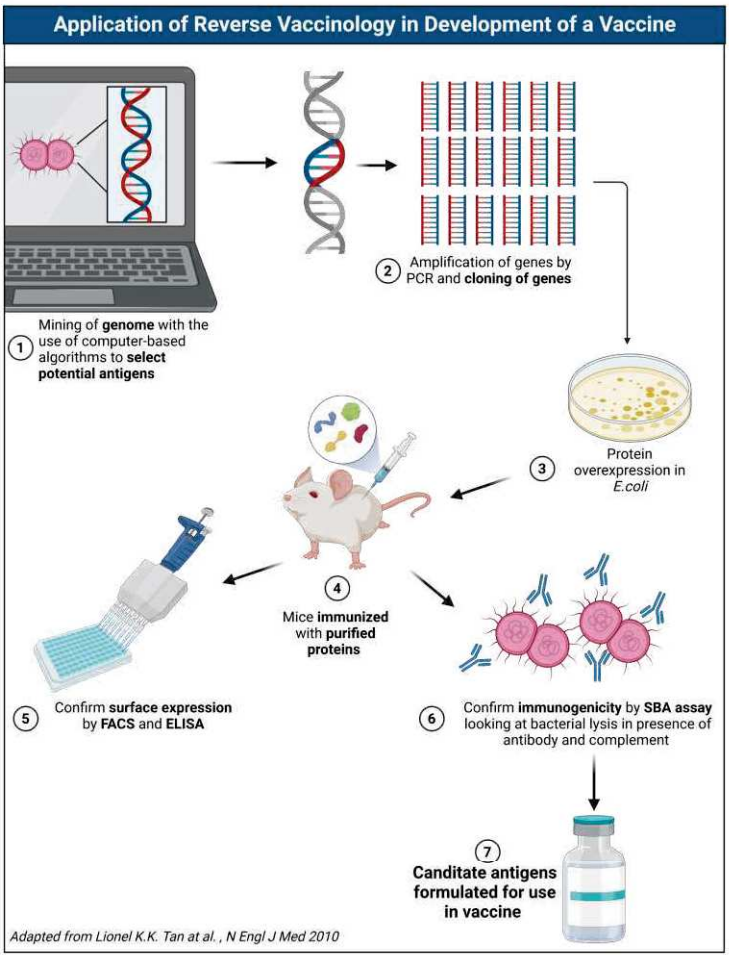


***Figure 2**: Reverse vaccinology is a technique for developing a vaccine from the genome sequence of a micro-organism(1). Once the genes of interest have been identified, these are cloned and amplified by PCR(2); the antigens are then cloned into appropriate vectors (E.Coli) to produce the specific proteins (3). The purified antigen are finally tested in an animal model (4). Recombinant protein candidates were then selected based on their surface expression (assessed by FACS), and ability to induce serum bactericidal antibodies (assessed by the SBA serum bactericidal assay(5-6). At the end the selected candidates are formulated in the final vaccine.(7)*

1.2 *Vaccine Adjuvants*

Safety improvement is a crucial parameter that has always driven the development of vaccine to involve a minimal health risk to vaccinees. Increasingly, modern vaccines are being developed based on rationally designed recombinant highly purified antigens, through structure based design, epitope focusing or genomic based screening, defined subunit vaccines[19]. Subunit vaccines allowed an evident increase in safety profile but a less immunogenic in comparison to the more traditional vaccines based on live attenuated or inactivated pathogens. Therefore, vaccine development becomes a complex fine-tuning between safety and efficacy of the final product. In this perspective adjuvants were introduced to balance the removal of natural immune-stimulatory components of the pathogens with the aim to enhance an immune response similar to the one triggered by the natural infection [20] while maintaining equilibrium between vaccine safety and efficacy. Adjuvants derived from Latin word *Adjuvare*, meaning "to help". The first evidence of this compounds was found in the studies of Ramon about diphtheria and tetanus toxins, which described as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone”. In general Adjuvants are molecules or compounds that boost the potency and longevity of the immune response to vaccines antigens, without causing increased toxicity. [21, 22]

The addition of these compounds in vaccines provides several advantages:

- Enhance the quality and magnitude of the immune response extending duration and potency of induced antibodies
- Quicker responses allow the antigen dose reduction or fewer doses
- Enable complex vaccine combinations overcoming competition among included antigens
- Overcome limited immune response in endangered population such as the elderly, young children, and immunocompromised people.

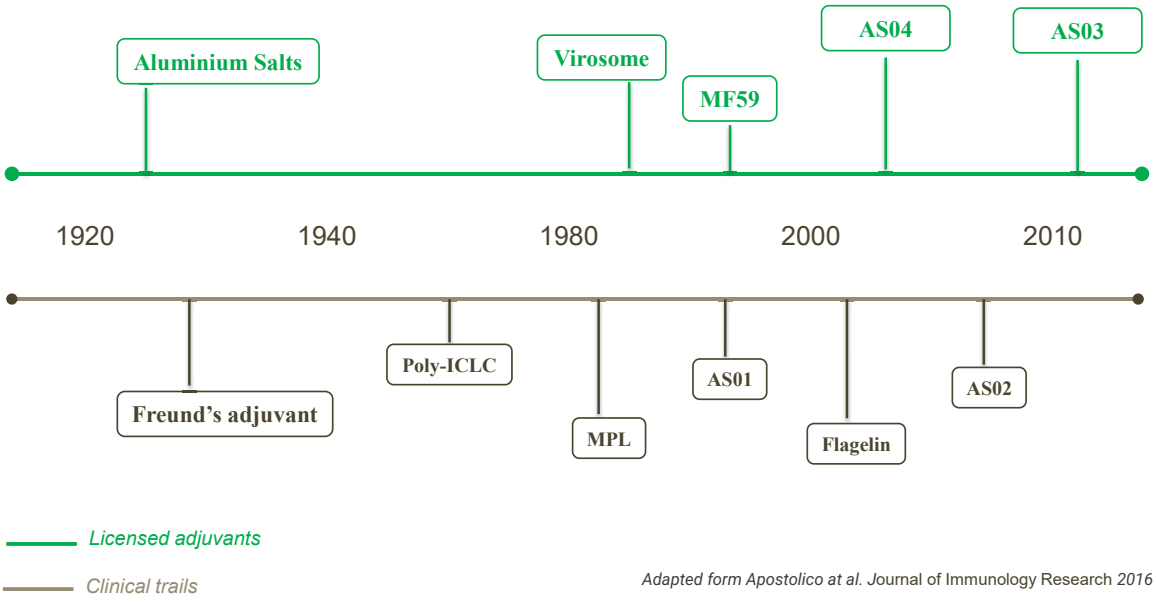


***Figure 3**:Timeline of vaccine adjuvants discovery.*

Although a variety of compounds with adjuvant properties, that seem to exert their functions through different mechanisms of action, few adjuvants are licensed for human use and several formulations [23] (see fig 3). Indeed, for almost 80 years Aluminum-base-adjuvants-approved in 1920s- were the only adjuvants licensed, only in 1997 MF59 approval for inclusion in an influenza vaccine. Aluminum salts and MF59 including in the 1st generation of adjuvants who feature is the delivery of antigens to immune cells. The 2nd generations have been developed combing a delivery system with an immunopotentiator molecule. An example is AS04 [24] that combines the lipopolysaccharide analog monophosphoryl lipid A (MPLA) with Aluminum salts and was the first to be approved for inclusion in a vaccine against Hepatitis B virus (Fendrix® from GSK) in 2005[25] and against human papilloma virus (Cervarix® from GSK) in 2007 [26]. However, as mentioned before, those are only a subset among the broad range of adjuvants described in literature, indicating that they are developing as slow and duty process, which overtasked many challenges from a safety and regulatory aspect.[19]

Surprisingly, despite the large use the adjuvants system the mechanisms of action of most adjuvants are complex and often remain poorly understood, although a growing number of research groups focused their attention and investments at deepening the knowledge in this area, following the identification of key receptors on cells involved in innate immunity that are activated by a variety of adjuvants.[27]

Formulation of adjuvanted vaccines plays a crucial step to mantain the integrity of the final compound without adverse effects on product/antigen stability and efficacy [28]; in this prospective a complex set of analytical characterization techniques is usually required for a complete characterization of formulated antigens and antigens-adjuvants interactions further complicate their application [29]. Inthe next section these challenges will be discussed focusing on Aluminum salts adjuvants which are one of the “main characters” of this thesis.

1.3 *Aluminum salts adjuvants*

Aluminum salts are most commonly used vaccine adjuvants in human vaccines, they have been included in many commercial products over many decades, and have been shown to be safe and well tolerated [19].Due to their long history they become a reference standard for the evaluation of new generation vaccines. At the beginning Aluminum salts were used for protein purification thanks to their high adsorbent capacity. Only in 1926 Glenny and Pope demonstrated the adjuvant effects of this material, who showed that Alum precipitated diphtheria toxoid was more potent than the antigen alone. This discovery inspired Ramons's adjuvant definition as cited before[5]. Subsequent other studies showed that Alum-precipitate diphtheria and tetanus toxoids enhanced immune response in humans. [30]

Aluminum adjuvants have been extensively studied during the twentieth century and included in many humans vaccines against infectious diseases tanks to their good safety profile (e.g. vaccines against hepatitis B, Haemophiles influenzae and human papilloma virus). However, how Alum works or what is the ‘mechanism of action’ is a big question that has been consistently "intrigued" scientists for many decades since the adjuvant was first introduced. One of the earliest hypothesis is that it works as "depot effect"[31]. Therefore, originally Alum adjuvants were thought to retain the antigen at the injection site due to slow release of the antigen and increasing the opportunity for interaction with recruited antigen presenting cells. This mechanism was supported by different studies that showed the possibility to immunize additional animals after the removal of the tissue granuloma induced by Alum adsorbed vaccine several weeks after administration and following maceration. This suggests the presence of antigen at the

site injection for a long period. However, the depot mechanism was subsequently downplayed by a different study, in particular by Holt[32] highlighting the importance to found alternative mechanisms. Nevertheless, the study during the years on this compound showed that the use of adsorbed Alum plays an important role to recruit many immunological components involved in the response [30].

Aluminum salts can be divided in two main types as adjuvants, based on their different chemical and physical compositions that influence antigens adsorption on their surfaces[33]:

- Aluminum hydroxide (Alum), chemically Aluminum oxyhydroxide, shown only hydroxyl groups on its surface and appears as crystalline structure composed of needle-shaped nanoparticles which tends to aggregate up to an average diameter of 10 μm. In the vaccine formulation, at neutral pH, Alum has a positive charge thanks its point of zero charge (PZC) of 11.4.

- Aluminum phosphate is chemically Aluminum hydroxy phosphate. On its surface hydroxyl and phosphate groups are present in ratio dependent on manufacturing conditions. The amorphous structure of alum phosphate is composed by particles size of 50nm that cause irregular aggregates. At neutral pH it takes a negative charge thanks to its PZC about 4.5 and 5.5. [34]

To develop a stable and efficacious adjuvanted vaccine, the entire physico-chemical properties of Alum salts compounds must be considered. The grade of antigens adsorption on their surface are one of the most crucial aspects to obtain strong antigen-specific responses. This lies in the previously citated theory, that shown how the antigen adsorption on adjuvants surface is crucial to promote the antigens retention at the injection site [35] improving the antigen stability and immunogenicity and facilitating the uptake of immune cells[36]. The antigen alum interaction is mainly affected by electrostatic forces, hydrophobic interactions, and ligand exchange. Thus, the chemical-physical characteristics of both the antigen and the two alum salts (e.g. isoelectric point) play a key role in the rational choice of the right adjuvant, to obtain a high grade of adsorbed antigen.

For such complex matrices, as antigen/Alum systems is needed a complex set of analytical tools to characterize adsorption, total content and integrity of antigen and Alum components. Most analytical tools commonly applied to monitor proteins (e.g. electrophoresis, Western blot, and liquid chromatography) cannot be used directly to the vaccine formulate due to the interference with adjuvant compound. Therefore, to characterize the antigens in the vaccine, it is necessary to completely recovery them from adjuvants surface through desorption procedure. Nevertheless, the high concentration of salts and surfactants, used in the desorption procedure, could result in incomplete recovery of the antigen, with the loss of some chemical and physical properties that compromise its integrity. In addition, common desorption procedures involve the use of centrifugation to recover desorbed antigens that are released in the aqueous phase prior to analysis, and some components of the desorption buffer may be present in the final matrix of the sample to analyze causing a possible interference with the assay (e.g. surfactants impact HPLC methods and Histidine buffers are not suitable for colorimetric assays) [13].

Thus the variety of analytical tools currently available for the characterization of soluble proteins is not applicable to adjuvanted vaccines in presence of adjuvant moreover, the antigen-adjuvant interactions can make the analysis of antigens in formulated vaccines to be an enormous challenge.[29] In this perspective, the development of analytical assays which allow the characterization of antigen-adjuvant interactions and the investigation of their effect on antigens stability and immunogenicity is continuously needed. It has been already observed that antigens undergo structural changes due to interaction and adsorption to Aluminum salts and the resulting effects on antigens physical-chemical stability due to changes in their environment have only recently started to be investigated [30].

For this reason, it is necessary to have analytical tools capable to fully understand any change in the physico-chemical properties of adjuvanted vaccines, and to establish whether antigen adsorption on adjuvants affects antigen structure. Indeed, the structural information of the antigen is not only important to study its immunogenicity, but also to understand the interactions between antigen and adjuvant and the mechanism by which the adjuvant can enhance the immune response.

Therefore, to fully characterize the components of adjuvanted vaccines without altering their physico-chemical properties, an analytical tool capable of being applied directly

to the final product, without further manipulation such as desorption, is required. During this work, we explored different analytical tools, both physicochemical, such as Capillary electrophoresis and Mass spectrometry, and immunoassay (In vitro relative potency) to catch the complexity of protein-Alum interaction in the final product.

1.4 *Recombinant protein*

Recombinant proteins: NadA; fHbp and NHBA were selected as antigens in the adjuvant system model studied in this work. The choice was dictated by the great mole of literature in which their functionality and immunological role are well characterized. Furthermore, different studies highlighted their role as virulence factors in meningococcal pathogenesis.[36, 37] These antigens were discovered through reverse vaccinology applied to develop a MenB vaccine from the genome of the MenB MC58 strain. They were selected from over 2000 predicted proteins revealed through the shotgun strategy due to their ability to induce a high degree of protection. The ability to induce a broad protection was assessed by the SBA (serum bactericidal assay) (i.e. against a diverse collection of strains) thanks to their ability to confer passive protection in animal models due to specific antigen antibody. Finally, the three antigens that met these criteria were identified and called: Neisseria genome-derived antigens (GNA) 2132 (Neisseria heparin-binding antigen, or NHBA), GNA1870 (Neisseria factor H-binding protein, or fHbp) and GNA1994 (Neisseria adhesin A, or NadA).[6, 38]

Factor H-binding protein (fHbp or GNA1870) was identified as a surface-exposed lipoprotein that evades the human complement system by binding to human factor H, an inhibitor of the alternative complement pathway.[36, 39] Nuclear magnetic resonance (NMR) and X-ray crystallography resolved the three-dimensional (3D) structure of fHbp. The final structure obtained by the two techniques reveals that the protein is composed of two domains: an N-terminal domain of 8 beta-strands forming a highly curved anti-parallel beta-sheet (approaching a beta-barrel) and a C-terminal domain that is a well-defined beta-barrel of 8 anti-parallel beta-strands[40, 41]. Minimal orientation flexibility exists between the two domains, due to a short linker that connects them together with several hydrophobic contacts between them. Several epitope mapping studies have been performed to identify the immunogenic region of fHbp, using NMR spectroscopy, scanning of the full-length protein via synthetic linear peptides and other ELISA-based techniques. These studies revealed that the amino

acids that contribute to the immunogenicity of the different fHbp variants are located in non-overlapping areas and identified how the protein binds Factor H binding sites in domains 6 and 7 of human factor H. [42, 43]

NHBA (Neisserial Heparin Binding Antigen, or GNA2132) is a surface-exposed lipoprotein that binds to heparin in vitro via an arginine-rich region[36, 44]. The interactions between heparin and the complement system are complex and involve several component proteins, including complement inhibitors such as factor H, C4b-binding protein and vitronectin.[6, 45] Complement inactivation occurs through the recruitment of the complement inhibitor by the NHBA-heparin complex on the meningococcal cell surface. The structure of NHBA consists of a primary amino acid sequence of approximately 470 residues. The N-terminal region (residues 1 to ~230) has been identified as an intrinsically unfolded region, whereas the carboxy-terminal region appears in a highly conserved level and consists of a single 8-stranded anti-parallel beta-barrel structure. Due to the lack of order in the N-terminal region, the structure's activities were concentrated on the C-terminal region, which was defined by NMR.[46] The structural model is shown in fig4.

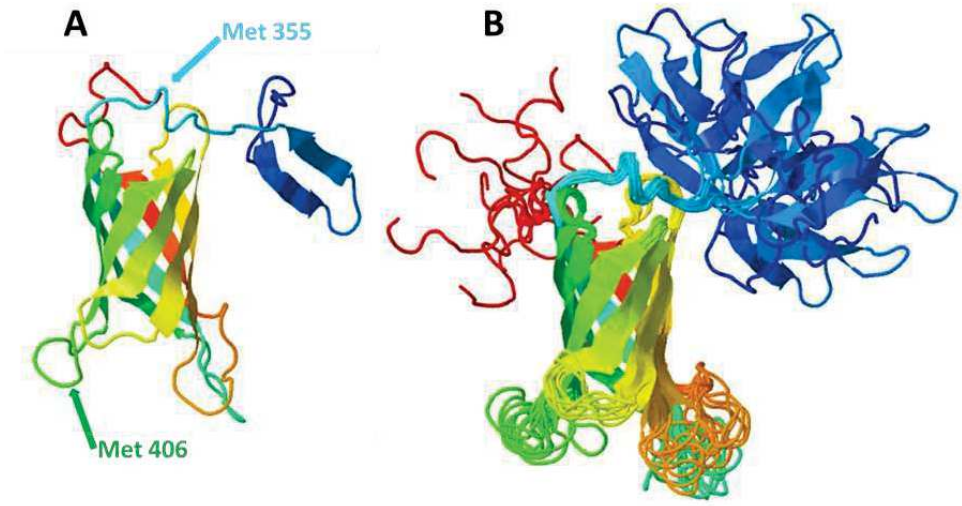


Figure 4: *Jmol cartoon representation of NHBA C-Term construct from Isoleucine residue 320 to final 470 from PDB 2lfu [46]. A) NMR structure Model 1; B) 10 conformer models superimposition. Met residues 355 and 406 spotted by colored arrows.*

The two methionine residues highlighted show that the Met335 residue is located in the low-structure linker portion connecting the β-barrel domains shown in the NMR structure, while Met 406 is located in a more rigid loop contained in the 8-strand structure (β1, 359-371; β2, 380-388; β3, 393-398; β4, 410-415; β5, 419-424; β6, 431-436; β7, 443-448; β8, 462-468) described by the NMR results[47].

NadA (Neisseria adhesin A or GNA1994), is a protein of the 'OCA' (oligomeric coiled-coil adhesin) family, which have a unique mechanism of secretion by an extracellular 'passenger' domain and subsequent trimerization on the bacterial surface.[6, 48] Thanks to the putative protein receptor, NadA trimers can enter the epithelial cell. The structure of the protein shows a folded 'head' domain at the N-terminus, which seems crucial for cell-binding activity in vitro. The 3D structure of the protein has not yet been determined, but knowledge about OCA provides some details on its organization and function. The N-terminal leader peptide of 23 residues, is followed by a ‘head’ domain of around 70 residues. The head domains followed by a predicted homotrimeric coiled-coil region formed by a of 200–250 residues. Nevertheless, NadA can be produced in a soluble and stable form, the large size of the trimeric, mature ectodomain of NadA makes it a challenging target for NMR spectroscopy. Instead, the long coiled-coil region appearance too flexible, which could be explain why a crystallographic structure of NadA has not yet been determine[6, 49]

1.5 *Aims of the project*

In this thesis, a set of analytical methods to study the two major components of adjuvanted vaccines were developed and investigated. Recombinant protein Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp) were selected as antigens, and aluminium hydroxide (Alum) was used as an adjuvant (fig5)

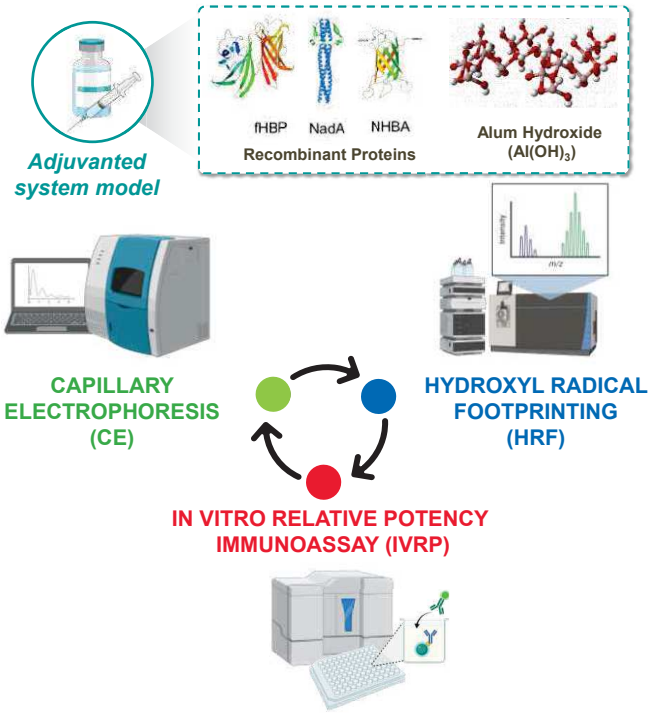


***Figure 5**: Project grafical abstract.*

This project was focused on two main areas: 1) the characterization of adjuvanted vaccines without altering their physical-chemical proprieties; 2) the impact of aluminium on the antigen structure and/or response versus their immunological target. The structure of this thesis is split into two main chapters:

- *Method for direct quantification of antigens in adjuvanted vaccines*. One of the main analytical challenges in the field of vaccines is the determination of antigen purity and quantification in the final product of adjuvanted vaccines. Most current methods used to characterize adjuvanted vaccines require the desorption of the antigen from model aluminum-containing formulations; there

is therefore a need for tools that are able to directly quantify adsorbed antigens. For this reason, in the first part of this work, the development of a generic method which could be applied with minimal product specific adaptation, ensure physical-chemical integrity of antigens (Fig.6) was carried out. In particular, we used capillary electrophoresis (CE) to simultaneously analyze multiple antigens adsorbed on the aluminium adjuvant in the final container of adjuvanted vaccines.

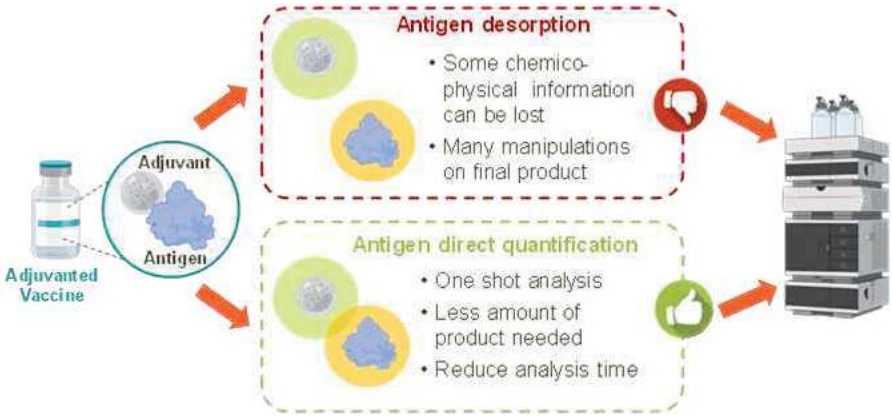


***Figure 6**:**Schematic representation of the analytical tool for antige-Alum formulates**: The most commonly used assay to analyze the quantity and purity of the adsorbed antigens, required the desorbing process. Desorbing process required several manipulations on the final product and could affect their chemical-physical proprieties. A direct quantification of adsorbed antigens could solve issues linked to desorbing and reduce the analysis time.*

The assay was developed for the simultaneous characterization of three recombinant protein: Neisseria adhesin A (NadA); Neisserial Heparin Binding Antigen (NHBA); and factor H binding protein (fHbp), formulated with aluminum hydroxide (Alum) as an adjuvant system.[6] Our results demonstrated how this technique is able to detect and quantify the antigenic components in a single run, but unfortunately the physical chemical characteristics of the adjuvanted components affected the robustness of the separation.

The impact of aluminium concentration on the antigen structure and resulting immunological response. The second part of this work was focused on two main analytical tools: in vitro relative potency (IVRP) and a mass spectrometry-based technique, with the aim of evaluating the antigen-aluminium interaction and the antigen structure in the presence of different aluminium concentrations.

1) An immuno-based technique to evaluate antigen response at different aluminium concentrations, focusing on in vitro relative potency (IVRP).
2) A mass spectrometry-based technique as a physical-chemical orthogonal assay to monitor epitope binding sites of the Alum formulate with recombinant proteins, hydroxyl radical foot printing (HRF).

Thanks to the capacity of these two assays to work directly on formulations without being affected by the presence of aluminium, they were used to study the influence of different aluminum concentrations on the antigen-aluminium interaction processes.

Chapter II.
METHODS FOR DIRECT QUANTIFICATION OF ANTIGENS IN ADJUVANTED VACCINE

2.1 Introduction: Analytical tools for adjuvanted vaccine

One of the major challenges in subunit vaccines research is the characterization of antigen-adjuvant interaction (association and de-association) to ensure optimal efficacy, safety and quality of final product. In comparison with the high mole of literature studies describing new antigens and/or adjuvants and their immunological profiles, only spared studies regarding physico-chemical characterization of antigen-adjuvant interaction are exiting. The reason lies in the high degree of complexity in the characterization of vaccine formulations and because the combination of low antigen doses and colloidal system poses a great analytical challenges [50]. Nevertheless, a deeply understanding of the physicochemical proprieties of the two components (Adjuvant-antigen) is an important step to monitor a critical parameter, such as the degree of antigen desorption and the colloidal stability that could have the impact on the efficacy, safety and the vaccine product shelf-life. The importance to combining physiochemical characterization with immunological studies to characterize antigen-adjuvant interaction, was first emphasizing by Stem Hem and co-workers, identifing guidelines critical for biological proprieties of adjuvant-antigen interaction, such as antigen association de-association and binding characteristics, that could be identified and monitored. To characterize Alum-antigen interaction, a panel of several analytical tools[50, 51], summarized in Table 1, is applied:

Interaction parameters	Techniques
Physical characteristic	Particle size and distribution (DLS) Zeta potential Sedimentation
Adsorption	Adsorption isotherms
Antigen integrity	DSC FTIR Intrinsic fluorescence CD
Binding assay:specific epitope integrity	ELISA Surface plasmon resonance Western blotting
Chemical stability	MS

***Table 1**: Analytical tools used to characterize Alum adjuvant-antigen interaction (adapted from M. Hamborg and C. Foged)*

However, these assays have several limitations: vaccine concentration is usually too low compared to the sensitivity of the method to monitor a structural integrity of antigen. Monoclonal antibodies, for binding assay, are difficult to produce and in some case extraction of the antigen from alum is needed. Nevertheless, is crucial to have an analytical tool or a set of analytical tools to monitor all these parameters ensuring both the antigen's and adjuvant's integrity. Furthermore, several studies have showed that the reactions such as oxidation and deamidation could disrupt specific epitopes which correlated with a reduced potency of the vaccine. This and other literature evidences highlight the importance to characterize the chemical stability upon adsorption[52] [53].

2.2 Capillary electrophoresis

In this perspective, capillary electrophoresis (CE) techniques have been exploited to improve and develop an analytical tool for the direct characterization of antigens in final vaccine formulations. Capillary electrophoresis is one of the most important separation techniques in analytical chemistry. The separation principle is based on the electrophoretic mobility that molecules present in a electric field, the separation occurs in narrow-bore capillaries, typically from 25- to 75-μm inner diameter (id), which are usually filled with buffer.[54] The pioneer of this technique was Tiselius in 1937, who first observed, placing a buffer solution with protein mixtures in a tube and applying an electric field, the ability of proteins to migrate in a direction and at a rate directly proportional to their charge and mobility. For his work in separation science Tiselius was awarded a Nobel Prize.[55] Due to low separation efficacy limited by thermal diffusion of the work of Tiselius had gone unnoticed until Hjerten introduced the use of capillaries in the 1960's. However, due to a spare availability of capillaries variety, his establishments were not widely recognized until Jorgenson and Lukacs published papers showing the ability of capillary electrophoresis to perform separations with 75-μm id fused silica capillaries. In addition, Jorgenson clarified the theory, described the relationships between operational parameters and separation quality, and demonstrated the potential of high performance capillary electrophoresis as an analytical technique.[54, 56] His innovation using a thin dimension of capillary, allowed to increase the surface to volume ratio, solving some common problems in traditional electrophoresis, as overheating by high voltage. The continuous studies and evolution of these techniques lead to obtain a constantly increased efficiency and the high separative capabilities spurred a growing interest among the scientific society in the analytical field[57]. Technical advancements, favoured by the advantages that CE offered over traditional separation techniques, as High-Performance Liquid Chromatography (HPLC) or gel electrophoresis, has allowed the CE to became a very suitable technique in the separation and characterization of biomolecules (proteins, peptides, DNA). Indeed, CE offers a different advantages respect the traditional liquid chromatography as:

- It uses a broad range of separative techniques: capillary zone electrophoresis (CZE); micellar electrokinetic chromatography (MEKC); capillary gel electrophoresis (CGE).

- High versatility using a several detectors such as: UV, Laser induced fluorescence (LIF) and various forms of mass spectrometry (MS)
- it requires small amounts of sample and consumes limited quantities of reagents.
- Easy to use, it is easily automated for precise quantitative analysis

- is applicable to a wide selection of analytes compared to

other analytical separation techniques.

- It can be used in different modalities using a single apparatus system

The CE system is relatively simple and comprises a high-voltage power supply, a capillary with online detection window and a autosampler (Fig 7). The separation take place trough a capillary made in bare-fused silica (BFS), with both sides dipped in vials containing the electrode and an electrolytic solution. When an electric voltage is appliedthe separation as result of different mass-to-charge ratios of each analyte in the background electrolyte (BGE) solution Occurs. In this condition the charge of analytes allows their migration toward electrodes with oppositecharge: (+) cations to the (-) cathode and (-) anions to anode (+). At the end of the capillary, near the cathode, there is a small window through which the analyte is revealed with an opportune detector.[54, 58]

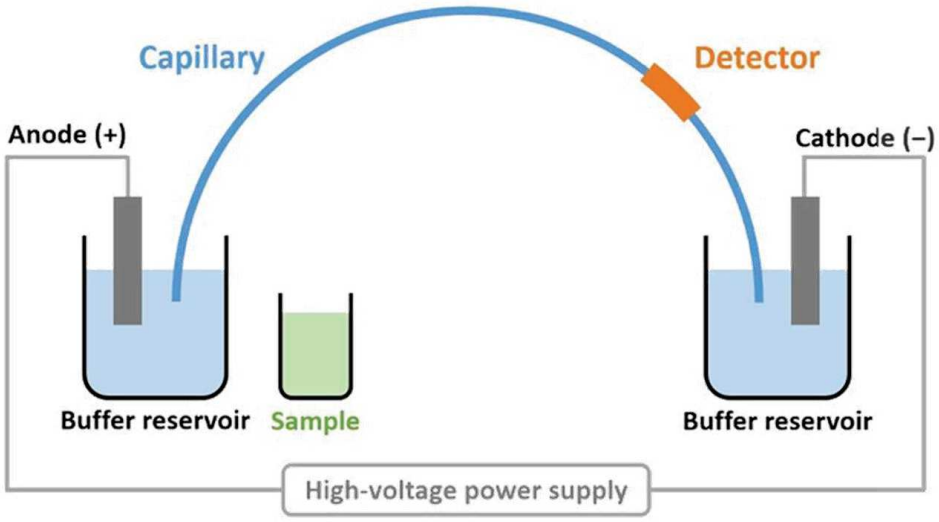


Figure 7: *schematic representation of a classical configuration of a CE system; adapted from Agilent book of Capillary.*

One of the most important parameters to consider in capillary electrophoresis is the mobility. The total (also named apparent) mobility of a sample molecule (μ_{TOT}) is the sum of the electroosmotic mobility and the electrophoretic mobility.

$$\boldsymbol{\mu_{TOT} = \mu_{EP} + \mu_{EOF}}$$

The magnitude of the electrical field $\boldsymbol{E}$, is directly proportional to the effective velocity ($\boldsymbol{v}$) of the ions, and can be determined by the equation 2:

$$\boldsymbol{v_{TOT} = (\mu_{EP} + \mu_{EOF})E}$$

Electrophoretic mobility to do the ions move under the influence of an applied voltage. The ion undergoes a force that is equal to the product of the net charge and the electric field strength and affected by a drag force that is equal to the product of, the translational friction coefficient, and the velocity. All this force can be reassumed in equation 3:

$$\boldsymbol{\mu_{EP} = \frac{q}{f} = \frac{q}{6\pi\eta r}}$$

where $\boldsymbol{f}$ for a spherical particle is given by the Stokes' law; $\boldsymbol{\eta}$ is the viscosity of the solution, and $\boldsymbol{q}$ and $\boldsymbol{r}$ are the ion charge and the ion radius respectively.

These relationships evidence how at high voltages the migration of the ionic species is acelereted. Moreover it isn evidenced that small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities.

A fundamental constituent of CE operation is electroosmotic, or electroendoosmotic flow (EOF). EOF is an effect caused by the ionization of the inner capillary surface and a the voltage applied to an electrolyte-filled capillary. When a BFS capillary is filled whit a electrolyte with a pH greater than 3, their silanol groups SiOH lose a proton to become SIO^- ions. In this way their surface will be negatively charged and will attract cations forming a characteristic diffuse double layer. Counterions, maintain charge balance and create a potential difference very close to the wall. This is known as the zeta potential. The voltage application across the capillary attracts the cations forming the diffuse double-layer toward the cathode creating a powerful bulk flow to form the

electroosmotic flow (EOF). The rate of the electroosmotic flow is governed by the following equation:

$$\mu_{EOF} = \frac{\epsilon\zeta}{4\pi\eta}$$

where ε is the dielectric constant of the solution, η is the viscosity of the solution, and ζ is the zeta potential[54]. Negatively charged particles are naturally attracted to the positively charged anode, but thanks to the EOF all species, regardless of charge, move in the same direction and can be separated. The zeta potential is essentially determined by the surface charge on the capillary wall and depends on pH and ionic strenght of the electrolyte, consequently the magnitude of EOF is depending on the same factors. At high pH, the EOF increases, while at high inic strenghts, EOF decreases. A large zeta potential between the cation layers, a large diffuse layer of cations to drag more molecules towards the cathode, low resistance from the surrounding solution, and buffer with pH value that all the SiOH groups are ionized, are the best condition for EOF (Fig8).

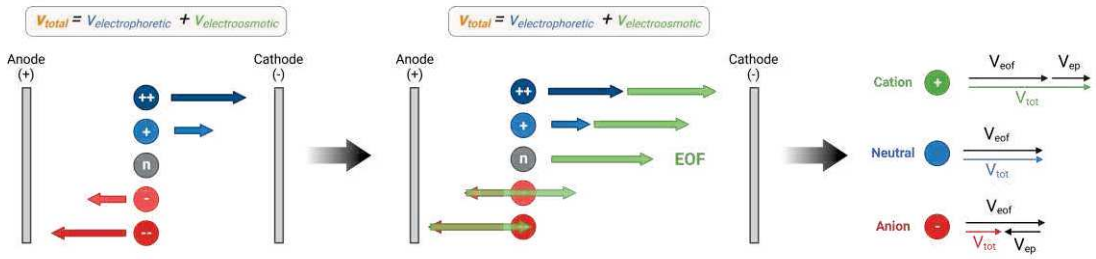


***Figure 8**: Schematic representations of the influence to EOF on the velocity of the ions. As illustrated at the left of the figure, cations and anions are separate based on their electrophoretic mobilities, which are related to their charge-to-size ratios. Neutral molecules do not have electrophoretic mobility and move with EOF.*

Furthermore, the flat flow profile of electroosmotic flow, gives major benefit, eluting component as narrow bands giving sharper peaks respect the pumped or laminar flow profile using in HPLC or turbulent flow of GC (fig 9)[58]. In HPLC the pumped flow produces broader peaks with lower efficiencies as the solutes in the center of the column move significantly faster than those at the walls. This is possible, because in CE the

smaller frictional drag near the capillary wall does not influence the peaks profiles giving higher efficiencies with less zone spreading respect the HPLC in which the solutes in the center of column move faster than those at the wall under the pumped flow, resulted in a broader peaks and lower efficiencies. Indeed, it is important, however, to maintain a constant EOF to avoid variable migration times and errors in peak identification and quantitation.[58]

Working in CE, a right compromise must therefore be reached between the EOF and separation time: a faster EOF results in shorter migration times and sharper narrower peaks;however, a short separation time could be causing a worst resolution of the solutes of interest.

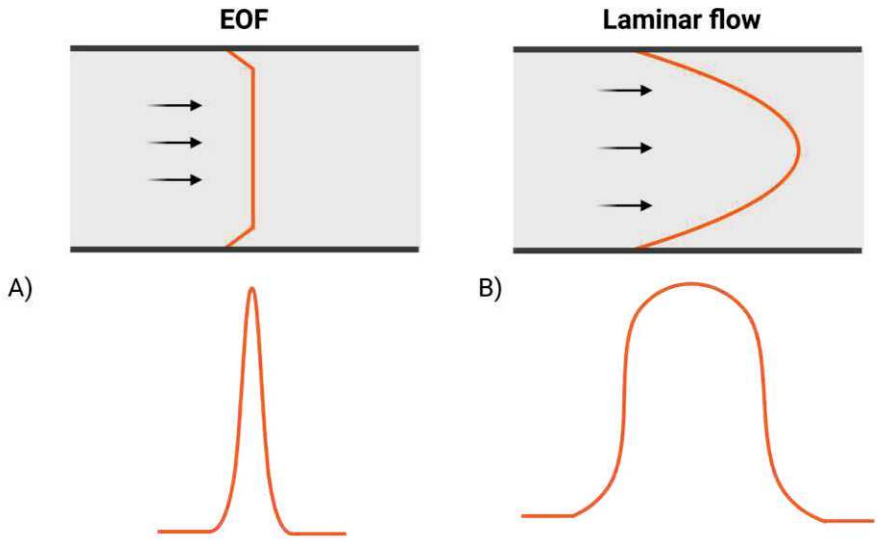


Figure 9*: Schematic representation between the laminar flow (B) using in HPLC system and EOF in CE (A)*

The electrophoresis modes can be classified into two main group: Continuous and discontinuous. A continuous system a background electrolyte acting throughout the capillary as a buffer. This can be subdivided in another two sub-groups: Kinetic in which electrolyte composition is constant, and steady-state, in which electrolyte composition varying.

A discontinuous system keep sample in distinct zones separated by two different electrolytes. Some CE modes with their classification are reported in fig10: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar

electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP).[59, 60]

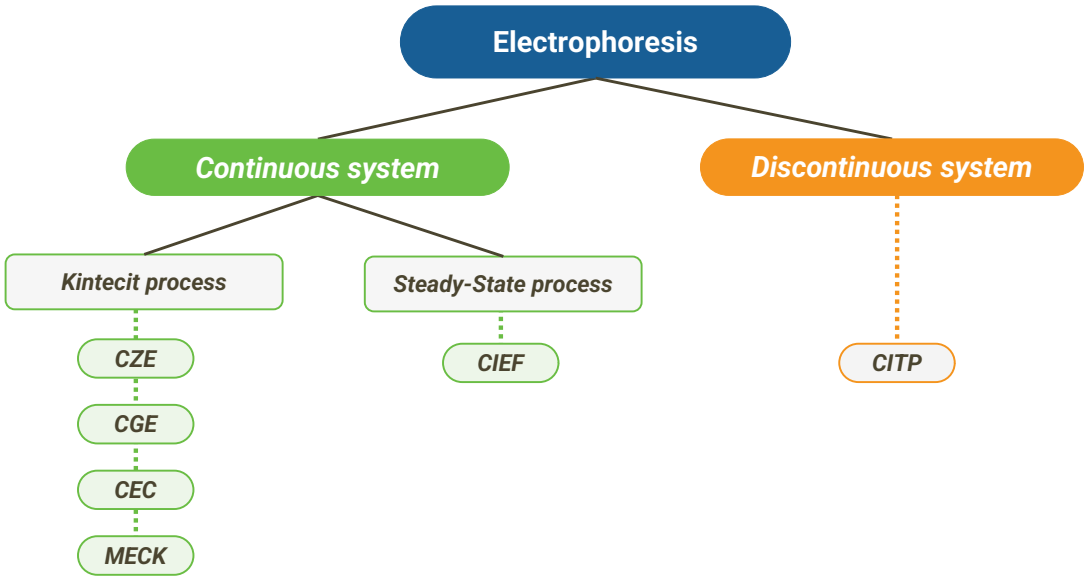


***Figure 10**: Diagrams of CE modes.*

CZE is one of the most commonly CE mode used, due to its simplicity of operation and its versatility. Its application range is very broad, extending from the analysis of amino acids, peptides, ions, to a wide range of enantiomers, and numerous other ionic species. In the field of proteins, CZE is applied for both for content and quality attributes (i.e. purity, screening of protein variants, and conformational studies). The characteristic of this techniqueis that the capillary is only filled with a buffer, and separation occurs thanks to the charge and size of analyte at different velocities. Separation of both anionic and cationic solutes is possible by CZE due to electro-osmotic flow (EOF). Typically, small highly positively charged, migrate toward the cathod prior to neutral that don't migrated and coelute with the EOF, followed by the negatively charged molecules that overcome the anodic attraction thanks to the EOF [60]

The second widely used CE mode is Micellar electrokinetic chromatography (MEKC or MECC). This technique was introduced by Terabe in 1984; MEKC is a combination of electrophoretic and chromatographic principles. This major benefit in the using a MECK technique is that it is able to separate neutral molecules as well as charged ones. The strength of separation is based upon solutes partition between micelles (acting as a

pseudo stationary phase) and the solvent of the running buffer [58]. Micelles form by surfactant when is added to a solution above the critical micelle concentration. The most commonly used surfactant is Sodium dodecyl sulphate (SDS), needs 8-9mM to reach the critical micelle concentration and forms micelles. Micelles structure has basically spherical with the hydrophobic component of the surfactant on the inside, oriented towards the center to avoid interaction with the hydrophilic buffer and the charged heads oriented toward the buffer. In this condition, hydrophilic molecules migrated faster through the solvent, compared with the hydrophobic molecules that, retained in the micelle, slow down their migration. [54, 60].

However, in both techniques some factors can be changed to improve the quality of separation and peak shape such as, for instance, the concentration and pH of used buffers. Several studies evidenced how the surfactant growing concentrations (example SDS) in MEKC, increase the analytes mobility in the capillary, improving peaks resolution and migration times on the electropherogram [29–31]. Otherwise, increasing the concentration of the buffer salt results in a higher ionic strength, which reduces the migration time of the analyte. Furthermore, the excessive heat derving from the higher current in the capillary, reduces the EOF, resulting in broadening peaks with less resolution due to the Joule heating effect. [61]. Another important parameter that impact shape and peaks distribution is the pH: in MEKC, a more basic pH reduces the run time and separation efficiency because of an increased EOF[62, 63]. A commonly method applied to increase resolution, is to add an organic solvent in the separation buffer, as it reduces the EOF.

2.3 Capillary technologies to direct quantify antigens in adjuvanted vaccine

In this work, capillary electrophoresis (CE) was exploited as innovative way to the simultaneous characterization adjuvant system model containing the three recombinant proteins Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp) as antigen and aluminum hydroxide (Alum) as adjuvant system. This adjuvant model was prepared on the literature based formulated vaccines (Table 2)[47]. CE approach was explored for the analysis of quantity and purity of antigenic components in Alum-based formulations avoiding physical separation procedures from the adjuvant. The purpose was to

develop a generic analytical method able to fully characterize vaccine antigenic components in a single analysis and without desorbing of antigens from the Alum..

Antigens	Amount per 0.5mL dose	Isoelectric point
fHbp	50 µg	5.1
NadA	50 µg	4.5
NHBA-GNA1030	50 µg	9.1
OMV	25 µg	~ 4.2*
Aluminium hydroxide**	1.5 mg	~ 9.1
Water for injection	up to 0.5 mL	
pH formulate: as for specification formulated vaccine pH 6.5±0.5		
*OMV pI on the basis of zeta potential measurements: ~4.2. Stable 6<pH<10.5; aggrgation pH<5		
** Aluminium hydroxide (Marburg); aggregate size 1-10µm		

Adapted form Esposito S at al. , Hum Vaccin Immunother. 2014

Table 2 composition example of a licensed vaccine formulated with Alum

The surface charges of Alum and colloidal particles, allow the application of CE for their characterization thanks to its ability to separate a mixture on nanoparticles (NPs) of different sizes. Additionally, different studies showed the possibility to apply the capillary technologies to quantify antigen with or without desorbing process from adjuvant.[64, 65].

Based on this evidence, a previous work in our laboratory was performed to establish the feasibility of this analytical tool for the quantification of both free and adsorbed antigens in one shot analysis. To achieve this goal, first attempts were performed using a MEKC method with SDS as surfactant in the buffer and trying to evaluate the behavior of antigen in the presence of Alum Hydroxide.

This study was performed using NadA protein as a case study. In detail, to understand the NadA adsorption behavior, the amount of antigen was kept constant at 0.3mg/ml, while the concentration of Alum was increased from 0.3 to 3.0 mg/mL to reach the final concentration in the vaccines. Experiments were performed with bare silica capillary, total length 30.2cm, effective 20cm, id.75um. Background electrolyte (BGE): borate 50mM pH 10.0 + SDS 12.5 mM. Instrument method was set using the following conditions: wash 10 psi 3 min with water, MeOH 30%, NaOH 0.1N, 10 psi 2 min with BGE. Autosampler at 25°C. Injection by pressure 0.5 psi 5.0 sec. Separation was performed at + 7kV.

Preliminary results showed that it was possible to monitor proteins both as free and adsorbed antigens. However, as shown in the figure below (Fig. 10), some issues were encountered at high concentrations of Alum. Indeed, collected data, showed the feasibility of this technique to identify two different specific peaks related to free antigen and adsorbed antigen up to the concentration of 1.0 mg/ml of Alum.

Unfortunately exceeded this concentration, Alum with adsorbed protein appears as a spike-like peaks causing a not quantifiable signal. This phenomenon was evaluated on the correct area of the two peaks, indeed, as reported in the graph below (fig 11 b), the decreasing area of free antigen is directly proportional to the Alum increase, exceptedt for the last concentration (3.0 mg/ml).

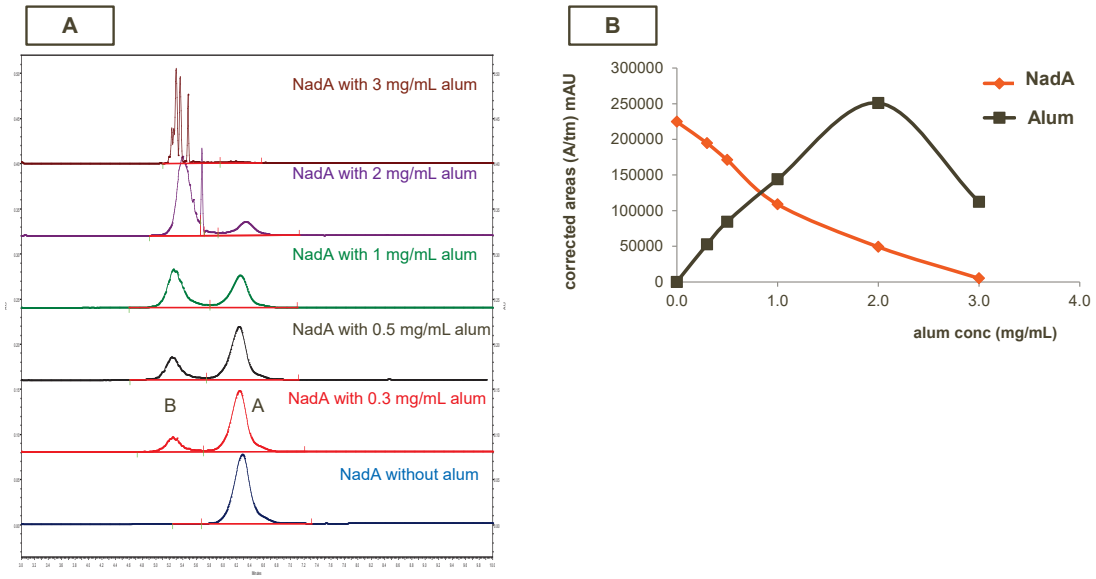


***Figure 11: Behavior adsoprtion of NadA**: keeping constant at 0.3mg/ml antigen amount and increasing Alum concentration up to 3 mg/mL. Peak A:Free antigen; Peak B: adsorbed antigen on Alum. Analytical method was performed with-bare silica capillary, total length 30.2cm, effective 20cm, id.75um. BGE: borate 50mM pH10.0 + SDS 12.5 mM. Wash 10 psi 3 min with water, MeOH 30%, NaOH 0.1N, 10 psi 2 min with BGE. Autosampler at 25°C. Injection by pressure 0.5 psi 5.0 sec. Separation 7kV.*

Moreover, the Alum aggregation that led to sedimentation in autosampler vials, gave not reproducible experiments and reliable data.

As reported in literature, to overcome a spike-like peaks issue, is crucial to reduce the interaction with capillary wall and analytes [66]. For this purpose, neutral capillary (linear polyacrilamide , LPA coated), was used because of to its peculiar characteristic to suppress the EOF and the interaction between the surface and the particles mainly according to steric repulsion forces.

Furthermore, others important parameters to considered are, concentration, and pH of the BGE. Therefore, new experiments have been set considering the following recommendations [49]:

- Avoid phosphate, borate, citrate, carbonate, and succinate: due to their ability to replace the hydroxyl groups of Alum with phosphate group, and changing Alum Hydroxide morphology and superficial net charge.
- Use inert buffers with an optimal range of pH for a stable Alum-protein interaction as:
  - ✓ TRIS, Histidine and MOPS buffers
  - ✓ Acetate 3.8- 4.8 optimal pH range; wavelength 200nm

- ✓ TRIS 7.3-9.3 optimal pH range; wavelength 220nm.

Keeping in mind the above literature evidence, we developed an analytical tool capable to direct quantify adjuvanted vaccines components. In particular, as said before, the three recombinant proteins (NadA; NHBA;fHbp) were selected as antigen while Alum was chosen as adjuvant system.

2.4 Materials used in the Capillary Electrophoretic experiments

Buffers were prepared using the following materials: $C_4H_{11}NO_3$ Tris (hydroxymethyl) aminomethane, Sodiumdodecylsulfate SDS, Methanol and Histidine purchased from Sigma Aldrich.

Hydrochloric acid fuming 37% HCl was purchased from Merck; H_2O_2 was purchased from GE Healthcare. Sodium Hydroxide, 50%(w/w) NaOH was purchased from J.T baker.

All the reagents were stored in accordance to manufacturer recommendation and used without further purification.

2.4.1 BGE solutions

- To prepare Tris Acetate buffers a proper weight of Tris(hydroxymethyl)aminomethane was dissolved in ultrapurified water to reach the final desiderated molarity. A proper volume of Glacial acetic acid was added to obtain the desired pH.
- To prepare Tris Acetate SDS buffers a proper weight of Tris(hydroxymethyl)aminomethane and SDS was dissolved in ultrapurified water to reach the final desiderated molarity. A proper volume of Glacial acetic acid was added to obtain the desired pH.
- Tris HCl buffer were prepared from a solution of Tris(hydroxymethyl)aminomethane inultrapurified water adding a proper volume of HCl to reach a desiderated pH
- To prepare Tris Acetate CTAB buffers solution a proper weight of Tris(hydroxymethyl)aminomethane and CTAB were dissolved in ultrapure water to reach the desiderated molarity. A proper volume of Glacial acetic acid was adding to obtain the desiredpH.

All the reagents were stored in accordance with manufacturer recommendation and used without further purification.

2.4.2 Antigens and Adjuvants

Aluminum hydroxide (Alum) adjuvant was obtained from GSK Vaccines (Marburg, Germany). Vaccine recombinant proteins were obtained from GSK Vaccines (Siena, Italy). Factor H binding protein (fHbp) fused with GNA2091, Neisseria adhesin A (NadA), Neisseria Heparin-Binding antigen (NHBA) fused with GNA1030, were used for the study. Isoelectric points (pI) were theoretically calculated as 4.6, 5.1, and 9.0, respectively.

2.4.3 Preparation of antigen formulation without Alum

The antigens formulation solution was a mixture of the three recombinant protein NHBA-NUbp; FHbp- GNA2091, NadA.

The solutions were formulated freshly by diluting the proper volume of the NHBA-NUbp; FHbp- GNA2091, NadA, drugs substances bulk (stored in aliquots at -20°C) to reach the final composition: 0.1 mg/ml of each in ultrapurified water.

While, the single antigens solution without Alum, was freshly formulated by diluting the proper volume of the each antigens NHBA-NUbp; FHbp- GNA2091, NadA, drugs substances bulk (stored in aliquots at -20°C) to achieved final composition: 0.3 mg/ml of each in ultrapurified water.

2.4.4 Preparation of Alum samples

The Alum samples were prepared by diluting of proper volume of Alum Hydroxide bulk to reach the desiderate concentration of 3.0 mg/ml.

2.4.5 Preparation of antigen formulation with Alum

The single antigen formulations with Alum, were formulated used the same protocol for each antigen:

- For the freshly formulation of selected antigen (NHBA-NUbp; FHbp-GNA2091, NadA), diluted with a proper volume, drugs substances bulk (stored in aliquots at -20°C) to reach the fixed antigen concentration of 0.3 mg/ml, was added to a proper volume of Alum stock solution to reach different Alum concentrations (0.3-0.5-1-2-3 mg/ml) in ultrapurified water. The solutions were stirred for minimum of 2 hour, to allow the protein adsorption on Alum.

2.5 Capillary electrophoresis instrument and separation methods

Experiments were performed with High-performance capillary electrophoresis system: PA800plus (AB Sciex). Two different capillaries were used during the experiments:

1. Uncoated fused silica column (SCIEX); inner diameter (ID): 50 μm; total capillary length: 70 cm (50 cm to detector).
2. Neutral capillary linear polyacrylamide (LPA) coated capillary (SCIEX.) with an inner diameter (ID): 50μm; total capillary length: 50cm (32 cm to detector)

Detection: PDA with wavelengths set at 200nm, 220 nm and 280 nm. Acquisition and data elaboration software: 32-Karat V10.1.

Before each run, neutral capillary was sequentially rinsed for 5 min with water at 50psi and 6 min with selected assay BGE. Samples were loaded into individual microvials autosampler. The samples were hydrodynamically injected in the capillary by a 60s at 0.5psi. Separation step was performed for 60 min by applying 12 kV constant voltage in reversed polarity plus 0.2psi.

Uncoated fused silica capillary, before each run was sequentially rinsed for 3 min with water, 3 min with MeOH 30% solution; 3 min NaOH 0.1M and 4 min in selected assay buffer, applying 10psi for each step. Samples were loaded into individual microvials autosampler and were hydrodynamically injected into the capillary by 0.15s at 0.5psi. Separation step was performed applying 15 kV constant voltage in normal polarity for 35 min.

Universal plastic vials for capillary electrophoresis and Universal rubber vials caps – blue were purchased from Sciex.

2.6 Results

2.6.1 Selection of the correct background electrolyte (BGE).

Based on the previous results obtained in our laboratory (see paragraph 2.3) and on the evidence reported in literature, we first focus our attention on the most critical parameter in CE: the selection of the most fit for purpose background electrolyte (BGE). Indeed, the selection of the proper background electrolyte (BGE) is key to a successful CE method.

As reported in paragraph (2.2) electrophoretic separation is based on migration of charged components in an electric field, so we first selected the BGE based on the properties of our analytes. In particular, to avoid interferences or desorbing effects during the run, we check the compatibility of BGE with the antigen-Alum interaction. Furthermore, to ensure that analytes are fully charged and able to migrate inside the capillary we selected the properly BGE pH on the basis of isoelectric point of analytes. Finally, different molarities of the running buffer were compared in order to obtain sharp peaks ensuring a precise and robust quantification[66]. In the Table 3 are summarized all the BGE conditions and capillary used to analyze each of the three recombinant proteins. The applicability of two different capillary techniques (CZE and MEKC) for the characterization of three different antigens was evaluate analyzing the three antigens separately but in the same condition. First of all, as reported in Table 3, the ability of the different buffer to detect a single antigen or Alum, was evaluated. In detail, to perform the experiment, each single antigen was diluted at 0.3mg/ml in ultrapure water, to mime a final condition, in which the total concentration of the three antigens is 0.3mg/ml.

Buffer	Molarity (mM)	pH	SDS mM	Mode	Capillary	Polarity	Separation comment
Tris Acetate	100	7.5		CZE	Neutral*	Reverse	*No*
	50			CZE		Reverse	*No*
	100		15	MEKC		Reverse	Only two antigens detected
	100		15	MECK		Normal	*No*
Tris Acetate	50	9.0	10	MECK	BFS**	Normal	*No*
	50	9.0	15	MECK		Reverse	Only one antigen detected with bad resolution
	50	9.5	15	MECK		Normal	*No*
	50	10	15	MECK		Normal	*No*
	50	**8.0**	**15**	MECK		**Normal**	**Detected all three antigens and Alum**
Tris HCl	100	7.5		CZE	Neutral*	Reverse	*No*

*Neutral capillary cannot be used at pH>8
* * Bare fused silica capillary

[1]Capillary zone electrophoresis (CZE)
[2]Micellar electrokinetic chromatography (MEKC)(withSDS)

Table 3*:*Summary of different BGEs and capillary: *To prepare Tris Acetate buffers a proper weight of Tris(hydroxymethyl)aminomethane was dissolved in ultrapure water to reach the desiderated molarity. A proper volume of Glacial acetic acid was added to obtain a fixed pH. Tris HCl buffer were prepared by a solution of Tris(hydroxymethyl)aminomethane in freshly water adding a proper volume of HCl to reach a desiderated pH. In the Table are reported the different buffers used for the analyses and a summary of the obtained separation results for the three recombinant protein.*

2.6.2 Method with Neutral capillary

The right compromise between proper BGE, capillary type and separation method, gave first encouraging results. In detail the single three antigens' solutions, prepared as reporter above, were analyzed performing a separation in a neutral capillary. Separation was performed using as BGE composition: 100mM TrisAcetate pH 7.5, SDS 15mM, and applying the separation conditions reported in the paragraph 2.4.1. The pH of BGE was properly selected based on analytes isoelectric point to assure that they were fully charged and able to migrate inside the capillary. The Isoelectric point of a protein is the pH at which the net charge of the protein is zero. Thus, different pH values can modify a protein net charge which became more positively or negatively charged due to the gain or loss, respectively, of protons (H^+)[67]. This mean that, proteins possess a net positive charge at a pH below their pI, whereas at above pI they have a net negative charge. Based on this evidence, thanks to the specific pI of analytes, reported in Table 4, at the selected pH of 7.5, NHBA and NadA possess a net negative charge while fHbp

possess a net positive charge.

Protein	pI
NHBA-NUbp	5.1
NadA	4.5
fHbp- GNA2091	9.1

***Table 4**:Summary of isoelectric points of three recombinant proteins used in this study.*

As reporter in the paragraph 2.2, the separation occurs thanks to the migration of charged analytes toward electrodes with opposite charge. CE instrument permit to change the polarity of electrode according to the needs. Therefore, considering the different charge of the three proteins, inverse polarity of CE electrode was applied (fig 12). In particular, the proteins separation occurred from cathode(-) to anode(+).With this type of coating in neutral capillaries, migration is mainly by electrophoretic flow as the EOF is minimized and not all species differently charged in a mixture are separated, on the other side the principal benefit provided by these capillaries is that they may dramatically reduce protein adsorption.

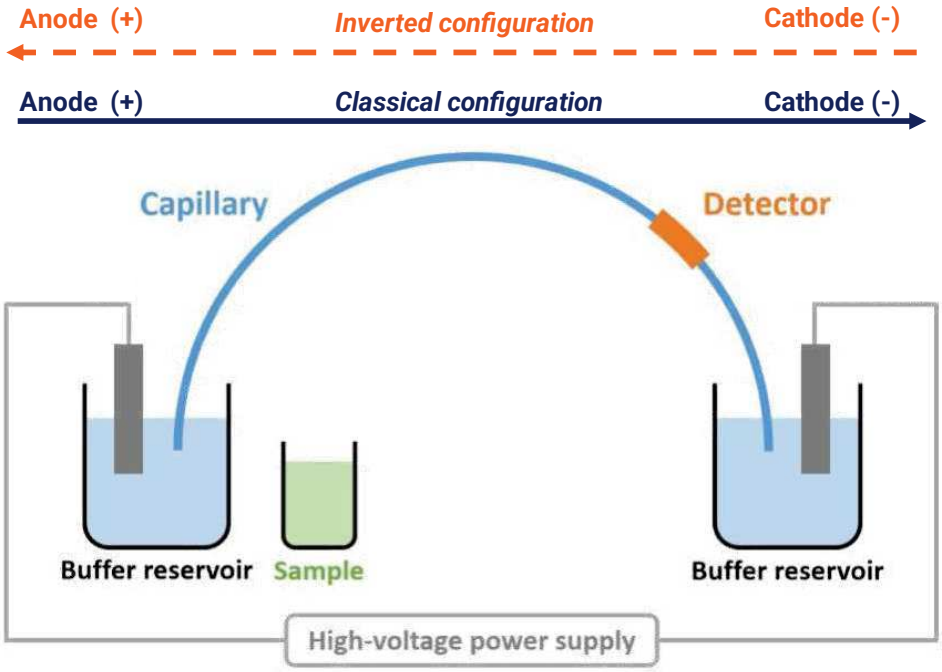


Figure 12:Schematic representation of capillary electrophoresis setting changing the polarity.:

This configuration, allowed to separate and detect NHBA and NadA, that at this pH possess a negative net charge, while fHbp was not detectable (fig 13). For this protein even the use of SDS was not enough to allow its migration. Probably, the positive net charge of fHbp, create an electrostatic force in the capillary that caused protein retention and the subsequent lack of migration over time.

Aonther attemp was done changing the polarity, but unfortunately fHbp remained undetectable.

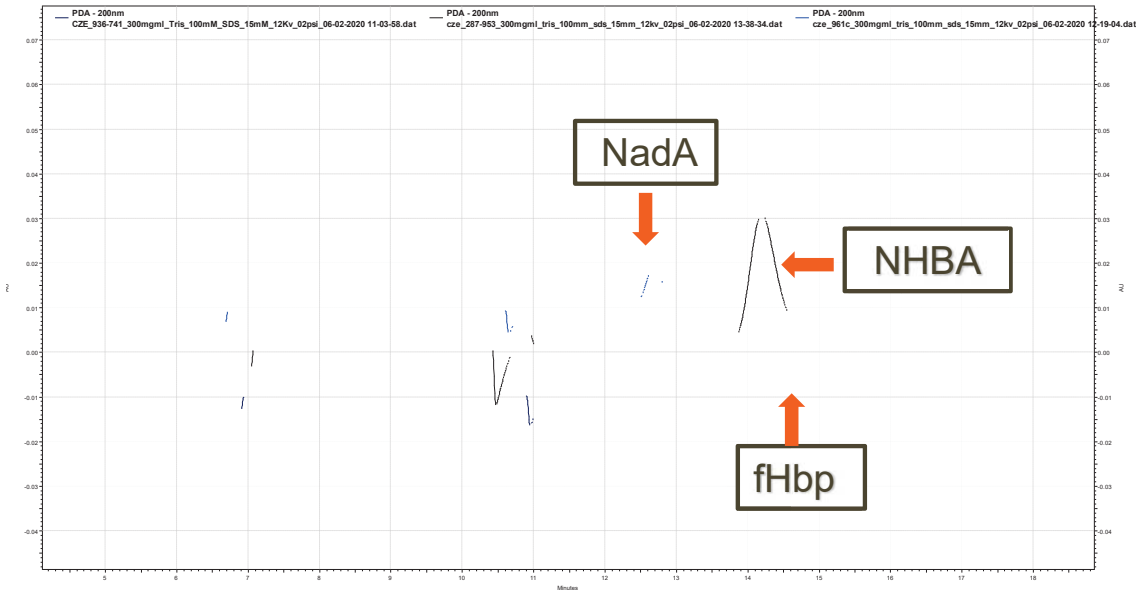


***Figure 13**: **Electropherogram of fHbp; NadA; NHBA, without Alum**. The three recombinant protein were diluted at 0.3mg/ml in freshly water. Analytical method was performed with neutral capillary, length tot. 50cm, id. 50um; BGE: Tris Acetate 100mM pH7.5 + SDS 15mM; Wash 50psi 5 min with water, 50 psi 6 min with BGE. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 60.0 sec. Separation 12.0kV (reverse polarity)+ 0.2psi.*

2.6.3 Adsorption kinetic

An intriguing aspect evaluated with CE was to understand the adsorption behavior of antigen on Alum. The NHBA antigen was selected as case study. To perform the experiments the amount of antigen was kept constant at **0.3 mg/mL** (as was the total amount of three proteins in the final formulates) while the Alum concentration was increased from **0.1 mg/mL to 0.5 mg/mL**. As shown in figure 14, at the increasing of alum concentration corresponds a decrease of NHBA peak area.

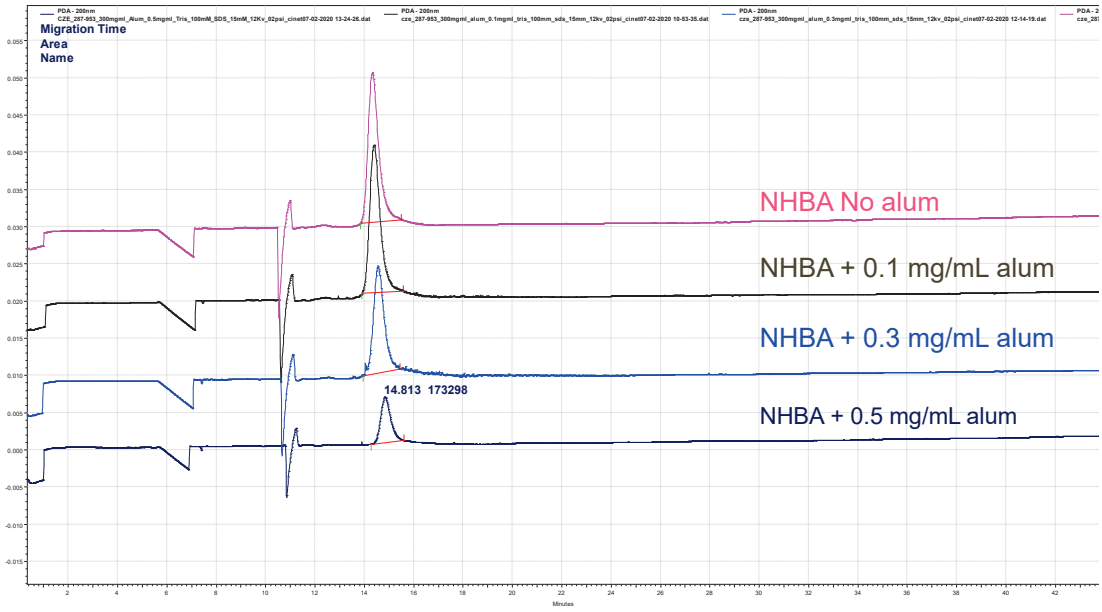


Figure 14: **Electropherogram of NHBA, with Alum at different concentrations:** Recombinant protein was diluted at 0.3mg/ml in ultrapure water and Alum hydroxide was added at a proper volume to achieve the desired final concentration of 0.1,0.3, 0.5 mg/ml . Analytical method was performed with neutral capillary, length tot. *50cm, id. 50um; BGE: Tris Acetate 100mM pH7.5 + SDS 15mM; Wash 50psi 5 min with water, 50 psi 6 min with BGE. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 60.0 sec. Separation 12.0kV (reverse polarity)+ 0.2psi*.

In order to assess whether the presence of Alum could have has an impact on the antigen, we have evaluated the change in peak area. The peak area is directly correlate to the amount of analyte so its decreasing or increasing represents, respectively, a decrease or increase of the analyte quantity. To obtain consistently data, we consider the correct area of the peak(rule generally applied in capillary electrophoresis measurements), that is calculated dividing the area of a peak by its migration time:

$$CA = (Area)/(Migration\ Time)$$

As shown in the graph reported in fig 15, at the increasing of Alum concentration corresponds a decrease of correct area of antigen. In this way it was possible to have a method able to determine the amount of antigen in solution that corresponds to not adsorbed antigen. Unfortunately, under these conditions the assay was not enough efficient to follow the complete adsorption process on Alum, as neither other species were detected a part the peak of not adsorbed antigen as shown in fig 14.

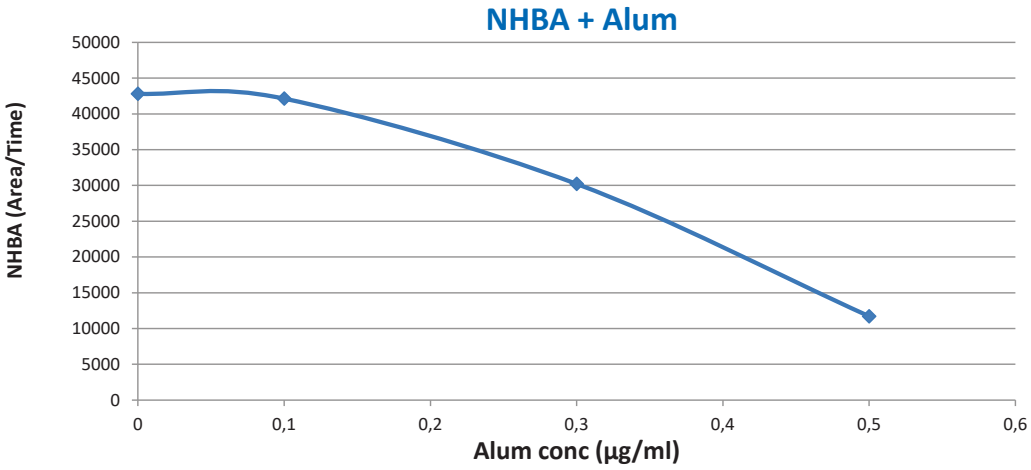


Figure 15: Correct Area of the NHBA in the presence of Alum at increasing concentrations: *The graph shows a decrease of corrected area of the protein, but it is not possible to follow the adsorption process on Alum. The correct area is a Area of a peak divided by its migration time.*

2.6.4 Method with Bare fuse silica capillary

To increase the sensitivity and efficiency of the assay, the second step was to investigate a different type of capillary for the study. As summarized in Table 3, the most promising results were obtained when bare fused silica capillary was used with Tris acetate buffer. Several types of BGE at different pH were screened and at the end the good compromise was found using Tris acetate at 50mM with 15mM SDS at pH 8.0. Under these conditions, the method was able to detect and separate both the antigen alone and the antigen adsorbed on the Alum.

The change in strategy was dictated by the need to have the possibility to detect and follow both the protein and Alum alone or in the complexes with each other. On this basis, we tried to verify whether, using these conditions, the assay had the ability to detect Alum.

Starting from a stock solution of the compound, a solution of 3.0 mg/ml was tested. As shown in figure 16, Alum appeared as a multi sharp peaks. As reported in literature, this effect may be due to the aggregation of the adjuvant caused by different Alum hydroxide populations formed as a consequence of poor polydispersity of the substrate [68]. Another major cause of these multi sharp peaks, could be the sedimentation of the Alum over time, which could cause a non-uniform withdrawal of the component in the microvial.

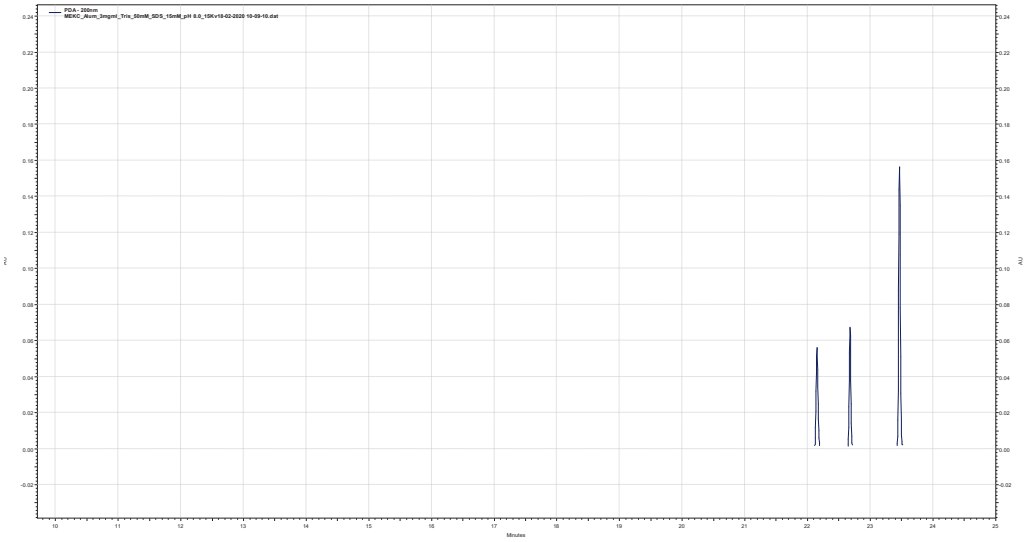


***Figure 16**:electropherogram of Alum at 3mg/ml in water. Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetate 50mM pH 8 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with MeOH 30%, 10 psi 4 min NaOH 0.1, 10.0 psi BGE.. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 5 sec. Separation 15.0kV 25minutes.*

Some suggestions to avoid the multiple peaks signal of Alum become from literature. The first action was the introduction of Hisitidne in the sample. Histidine is an excipient and one of the components of the vaccine formulation. It plays a crucial role both as a 'stabilizer' and as ionic strength modulator[69]. Indeed, it is mainly used to preserve the structure and integrity of recombinant proteins. Another important role is the ability of this excipiens to 'disperse' Alum, preventing its aggregation. Furthermore, as reported in literature, the exposure time of the antigen to the Alum was evaluated [70].

On this basis, a new protocol (fig *17)* was developed. In detail, a bulk of antigen was prepared and mixed with a bulk of Alum and Histidine at a final concentration of 107 mM, the solution was left to stir for 2 hours, then mixed and injected.

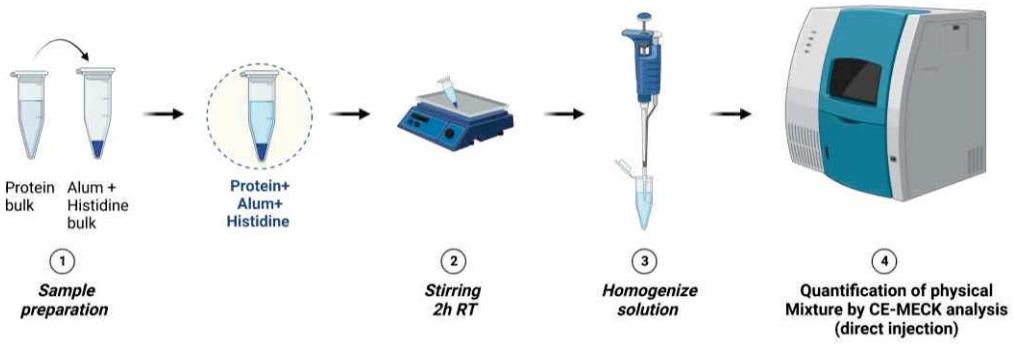


Figure 17:New protocol with Histidine. A bulk of antigen is prepared and mixed with a bulk of Alum and histidine at a final concentration of 107 mM, the solution is left to stir for 2 hours, then mixed and injected.

To avoid the sedimentation issue in micro vial, instrument method was changed as shown figure 18: first performing the separation as first step, reducing the run time from 35 minutes to 25 minutes, followed by the capillary reconditioning step, to leave the sample in the vial for as minor time as possible, avoiding sedimentation and allowinga uniform withdrawal.

Normal Method

Capillary rinsing

Sample	Value	Duration (min)
H_2O	10.0 psi	3.00
MeOH 30%	10.0 psi	3.00
NaOH 0.1	10.0 psi	4.00
Buffer	10.0 psi	4.00

Separation

Sample injection	0.5 psi	0.05
Separate voltage	15.0 KV	35.00

Modified Method

Separation

Sample	Value	Duration (min)
Buffer	10.0 psi	4.00
Sample injection	0.5 psi	0.05
Separate voltage	15.0 KV	25.00

Capillary rinsing

H_2O	10.0 psi	3.00
MeOH 30%	10.0 psi	3.00
NaOH 0.1	10.0 psi	4.00

Figure 18:Alternative method to avoid sedimentation issue in micro vial. *As compared to Classical method (a), the alternative method (b) involves first the separation step. The run time is reduced from 35 minutes to 25 minutes and is followed by a capillary reconditioning step.*

Appling these conditions, was possible to evaluate the antigen adsorption behavior at increasing Alum concentration. The experiments protocol followed was the same applied before. Single antigens were tested at 0.3mg/ml constant concentration while the Alum concentration was increased from 0.3mg/ml to 1.0 mg/ml. As reported in figure 17, each sample was prepared starting from protein bulk mixed with the proper volume of Alum bulk in which was introduced a fixed Histidine concentration to obtain the following samples: 0.3mg/ml of protein different Alum concentrations (0.3-1.0 mg/ml) and 107 mM of Histidine (as concentration in the final formulate) then the sample was stirred for 2h and injected for the analysis. The experiments were performed for each three antigens separately.

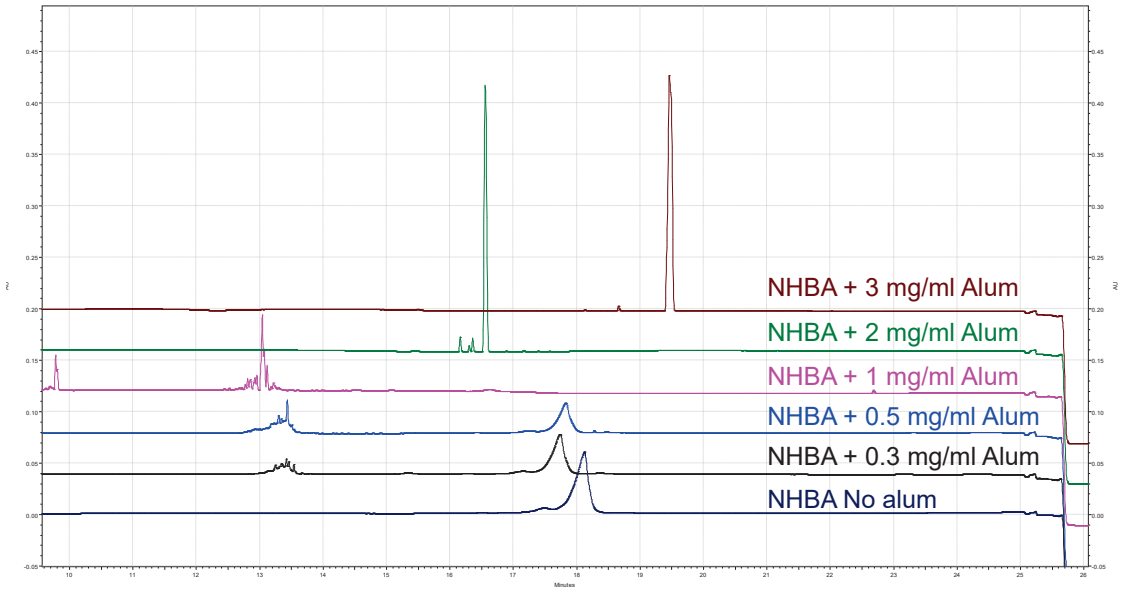


***Figure 19*: Electropherogram profiles of NHBA, with Alum at different concentrations:** Recombinant protein was diluted at 0.1mg/ml in ultrapure water and Alum hydroxide was added at proper volume to achieved the desiderate final concentration of 0.1,0.3, 0.5 mg/ml . Analytical method was performed with b*are fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetate 50mM pH 8 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with MeOH 30%, 10 psi 4 min NaOH 0.1, 10.0 psi BGE.. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 5 sec. Separation 15.0kV 25minute*

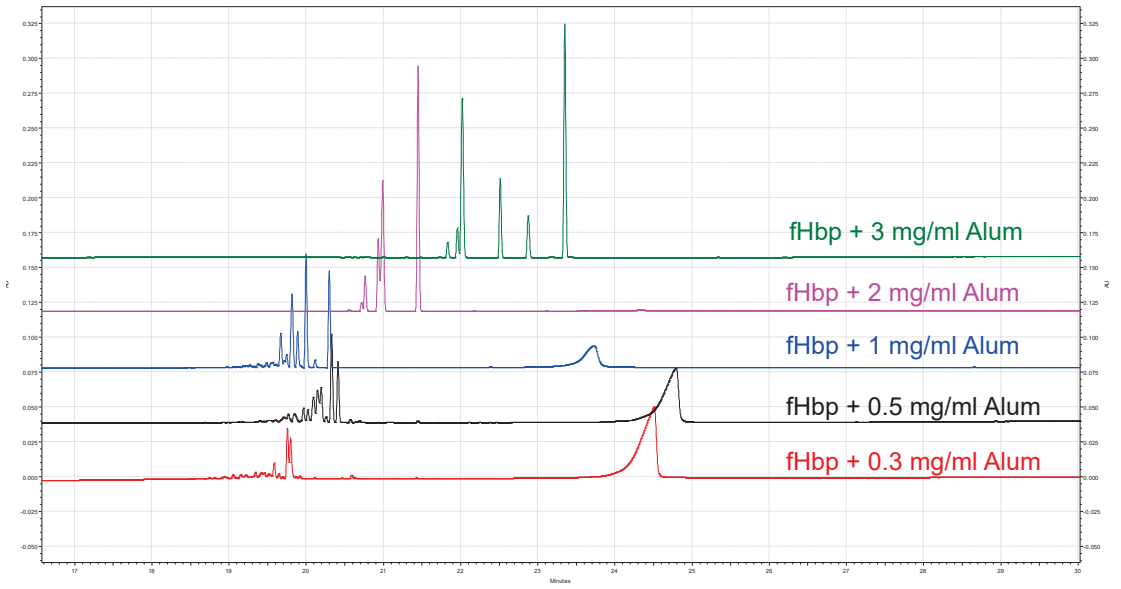


***Figure 20:* Electropherogram profiles fHbp, with Alum at different concentrations:** Recombinant protein was diluted at 0.1mg/ml in ultrapure water and Alum hydroxide was added at proper volume to achieved the desired final concentration of 0.1,0.3, 0.5 mg/ml . Analytical method was performed with b*are fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetateo 50mM pH 8 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with MeOH 30%, 10 psi 4 min NaOH 0.1, 10.0 psi BGE.. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 5 sec. Separation 15.0kV 25minute*

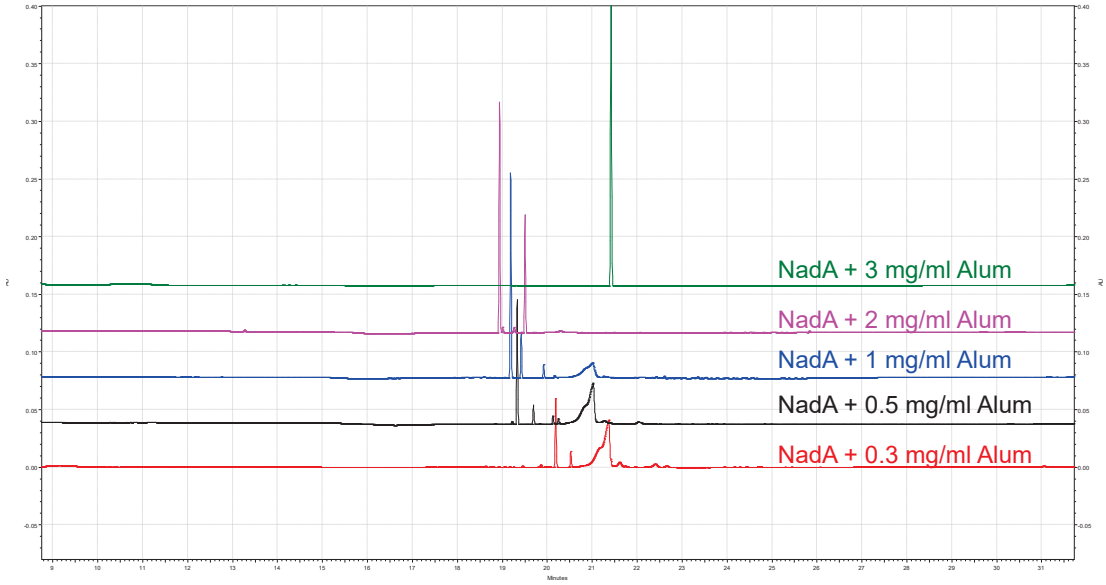


Figure 21: **Electropherogram profiles NadA, with Alum at different concentrations:** Recombinant protein wasere diluted at 0.1mg/ml in ultrapure water and Alum hydroxide was added at proper volume to achieved the desired final concentration of 0.1,0.3, 0.5 mg/ml . Analytical method was performed with b*are fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato 50mM pH 8 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with MeOH 30%, 10 psi 4 min NaOH 0.1, 10.0 psi BGE.. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 5 sec. Separation 15.0kV 25minute*

As reported in fig 19-21, in these conditions the assay was able not only to detect the three antigens but also to detect the different Alum species.

. Furthermore, also in this condition was observed a decrease of free antigens area at increasing of Alum concentration. Unfortunately, also in this case Alum appears as a multi sharp peaks, and in same case, like for fHbp antigen, at high concentrations spike peaks, caused difficult the differentiation of the Antigen-Alum compounds.

To better understand the correlation between the decrease antigens area, and the increasing of Alum concentration, the Trend of corrected areas was calculated considering the sum of single peaks derived from Alum compounds (reported in fig 22):

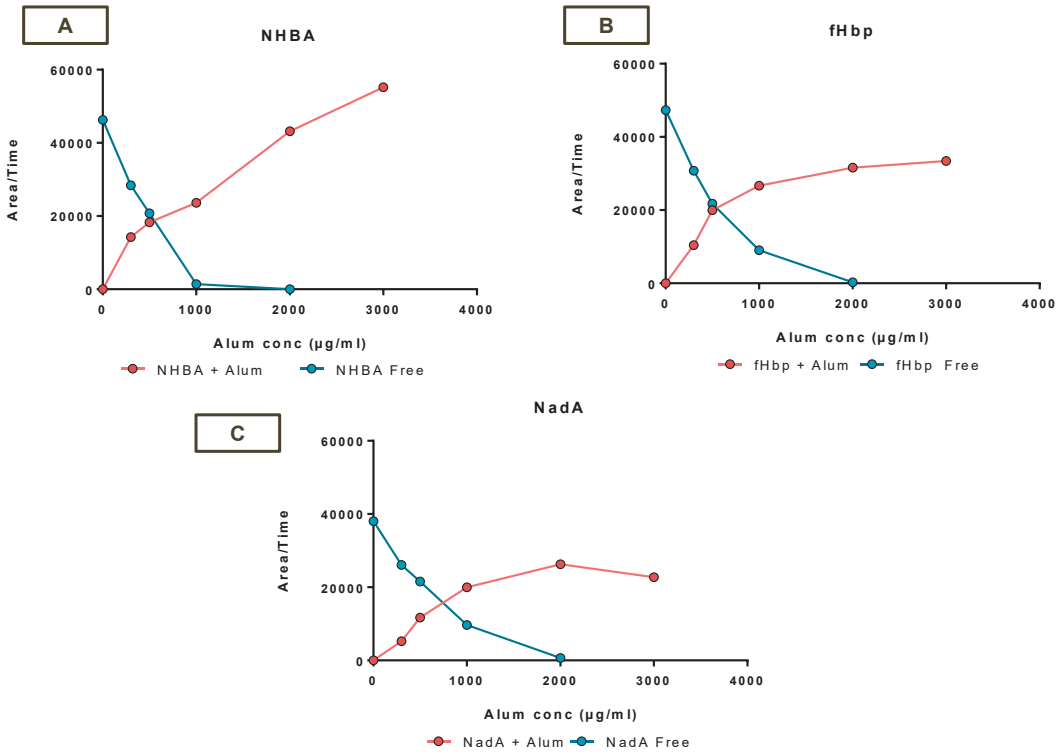


Figure 22: Trend of correct areas in function of Alum concentration: *Trend of correct areas of each antigen was calculated as sum considering each single observed peak*

Results suggested an effective adsorption of protein demonstrating by a decrease of antigen's area at increasing Alum concentration. Unfortunately, as graph shown, it was not achieved a linear correlation for areas of antigen-Alum compounds.This suggested that in this way Alum was not correctly separated. Furthermore, the presence of spike like signals, as in case of NadA antigen, indicated the possible wall interaction of Alum compound.

The incoming of this new issue, led us to evaluate the introduction of a stronger capillary wash step, displacing MeOH 30% with HCl 0.1M and increasing the washing run time to improve the separation (fig 23):

Capillary rinsing		
Sample	Value	Duration (min)
H_2O	10.0 psi	3.00
MeOH 30%	***10.0 psi***	***3.00***
NaOH 0.1	10.0 psi	4.00
H_2O	10.0 psi	3.00
Buffer	10.0 psi	4.00
Separation		
Sample injection	*0.5 psi*	*0.15*
Separate voltage	15.0 KV	40.00

Capillary rinsing		
Sample	Value	Duration (min)
H_2O	10.0 psi	1.00
HCl 0.1	***10.0 psi***	***3.00***
NaOH 0.1	10.0 psi	1.00
NaOH 0.1	10.0 psi	3.00
Buffer	10.0 psi	4.00
Separation		
Sample injection	*0.5 psi*	*0.15*
Separate voltage	15.0 KV	40.00

Figure 23: Changing wash step: *replacing of MeOH 30% with HCl 0.1M in the wash steps*

The new approach allowed the detection and separation of the three antigens mixed together (see paragraph 2.5) in one shot analysis. In fact, as shown in fig 24, the three antigens appear as a sharp single peak with good resolution and reproducibility. In the specific experiment a mixture of the three antigens at 0.1mg/ml each was tested.

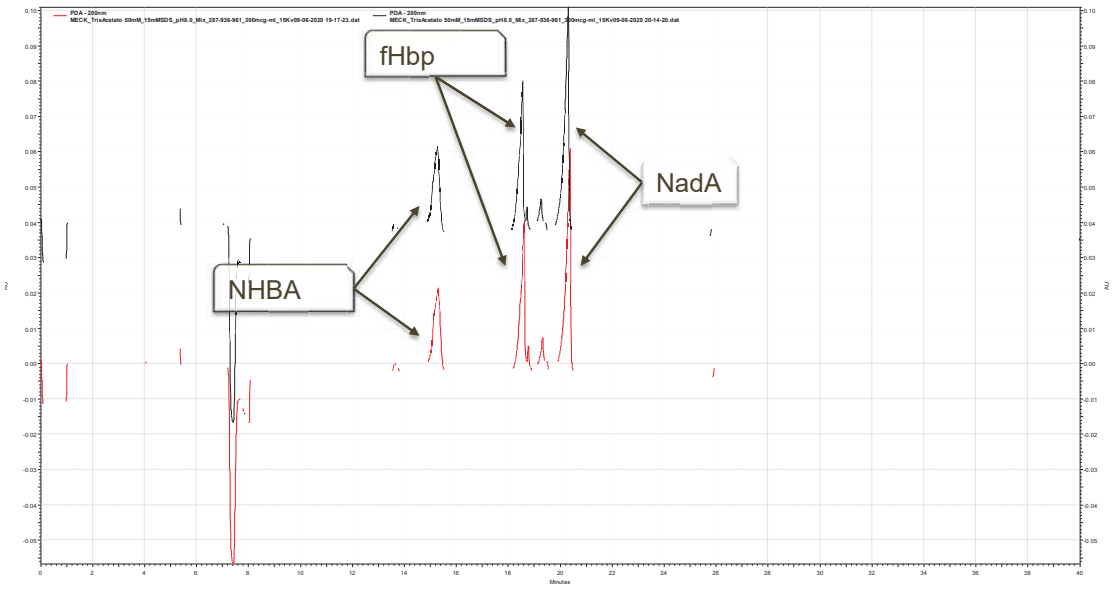


Figure 24: Electropherogram profiles of fHbp; NHBA; NadA: *Three proteins were mixed in ultrapure water to reach a final concentration of 0.1mg/ml each. Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetateo 50mM pH 8 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE.. Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute. Method shown good separation reproducibility and resolution between the three antigens mixed together.*

The next step was to evaluate the behavior of each antigen in the presence of Alum. To immediately assess the impact of the proposed new approach, it was analyzed each

individual antigen at a concentration of 0.3mg/ml with Alum 3mg/ml, following the protocol described above for sample preparation. To perform experiments, it was analyzed in the same analytical sessions, each antigen alone, and then the antigen mixed with Alum. To evaluate the repeatability antigen-Alum sample was injected twice. The results captured in fig 25, demonstrate the assay ability to detect both free and complexed antigens with Alum. As can be seen in detail in figure 25 (panel C), in the presence of Alum compounds don't have reproducibility. On this last point an experiment was carried out to assess the reproducibility of the compounds, mixing the three antigens together at a concentration of 0.1mg/ml each with Alum 3.0mg/ml including Histdine. (as per protocol in section 2.6).

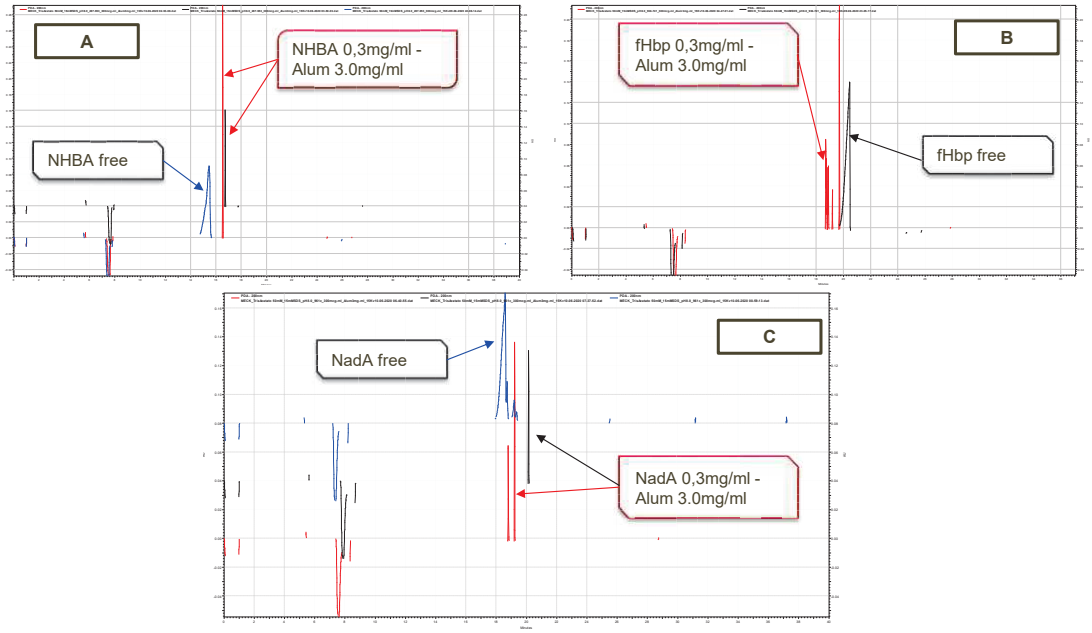


Figure 25: Electropherograms of NadA, fHbp, NHBA protein mixed with Alum*. Each protein was mixed with Alum stock solution to achieved final concentration of 0.3mg/ml for antigen and 3.0 mg/ml for Alum. Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato 50mM pH 8.0 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute A) The blue electropherogram is relative to the free NHBA, red and black are related to the two replicates of NHBA mixed with Alum; B) The black electropherogram is related to the free fHbp, red is related to the fHbp mixed with Alum; C)) Blue electropherogram related to the free NadA, red and black related to the two replicates of NHBA mixed with Alum;*

To evaluate the reproducibility, in the same analytical session the following samples were injected: a freshly mixed antigens-Alum sample twice (1° and 2° replicate in the fig 26). Let Alum sedimentation in the microvial and injected the sample (Sedimented samples in fig 26) and re-injected of *sedimented* sample applinged a 0.2psi pressure during separation steps to compact a multi sharp peaks in a single peak.

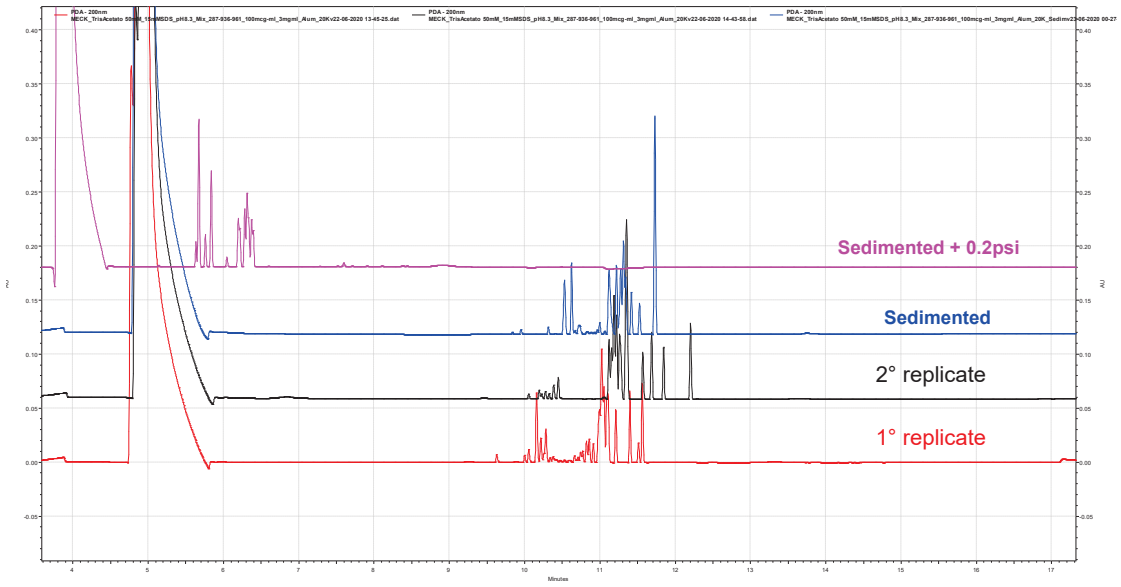


Figure 26: Electropherograms of NadA, fHbp, NHBA protein mixed with Alum*. Each protein was mixed with Alum stock solution to achieved final concentration of 0.3mg/ml for antigen and 3.0 mg/ml for Alum. The 1° and 2° replicate represent the freshly prepared sample. Sedimented, Alum was allowed to settle in microvial.Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato 50mM pH 8.0 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute.*

Obtained results are shown in figure 25. As clear by the electropherograms the new approach did not give improvements, as all samples in the presence of Alum appear as a multi sharp like spike peaks, causing a difficult to associate a specific peak with specific antigen or specific antigen-Alum complex. The same results were obtained also when the same experiments were performed on one single antigen (NHBA was chosen as case study) as reported in figure 27.

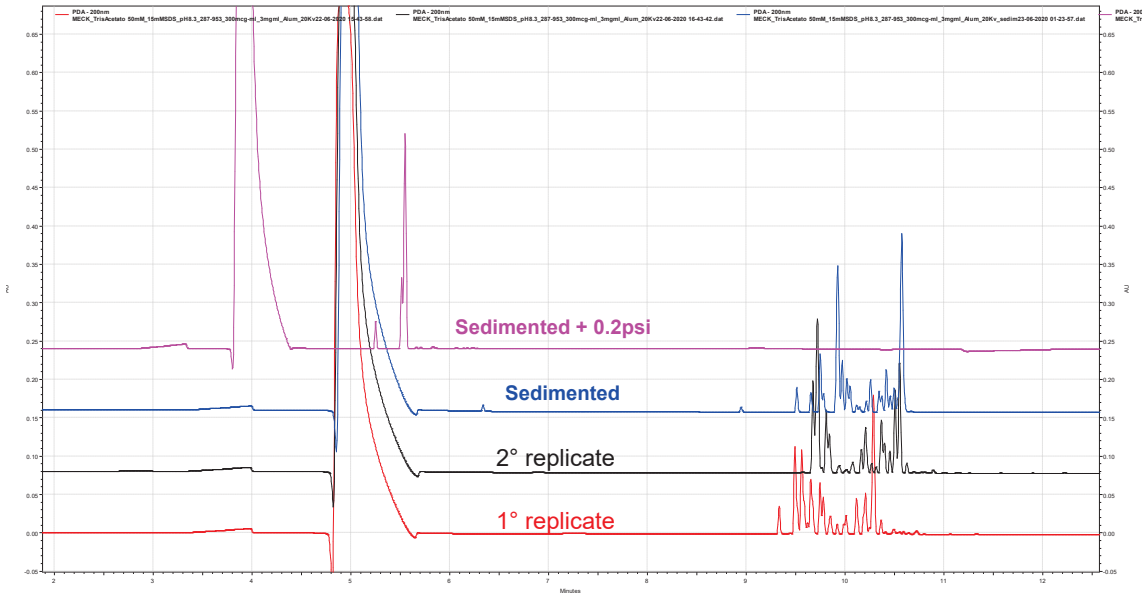


Figure 27: ***Electropherogram profiles of NHBA protein mixed with Alum****. Protein was mixed with Alum to achieved final concentration of 0.3mg/ml and 3.0 mg/ml respectively. Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato 50mM pH 8.0 + SDS 15mM; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute*

To improve the analytical tool and try to understand if it could be suitable for the goal, several experiments at different SDS concentrations and pH value were performed. The scope of thesestudies was to evaluate if changing the ionic force of BGE could be affected the separation and improved the assay resolution and repeatability.

To perform the experiments three BGEs of Tris Acetate 50mM pH 8.0 at different SDS concentrations (15mM-25mM-50mM) were formulate. The three BGEs used to testing the same sample, performing two different methods. One without applied pressure during the separation run and a second appling 0.2psi pressure in order to reduce the resolution and try to compact the multi peaks. For the testing it was selected the antigen NHBA-NUbp formulate at 0.3mg/ml in 3mg/ml of Alum that was tested with all BGEs (results are reported in fig 28).

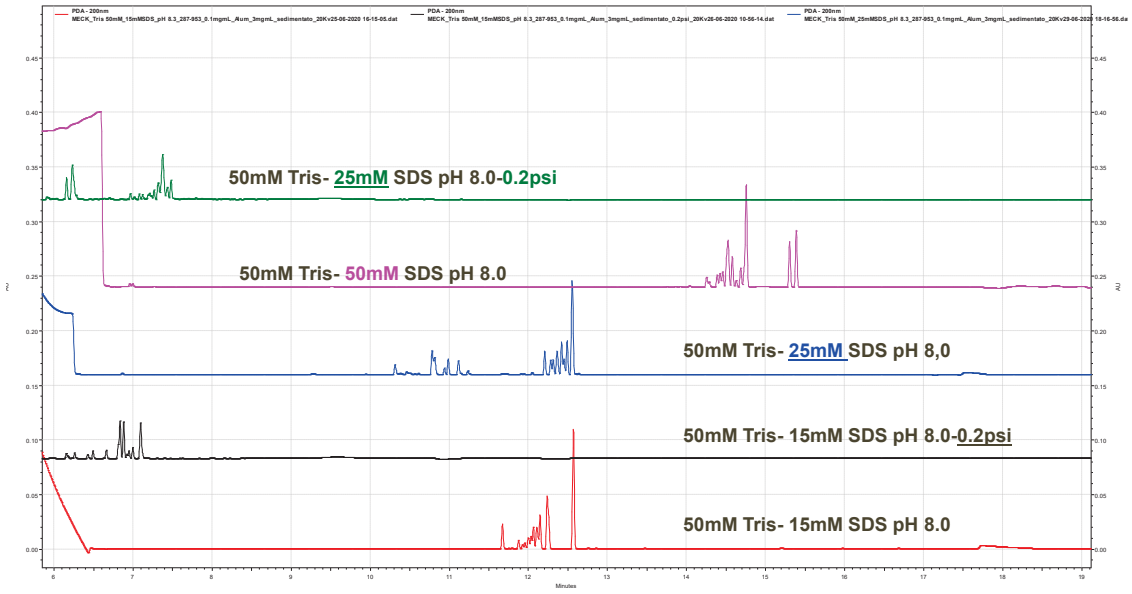


Figure 28: Electropherograms of NHBA protein mixed with Alum analyzed with BGE at different SDS concentrations: *. Protein was mixed with Alum to achieved a final concentration of 0.3mg/ml and 3.0 mg/ml respectively. Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute*

The same sample and two methods were used to testing BGE Tris Acetate 50mM SDS 15mM at different pH values (7.7-8.3) (fig 29). In both case results shown that the issue related the Alum compounds was not resolved:. peaks appears always as multi sharp and without reproducibility.

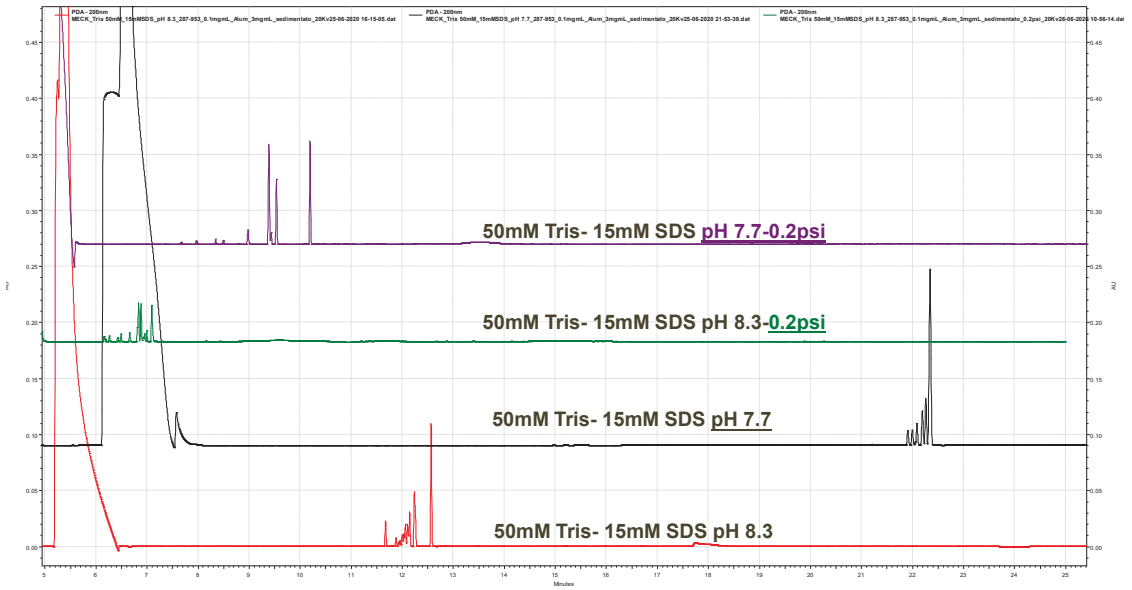


Figure 29: Electropherograms of NHBA protein mixed with Alum analyzed with BGE at different pH values: *Protein was mixed with Alum to achieved final concentration of 0.3mg/ml and 3.0 mg/ml respectively. Separation*

method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato ; 10psi 3 min with water, 10 psi 3 min with HCl 0.1M, 10 psi 4 min NaOH 0.1, 10.0 psi BGE Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 15.0kV 40 minute

The presence of multi peaks of the Alum-Antigen compounds can be attributed to different factors. As mentioned earlier, the polydispersion of it or the lower homogeneity of the solution could be the limiting factor. The other problem related to the method could be the possible interactions of the analyte with the capillary wall that cause a non-homogeneous separation within the running buffer creating spikes. Literature once again comes to our aid: A new efficient cationic micellar CE method was found in literature. [54]. The strength of this new method is its ability to separate protein in complex samples without any modification for the capillary. Studies shown how to be increased concentration (>or=2 mM) of CTAB (Hexadecyltrimethylammonium bromide) is an additive that into electrophoretic buffer, it shields the negative charges on the capillary wall and thereby reserves the EOF orientation. Furthermore, was reported that decreasing pH value in buffers from 6.0 to 3.0 the separation was significantly improved.

Buffer	Molarity (mM)	pH	CTAB mM	Capillary	Polarity	Separation
Acetato	150	4.0	1.0	BFS**	Reverse	936-741 and 961c
	150	4.0	2.0		Reverse	*3 antigen bad resolutin*
	150	4.0	10.0		Reverse	*3 antigen with $Al(OH)_3$*
	150	3.5	2.0		Reverse	*No*
	150	3.5	4.0		Reverse	*No*
	150	3.0	4.0		Reverse	*NO*
	50	3.0	2.0		Reverse	*NO*

Table 5: Summary of BGE at different CTAB concentrations: *To prepare a different Tris Acetate buffers a proper weight of Tris(hydroxymethyl)aminomethane and CTAB (Hexadecyltrimethylammonium bromide) was dissolved in ultrapure water to reach the final desiderated molarity. A proper volume of Glacial acetic acid was added to obtain a fixed pH. In the Table are reported the different buffers used for the study and it is summarized the obtained separation results for the three recombinant proteins.*

On this basis a several BGEs at different CTAB concentrations and pH values wereas screened, in order to analyze the three antigens alone and complexed with Alum. As resumed in Table 5, only one of the tested conditions allowed the detection of the three antigens and antigen-Alum complexes. In other case unproficient separation was obtained or not complete detection of the whole species. The best result was obtained with the following BGE: Tris Acetate 150mM pH 4.0 at 2.0mM of CTAB concentration. Unfortunately, also in this case the assay didn't reach the final goal as shown in figure 30. Also, in this case not a single o sharp peaks associated to Alum or Alum-antigen species was observed.

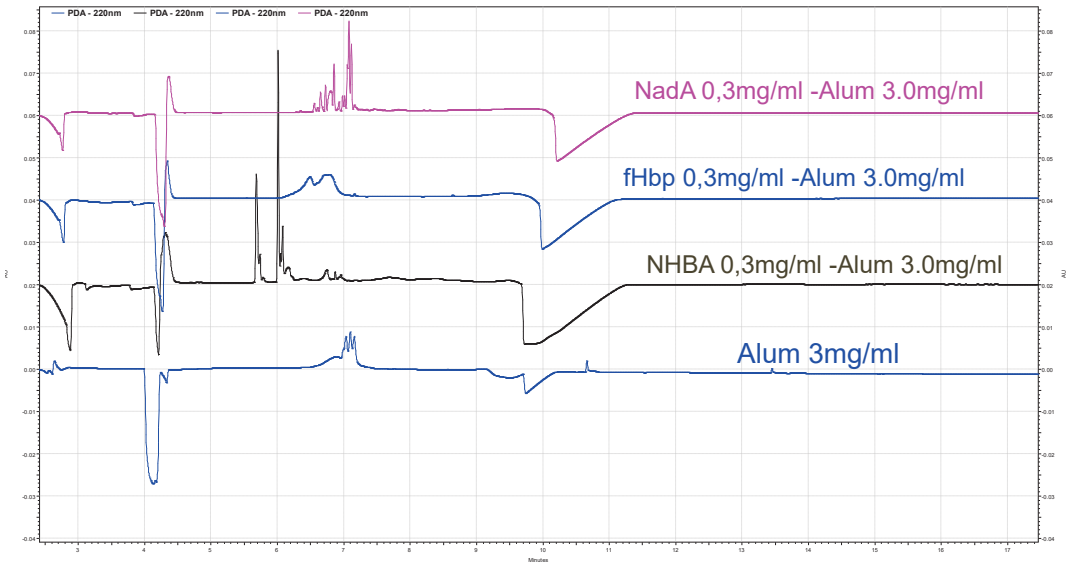


***Figure 30**: **Electropherograms of NHBA, fHbp, NadA, protein mixed with Alum and Alum:** Protein was mixed with Alum to achieved final concentration of 0.3mg/ml and 3.0 mg/ml respectively. Alum diluted in ultrapure water to achieved the final concentration of 3mg/ml Separation method: Bare fused silica capillary, length tot. 70cm effective 50cm, id. 50um; BGE: Tris Acetato 150mM pH 4.0 + CTAB 2.0mM; BGE 10.0 psi for 40 minute; Autosampler temperature at 15°C. Injection by pressure 0.5 psi 0.15 sec. Separation 30.0kV 60 minute*

2.7 Conclusions

Adjuvant and antigen are the two main components of adjuvanted vaccines. One of the main analytical challenges is the determination of the purity and quantification of the antigen in the final product of adjuvanted vaccines without sample pretreatments. A major part of methods commonly used for characterizing adjuvanted vaccines, in fact, requires the desorption of antigens from model Aluminum-containing formulations, so there is a big need for tools able to directly quantify adsorbed antigens. The object of

this study was to investigate the feasibility to directly quantify antigens in the final formulate of adjuvanted vaccines, without any further manipulation, like desorption. With this aim, we focused our attention on the development of a generic method which can be applied with minimal product specific adaptation, ensuring physical-chemical integrity of antigens. In particular, we exploited the potential of Capillary electrophoresis (CE) technique to analyze simultaneously multiple antigens adsorbed on adjuvant in the final container of adjuvanted vaccines. The selection of CE as separative technique, was driven by its high versatility. Indeed, it presents a wide range of application from small ions to virus particles and can be considered an orthogonal technique compared to chromatography. One of the greatest advantages compared to chromatography, is that the separation does not occur inside a solid phase but in a silica bore capillary, feature that can make it suitable for a complex matrix as Alum based formulations [71]. CE was then as techniques for our adjuvant system model in which we selected three recombinant proteins: Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp) as antigens and Aluminum hydroxide (Alum) as adjuvant system.

As described before, the selection of the proper background electrolyte (BGE) is a key point to a successful CE method. Electrophoretic separation is based on migration of charged components in an electric field, so we first selected the BGE based on the physical-chemical properties of our analytes.

BGE composition was chosen on the basis of its compatibility with the antigen-Alum interaction, to avoid interferences or desorbing effects during the run. The pH of BGE was also properly selected on the basis of isoelectric point of analytes to assure that they are fully charged and able to migrate inside the capillary. In the first attempts, described in paragraph 2.6, the selected buffer does not have a right pH value to assure that all three antigens were fully charged and migrate in the capillary. In fact, in the first relevant result we have obtained, the assay was able to detect only two antigens on three. Furthermore, no indication on Alum compound was obtained neither when we tried to analyze the sample alone, nor when tried to monitor the antigen-Alum complex.

Improvements of the assay model were performed screening different BGEs, with different capillary types and protocols, both for the final scope of the anitgens quantification and for a chaarcterization of the antigen adsorption process on Alum Hydroxide. Finally, different molarities of the running buffer were compared to obtain sharp peaks assuring a precise and robust quantification. [66]

Results showed that with this technique it is able to separate, detect and quantify the antigenic components in a single run moreover is able to detect changes in amount due to adsorption of antigen but unfortunately the chemical physical characteristics of the adjuvanted components have a negative impact on the robustness of the separation. In fact, data shown the reproducibility and specificity of the assay for the antigen component, both alone that mixing together, that appear as a single sharp peak with a specific migration time. Unfortunately, this was not the same for the Alum compound.

One important obstacle is related to Alum sedimentation, that causes not homogenous matrix in the final sample. Another issue of the analysis was that Alum with the adsorbed proteins appears as multiple peaks presumably due to different Alum populations: the peaks distribution differs from run to run, causing not reproducible quantifications.

To overcome this major encountered issue, the first action could be maintained Alum solution constantly in agitation until its injection in the capillary, to have a more homogenous solutions and remove the sedimentation. To do this a shaker could be added in the autosampler but unfortunately the system, now, doesn't allow the implementation of this device. So the presence of not reproducible multi peaks remained a great open point[69].

Chapter III.
IMPACT OF ALUM ON THE ANTIGEN STRUCTURE

3.1 General introduction

As mentioned in the previous chapter of this work, ensuring the integrity and purity of the antigen in the final adjuvanted vaccine product over time, is a crucial parameter to monitor in order to obtain an efficacious product. The complexity of the physico-chemical characteristics of Alum makes hardly to apply the commonly used analytical tool to monitor the proteic antigens (e.g. electrophoresis, Western blot, and liquid chromatography), directly on the vaccine product. Indeed, a major part of methods requires the desorption of antigens from model Alumium-containing formulations, to ensure the fully characterization and the evaluation of their stability over the time. The results previously showed, highlighted the challenges caused by the Alum interference that are encountered into the development of an analytical tool able to work on formulates able to follow both the Alum-antigen complex stability and to establish the purity and integrity of the adsorbed antigens. The need for an analytical tool able to directly characterize adsorbed antigen without desorbing, lies in the fact that, while the role of Alum in the immune response is well documented, its effect on antigen stability is one of the most debated topics. Indeed, the stability of adsorbed proteins is one of the main factors that could be influenced by structural changes, as for instance protein unfolding, that occurs after adsorption onto Aluminium adjuvant particles.[72, 73] Several papers highlighted that adsorption processes could cause structural changes in the protein. Specifically, during the adsorbtion on a solid surface, they could change their conformation to maximize the surface interaction, affecting in this way the conformation of epitopes and the stability of the protein [74]. Moreover, these changes could depend on the structure of the protein itself, the tightness of binding to the

surface, and the pH, among other factors. Indeed, the main chemical processes of protein degradation, which impact their structure and stability, as deamidation and hydrolysis or oxidation, are pH dependent.[75] This means that the pH of the microenvironment in which the protein is located plays a crucial role in its stability. In the case of Alum, the attraction of negatively charged ions of solvent by its positively charged surface formed the Stern layer near the particles. This caused an increase in pH relative to the bulk pH of the vaccine formulation. Therefore, antigens adsorbed on Alum are exposed to a different pH than antigens in solution, leading to promote the deamidation and oxidation.[76] Mostly of these effects are time-dependent and could be related to the vaccine ageing[77], but it is unclear whether they are reversible or irreversible and whether there is a negative effect on immunogenicity. Indeed, the structural protein changes could prevent the capability to induce an immune response due to the irreversible aggregates formation.[78] From this perspective the second part of this Thesis, was devoted to deeply understanding the behavior of recombinant protein in the presence of Alum evaluating if Alum could affect the structure of adsorbed antigen impacting their immunogenicity. Validated assays commonly used in routine testing for commercial products are immuno-based assays, then, the in vitro relative potency assay (IVRP) was explored for our scope. This is a Elisa-based assay used to measure the relative antigenicity of the vaccine formulation based on the ability of monoclonal antibodies (mAbs) to bind a specific antigen in the formulated vaccine.[79] The great advantage of this test is that it can be applied directly to our vaccine formulation model without antigen desorption and with low material consumption. In addition, the ability of Luminex technologies to handle multiple analytes in a single test was exploited to perform IVRP in our model vaccine, in which the three recombinant proteins (fHbp., NadA, NHBA) represent the antigen and Alum the adjuvant system.

3.2 Luminex Technologies

Luminex's xMAP technology is a multiplexed immunoassay system based on microplate format beads. This technology was exploited for its great ability to handle multiple analytes in a single test.[80] This could allow simultaneous analysis of the identity, quantity, and stability of each formulated antigen, unlike traditional analytical methods that require a separate assay for each component of the immune response. Microspheres, commonly called 'beads', are the basis of Luminex technology. The

internal labelling of the beads with different ratios of red and infrared fluorophores creates a unique spectral color code for each microsphere, called “region”. Therefore, when different beads of different colors are mixed, they cloud be identified thanks to their unique spectral addresses. Hence, the great force of Luminex is to allow several simultaneous measurements in the so-called 'multivalent approach'. Functionalized carboxyl groups, present on the surface of the beads, were activated using N-hydroxyisµlfosuccinimide (sµlfo-NHS)-mediated conjugation chemistry. These allow covalent coupling of analytes such as proteins, antibodies and target-specific nucleic acids. Using fluidics, in the Luminex system the microspheres are aligned and pass into the detection chamber where a red laser excites their internal dyes allowing them to be identified, while a green laser quantifies the bio-molecular interaction occurring on the surface of the microspheres. The internal software sorts the recorded events according to lateral dispersion, thus excluding events larger or smaller than a single microsphere and aggregated microspheres. The ability to analyze simultaneously multiple analytes has proved invaluable in a variety of fields, from diagnostics in clinical to the immunological response of vaccines.[81-83].

In this study, Luminex technology was exploited for the simultaneous characterization of three recombinant proteins (fHbp, NadA, NHBA) adsorbed on Alum. The Luminex approach was applied to assess the quality of the adsorbed antigen by avoiding physical separation procedures from the adjuvant. The aim was to understand whether the presence of Alum could cause structural changes of the antigen by reducing its immunogenic response. To achieve the scope, we tested different formulations (keeping in mind the formulation model seletced in all this work) varying the Alum concentration while keeping all other components constant. For this PhD project the indirect Luminex approach was used. In particular, the IVRP test is conducted by running a test sample in parallel with a reference sample. Each sample was prepared in a dilution series and made to react with fixed amounts of antigen-specific mAbs. The binding of appropriate mAbs to their respective antigens is followed by isolation of unbound mAbs by a centrifugation step and the unbound mAbs present in the supernatants are detected and quantified. This approach involved the coupling of

antigens on the beads surface, in this way a reduction in the fluorescence signal of the beads is indirectly proportional to an increase in the amount of antigens in the vaccine.

3.3Materials and Method:

3.3.1 Monovlaent Formulate

The Alum monovalent solutions were formulated by dilution of the proper volume of the NHBA-drug substance bulk (stored in aliquots at -20°C) to reach the final composition: 100 µg/ml NHBA in 10 mM L-Histidine pH 6.3 buffer plus 6.25 mg/ml NaCl, 2% (w/v) sucrose. Alum concentration was varying from 0,5 to 3mg/ml as target (0,5-1.0-2.0-3.0 mg/ml). The monovalent freshly formulated at 3.0 mg/ml of Alum were used as reference when compared to the monovalent at different Alum concentrations.

3.3.2 Multivalent Formulate

The Alum multivalent solutions were formulated freshly by dilution of the proper volume of each drug substance recombinant proteins: NHBA-fHbp and NadA bulk (stored in aliquots at -20°C) to reach the final composition: 100 µg/ml of each recombinant proteins in 10 mM L-Histidine pH 6.3 buffer plus 6.25 mg/ml NaCl, 2% (w/v) sucrose, Alum concentration was varying from 0,5 to 3mg/ml as target (0,5-1.0-2.0-3.0 mg/ml). The multivalent sample formulated at 3.0 mg/ml of Alum was used as reference when compared to the multivalent or monovalent formulates at different Alum concentrations.

3.3.3 Antibodies

Monoclonal antibodies reported in Table 6 were provided by GSK Immunoassay group. Phycoerythrin-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:400) was used as secondary detection reagent.

mAb SELECTED						
Antigen	mAb clone	Provider #Batch	Isotype	Source Purity	Working Dilution	working Conc. µg/ml
fHbp	12C1/D7	Takis #20150701	IgG2b	Hybridoma Purified	1:2000	1
NadA	6E3/39	Takis #20150701	IgG1	Hybridoma Purified	1:500	1
NHBA	10E8/A5	Takis #140618	IgG2b	Hybridoma Purified	1:12000	1

Table 6:mAb selected for IVRP assay

3.3.4 Luminex assay procedure

The three recombinant proteins (NadA; fHbp; 40µg/ml each and NHBA 80µg/ml) were coupled to the carboxyl groups of 2.5 x 106 MagPlex microspheres (Luminex Corporation, Austin, TX) following manufacturer's instructions. Each antigen was coupled to a microsphere set, identifiable through its unique spectral signature.

Samples were diluted 1:4 in assay buffer (1x PBS 1% Candor 0,05% Tween20) and transferred (600 µl/w) in 96 deep-well plate from 2ml. A serial step two dilution (300µl/w) for eight point was performed in assay buffer for eight points, and mAbs at working dilution were added in each well (300µl/w). After 30 minutes incubation step at 37°C, plates were centrifuged for 20 minutes at 1000g acceleration 9 brake 3. Supernatant, containing the unbound mAbs (100µl/w), were collected and transferred in 96-well flat-bottom plates, while Alum-antigen-mAbs complex remain on the well bottom. Following this step Ag-conjugated beads mix was added in each well and incubated for 60 minutes at room temperature, on shaker plate settled at 150-160 rpm/minute. Following incubation plates were washed with 1x PBS in by an automatized magnetic washer HydroSpeed 96i (Tecan, Männerdorf, Switzerland). R-Phycoerythrin affine pure F (Ab) 2 fragment goat anti-mouse IgG-PE (Li StarFish cat n° 115-116-072) diluted 1:100 in PBS was used for detection in a 30 min incubation step at room temperature, on shaker plate settled at 150-160 rpm/minute. After plates were washed with 1x PBS in by an automatized magnetic washer HydroSpeed 96i (Tecan, Männerdorf, Switzerland). The resulting unreacted mAbs complexed with beads-antigen were resuspended 1x PBS (100 µl/w) and and analyzed on the Luminex LX-200 system. The procedure was schematized in fig 31.

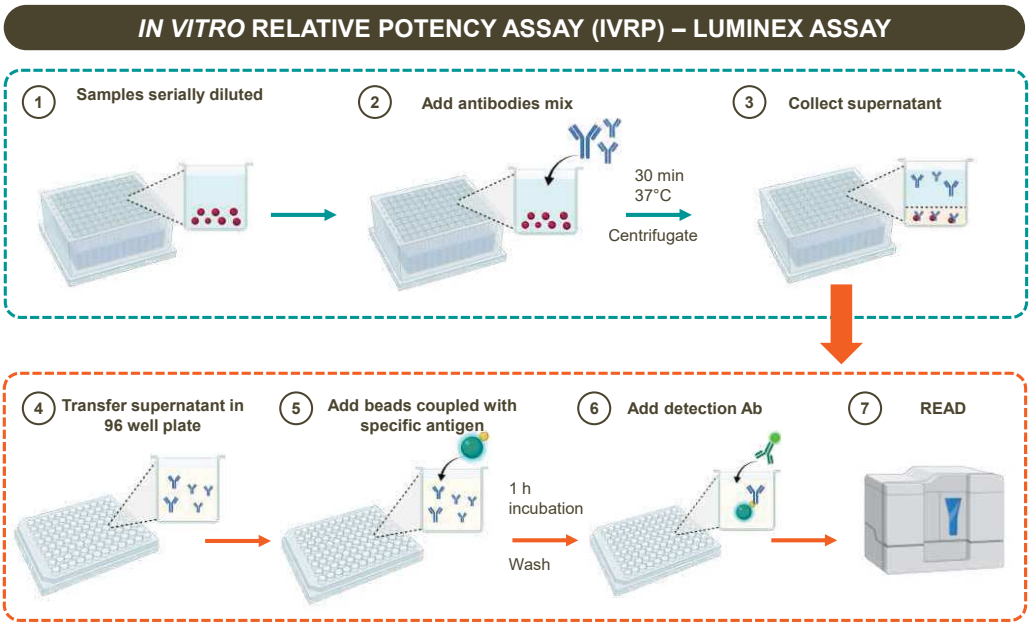


***Figure 31**: Schematic representation of IVRP-Luminex workflow.*

3.3.5 Data analysis

The raw data, Median Fluorescence Intensity (MFI), coming from the sample eight serial dilution (Reference and Test) were analyzed using the software Combistats (Version 6.1 or followings, EDQM – Council of Europe) [84]applying a Parallel Line assay (PLA) model on the least 4 consecutive dilution points, and assuming for reference test always value equal 1. The mathematical model in CombiStats 6.1 was parametrized according to the following formula:

$$\ln(y) = ci + b*ln(x)$$

where c is the intercept of sample i (reference or test), b is the common slope, x is the sample concentration and y are the MFI. The mathematical model was equivalent to the Parallel-Line Concentration-Response Models in USP:

$$Y = \alpha i + \beta * \log(z) + \varepsilon$$

In which αi corresponds to ci, z to x and β to b.

The Relative Potency (RP) was calculated as $(\frac{(\alpha t-\alpha s)}{\beta})$ where αs are the intercept factor estimated on the test sample, while αt are the intercept factor estimated on the reference and β are the common slope.

3.4 Results and Discussion

The great ability of IVRP assay to be directly applied on formulates without being affected by the Alum presence, gave us the change to employ it to study the influence of different Alum amounts on the antigen-Alum interaction process. The three different digital signatures regions of magnetic beads (respectively: MC10044, MC10046, MC10057), were used to coupled respectively the three recombinant antigens, fHbp, NHBA, NadA.

The PLA model was used to relate the linear portion of the dilution curve for each antigen of the test samples with the corresponding curve obtained for the reference samples and potency is expressed as a relative value (Relative Potency, RP) of each antigen in the test compared to the corresponding antigen in the reference. Three specific mAbs anti fHbp; NadA and NHBA were used, whereas R-Phycoerythrin affine pure F (Ab) 2 fragment goat anti-mouse IgG-PE used ad detection reagents.

The first evaluation was to understand if the amount of Alum can affect the antigen structure or potency over the time. To achieve the scope and with the literature evidence of the protective effect of Alum on antigen stability [85], we compared three multivalent formulates varying the Alum concentration from 0.5mg/ml to 2mg/ml (0.5-1.0-2.0 mg/ml) with the formulate target at 3mg/ml and testing at three different time points (2weeks-2months-4months). In order to create and maintain the final vaccine microenvoirment, all the other components (excipients, salts and other protein) were kept constant. (fig32).

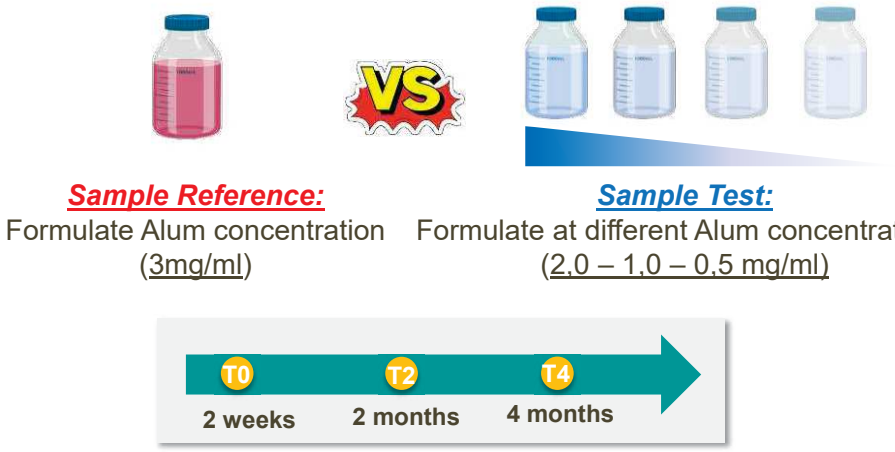
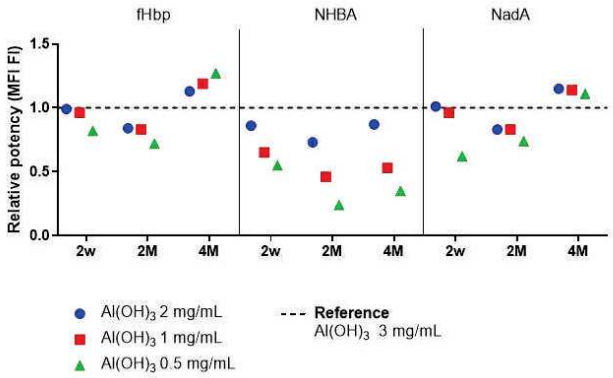


***Figure 32**:Target formulate at 3.0 mg/ml of Alum was compared with the formulate at different Alum concentrations at three different time points.*

The reported data in fig 33 highlighted the peculiarity behavior of NHBA antigen at different Alum concentrations. In detail, correlating the RP values obtained with formulate at 3 mg/ml (our reference) and the formulate at decreasing alum concentrations as our test, it was clear that at low Alum concentration NHBA antigen showed weaker potency respect at the target concentration.



		$Al(OH)_3$ 2 mg/mL	$Al(OH)_3$ 1 mg/mL	$Al(OH)_3$ 0.5 mg/mL
fHbp	2w	0.99	0.96	0.82
	2M	0.84	0.83	0.72
	4M	1.13	1.19	1.27
NHBA	2w	0.86	0.65	0.55
	2M	0.73	0.46	0.24
	4M	0.87	0.53	0.35
NadA	2w	1.01	0.96	0.62
	2M	0.83	0.83	0.74
	4M	1.15	1.14	1.11

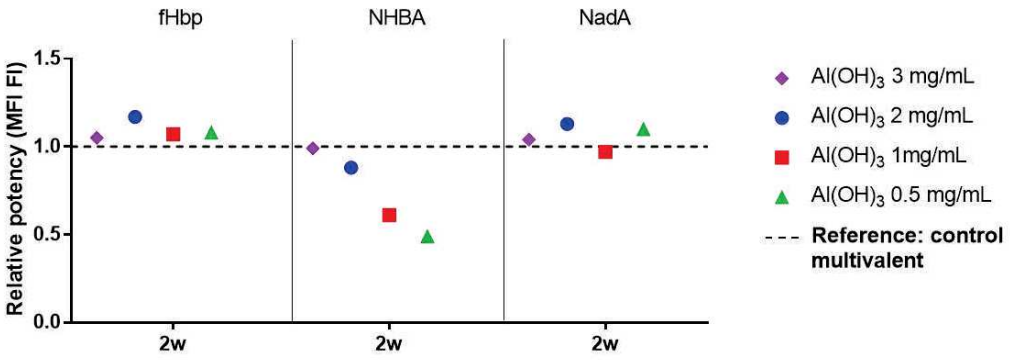
***Figure 33: Representation of relative potency (RP)** data obtained at three different time points for multivalent formulations (fHbp; NHBA; NadA) at different alum concentrations. The data were divided for each antigen at the different time points(2week-2months-4months). The formulations at Alum concentrations (2.0-1.0-0.5 mg/ml) were tested against the formulation at 3.0mg/ml. The reference as such has an RP=1. The relative potency values obtained for each antigen are shown in the table on the right.*

This means that decreasing the Alum concentration, during the binding phase, fewer mAbs were able to bound to a specific adsorbed NHBA antigen traducing in the higher detection of unbound mAbs presented in the collected supernatant. As was clear from the data, this aspect was not directly correlated with the antigen quantity or the aging time. Indeed, the same behavior was observed in the three tested time points.

On the other hand, the other two antigens didn't show this characteristic. Indeed, the data showed that at different Alum concentrations and at different time points, the potency remains the same. The small variations in the RP value can be due to the analytical variability.

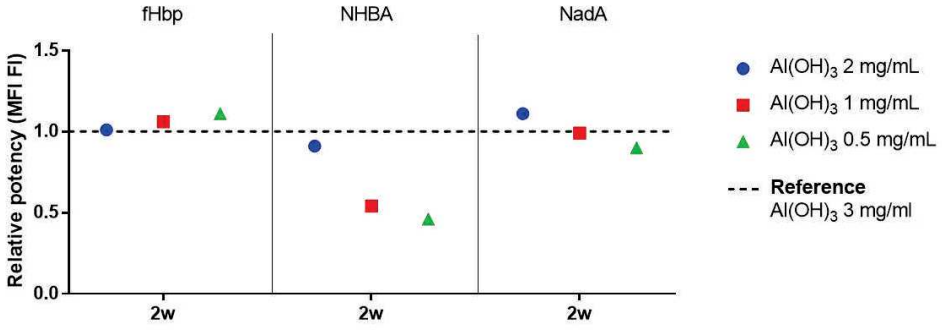
Based on these results we focused our attention on NHBA to better characterize its peculiar behavior. In detail we compared the freshly multivalent formulates at different alum concentrations (0.5-1.0-2.0-3.0 mg/ml) with the monovalent-NHBA formulates at the same Alum concentrations. As control we introduced in the analytical panel the previously used multivalent formulated at 3mg/ml of Alum (our formulation model). To perform our experiments, we designed a multiplex protocol in which in each plate we had: Control multivalent 3mg/ml of Alum and tested freshly and monovalent samples at the others Alum concentrations (2.0-1.0-3.0mg/ml) in duplicate.

As shown in fig. 34, the three antigens' RP obtained with the new multivalent formulated at 3mg/ml of Alum (reference), is comparable to the previously value obtained with the old reference (control multivalent). Moreover, the relative potency related at NHBA decreases at the decreasing of Alum concentration, confirming the early results. The same data were obtained on the multivalent formulate at 3mg/ml of Alum as reference. (fig35).



	$Al(OH)_3$ 3 mg/mL	$Al(OH)_3$ 2 mg/mL	$Al(OH)_3$ 1 mg/mL	$Al(OH)_3$ 0.5 mg/mL
fHbp	1.05	1.17	1.07	1.08
NHBA	0.99	0.88	0.61	0.49
NadA	1.04	1.13	0.97	1.1

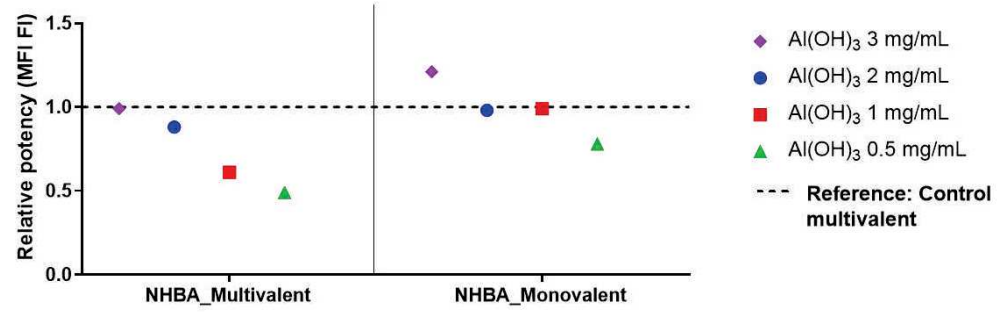
Figure 34: Relative potency (RP) for freshly multivalent *formulations (fHbp; NHBA; NadA) at different alum concentrations. The relative potency for each antigen in the presence of different alum concentrations (3.0-2-0-1-0-0,5 mg/ml) were compared to the RP =1 reference old multivalent formulate at 3.0mg/ml. The data shown as the freshly 3.0mg/ml formulate has the same potency of the old.*



	$Al(OH)_3$ 2 mg/mL	$Al(OH)_3$ 1 mg/mL	$Al(OH)_3$ 0.5 mg/mL
fHbp	1.01	1.06	1.11
NHBA	0.91	0.54	0.46
NadA	1.11	0.99	0.90

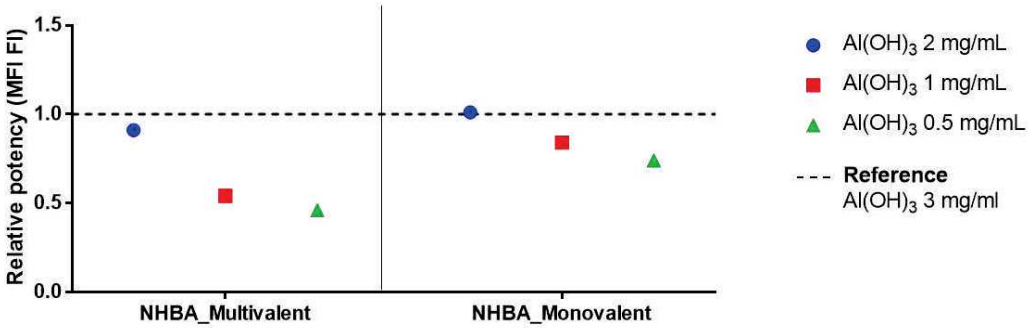
Figure 35: Relative potency (RP) for freshly multivalent formulations *(fHbp; NHBA; NadA) at different alum concentrations. The relative potency for each antigen in the presence of different alum concentrations (2-0-1-0-0,5 mg/ml) were compared to the RP =1 reference freshly multivalent formulate at 3.0mg/ml. In the table below were reported the RP value obtain for each antigen*

For data consistency, the monovalent-NHBA formulate at the same condition of multivalent was tested in the same analytical panel and session, obtaining the following results (fig36-37):



	$Al(OH)_3$ 3 mg/mL	$Al(OH)_3$ 2 mg/mL	$Al(OH)_3$ 1 mg/mL	$Al(OH)_3$ 0.5 mg/mL
NHBA_Multivalent	0.99	0.88	0.61	0.49
NHBA_Monovalent	1.21	0.98	0.99	0.78

Figure 36: Relative Potency (RP) obtained for multivalent formulate and monovalent of NHBA *antigen at different Alum concentrations. The RP of multivalent and monovalent NHBA were compared to the RP=1 of the reference old multivalent formulate at 3mg/ml of Alum.*



	$Al(OH)_3$ 2 mg/mL	$Al(OH)_3$ 1 mg/mL	$Al(OH)_3$ 0.5 mg/mL
NHBA_Multivalent	0.91	0.54	0.46
NHBA_Monovalent	1.01	0.84	0.74

Figure 37*: **Relative Potency (RP) obtained for multivalent formulate and monovalent of NHBA*** *antigen at different Alum concentrations. The RP of multivalent and monovalent NHBA were compared to the RP=1 of the reference formulates at 3mg/ml of Alum.*

Once again, the results demostrated the peculiar behavior of NHBA in both multivalent and monovalent samples. Indeed, as shown in Fig. 36, for monovalent formulations a decrease in Alum concentration corresponds to a decrease in relative potency compared to the reference formulation. The data for monovalent-NHBA showed a smaller difference in the relative potency value at the different Alum concentrations compared to the multivalent formulation. This effect was also observed using the DP formulation

at 3.0 mg/ml Alum as the standard for calculating the relative potency of the sample head formulation. (Fig37).

3.5 Discussion

In order to evaluate the ability of an analytical method to monitor the antigen stability and immunogenicity in the presence of Alum, IVRP assay was explored. The great effort of the assay is its capability do direct apply on the final product without being affected by the presence of Alum. To characterize the behavior of the antigens we tested different formulations varying the Alum Hydroxide concentration while keeping all other components constant. Relative potency was measured between formulate of our model system at 3mg/ml of Alum used as a reference and formulates at decreasing Alum concentrations (0.5-1.0-2.0 mg/ml).

First of all, we evaluate the effect of alum on antigens during the time. For this scope, three different time points (2week-2months-4months) were selected. Collected data showed that out of the three antigens studied, only NHBA exhibited a distinctive behavior. Indeed, the NHBA relative potency decreases at the decrease of alum concentration, independently from the aging time. This means that in the supernatant more unbounded mAbs were detected, resulting in fewer mAbs previously bound to the antigen-alum complex in the early phases of the assay.

One of the hypotheses to explain the NHBA behavior can lies in the antigens' competition for the adsorption on alum surface. Indeed, the distinctive behavior of NHBA, has been observed at lower concentration of Alum where the available adsorption surface area decreases. However, this effect does not occur for the other antigens studied, which show no change in RP values as the concentration of Alum changes. However, as reported in the literature, proteins have the ability to change their conformation to maximize adsorption on the Alum surface. This suggests that the weaker RP observed may be due to a masking of the conformational epitope, which makes binding to specific mAbs difficult. To find out whether this effect is mainly due to the presence of other antigens or an effect of NHBA's own, formulations containing all antigens were compared to the monovalent formulations containing only NHBA, compared at the same concentrations of Alum. In addition, a reference vaccine was introduced into the analytical panel as internal control. Even in this condition, NHBA alone showed distinctive behavior, both using formulate at 3mg/ml or vaccine as reference. However, this effect was less evident, in the monovalent sample, where the NHBA was formulated alone, as compared to the multivalent product where was in the

presence of all the other antigens. This confirmed the hypothesis that the lower presence of Alum may cause a protein adaptation to maximize its adsorption on Alum by making structural changes to better occupy the available surface area. This effect was most evident in NHBA antigens, possibly due to the presence of the epitope in the Arg-rich region located in the flexible loop between the beta-barrel of the C-terminus and the N-terminus region. [86, 87].Thus, this could lead to less accessibility of mAbs to antigen epitopes.

To deeply understand the observed behavior of the NHBA protein when adsorbed onto the Alum surface, it was decided to explore an orthogonal assay. Our decision was prompted by previously internal data in the contest of PhD work, in which was described a mixture of different mass spectrometry techniques, including Hydroxyl radical footpriting (HRF), to study conformational changes resulting from the characterization of adjuvants in vaccines. Furthermore, in the HRF was exploited to study the behavior of NHBA in the presence of Alum. This allowed to apply an already optimized technique to compare and confirm the peculiar behavior of the NHBA protein observed during the IVRP test.

3.6 A chemical -physical orthogonal assay to monitor epitope binding sites of the Alum formulate with a recombinant protein

3.6.1 Introduction

As described above, aluminum salts are the most commonly used vaccine adjuvants in human vaccines. Research on these adjuvants has grown exponentially over the years, mainly in three main areas: 1) understanding the effects of these adjuvants on the immune system[88, 89]; 2) studying antigen-adjuvant interactions;[34] and 3) improving vaccine stability over time. [90] Despite the extensive literature, particularly regarding their efficacy in enhancing the immune response, their impact on the stability of the adsorbed antigen remains an open question. Indeed, as reported in the literature, structural changes in proteins - primary-secondary-tertiary structure - could impact their immunogenicity. Therefore, establishing whether antigen adsorption on adjuvants could be inducing changes at the level of the four protein structures became the crucial importance for the science community to ensure effective products. [91]

The main structural changes generally occur at the level of the protein's tertiary structure, which can easily change depending on the environment. Several studies have shown that proteins were able to change their structure when adsorbed onto a solid surface[92-94]. Some of these changes may cause a lack of immune response or a difficult prediction of the protein release profile, such as the formation of irreversible aggregates, that may potentially constitute risk to patients. As already noted, only a few analytical techniques have been able to be applied without being affected by alum perturbation. For example, main of this techniques: the attenuated total reflectance in Fourier transform infrared (FTIR-ATR),differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were applied to obtained structural information.[95, 96]

In this field, the great advantages of mass spectrometry to reliably sequence and identify protein fragments and their modified products have proved invaluable for studying structural changes in protein molecules[97]. Unfortunately, most structural studies have been performed on desorbed antigens, causing a lack of knowledge of structural studies on adsorbed antigens[53]. Therefore, the last part of this work focused on applying the MS HRF technique, optimized in previously internal PhD work, to fully understand the behavior of protein antigen NHBA when adsorbed on Alum. Specifically, we tried to apply the technique to different multivalent and monovalent formulations at different Alum concentrations.

3.6.2 Hydroxyl Radical Footprinting (HRF) :

Hydroxyl Radical Footprinting (HRF-MS) is a powerful technique that exploits the solvent accessibility of amino acid side chains to measure protein structure by providing detailed information, when used in comparative analysis. The first accepted idea, was proposed by Tullius and Dombroski (1986)[98], which introduced the Fe(II) ethylendiaminetetraacetate (EDTA) Fenton-Haber-Weiss chemistry to generate radicals to covalently modified macromolecules. The radiolysis chemistry of available amino acids has increased exponentially over the years. In fact, a summary of all the methods by which it is possible to generate Hydroxyl radical can be found in the literature, such as lysis of H_2O via gamma-rays and X-rays, or the high-flux synchrotron X-rays, that was enabled millisecond timescale footprinting, which was used to examine Mg2+-dependent RNA folding[99]. But the first optimized method of protein footprintign via chemical modification, was obtained in the 1994 thanks to the work of Hanai and Wang. In 1994, using acetylation as a chemical modification, they succeeded in determining the reactivity of lysine residues to chemical modification by mapping their solvent accessibility.[100] In the last years. structural MS techniques, such as hydrogen-deuterium exchange (HDX) and HRF, are increasingly used in research as bottom-up MS approaches to characterize the higher-order structure (HOS) of proteins[101-103]. Although HRF is not an established technique like HDX, it has proven to be very valuable for studying the tertiary and quaternary structure of proteins. The common approach of HRF techniques is to determine the residual solvent accessibility of protein side chains through irreversible covalent labelling[104, 105], and provide information on the identity and quantity of analyte, when combined with liquid chromatography-

MS (LC-MS) and enzymatic digestion. The hydroxyl radicals generated during protein footprinting experiment, have a great advantages respect over chemical reagents. First, they can penetrate all solvent-accessible areas due to its water-like size. Second their high reactivity can modify many amino acids side chains and their chemical selectivity is well understood. Third, they can be generated safely and conveniently under a wide range of solution conditions. All this condition making them an excellent probe for structural studies.[106, 107]

Nevertheless, the correct generation of hydroxyl radicals and the control of their reactive species are important parameters to control for the success of the method. Indeed, during HRF experiments two main chemical events occurred: the generation of hydroxyl radicals and the 'fate' of reactive hydroxyl radical species. In fact, hydroxyl radical reactive species can act in several ways:

1: Oxidation of the protein analyte (protein labelling).

2: Scavenging effect; oxidation of buffer/additive components

3: Generation of H_2O_2, through recombination with another hydroxyl radical (radical-radical interaction).

4: Radical propagation, resulting in the formation of unwanted reactive oxygen species.

Therefore, it was clear that it is crucial to monitor radical reactions to ensure proper oxidation of the protein to enable detection, while avoiding secondary labelling of primary labelling-induced states such as aggregation.[108]

Fenton Fe(II)-EDTA chemistry is the most commonly accessible method for generating hydroxyl radicals. The great advantage among other techniques lies in the use of commonly available, inexpensive and easy-to-handle chemicals, and the use of EDTA as a chelating agent to neutralize the positive charge of transition metals. The footprinting approach using a hydroxyl radical as a probe of the protein surface, combined with quantitative mass spectrometry analysis, has made possible to study protein structure, protein-ligand, and protein-protein interaction.[109]

3.6.3 Relative Reactivity of Amino Acid Side Chains:

The amino acid side chains of proteins in solution were the target of radiolytic chemical modification. The degree of the modification was directly correlated on their ability to react with hydroxyl radicals and their accessibility to the solvent. MS detection,

identified the relative reactivity of the side chains under areobic conditions and classified as follows: Cys > Met > Trp > Tyr >Phe > Cystine > His > Leu ~ Ile > Arg ~ Lys ~ Val > Ser ~ Thr ~ Pro > Gin ~ Glu > Asp ~ Asn > Ala > Gly.

In summary the residues containing Sulphur- and aromatic (Met and Cys) are the most reactive undergo hydroxyls radicals, and their products are easy to detect in MS. While other residues, such as Gly, Ala, Asp and Asn, generate a weak ionic signal that is undetected in MS. Therefore, these amino acids, are not used as informative probes in the footprinting experiments. Nevertheless, the more reactive residues, are susceptible to the secondary oxidation, due to the high reactivity of Sulfo, that could generate less reactive species such as hydrogen peroxide and related peroxide radicals. Thus, is crucial to block this side reaction, in order to not increasing the oxidization of the sample after radiolysis. For example, in the Fenton chemistry hydrogen peroxide must be removed or quenched immediately. One of the major residues susceptible at this extra oxidation is the Methionine and the addition of reducing species, is fundamental to avoiding the secondary oxidation. after completion of the reaction. The addition of compound such as methionine in its amine or amide (excess) forms is generally used to compete with the undesirable secondary oxidation of methionine and cysteine residues in digested peptides or protein species[110]

3.6.4 Mass spectrometry approaches for quantitative protein footprinting:

As already mentioned, HRF is based on the covalent modification of amino acid side chains that are accessible to the solvent. Accessibility is assessed by means of a rate constant that describes the extent of oxidation for each amino acid site. For example, if a comparison of a ligand-free with a ligand-bound site shows a reduction in the rate constant in the ligand-bound site, it will mean that the site probed by the HRF is close to the binding interface with the ligand.[111]

The HRF method has been summarized as follows (fig37). In the protein in its native state, some residues are buried, while others are more exposed and accessible to solvents on the protein surface. The more exposed residues can be monitored by HRF, in which the exposed side chains of amino acids that are exposed to the solvent can be covalently modified by hydroxyl radicals, which are produced isotopically in

solution[106, 112, 113]. Subsequently, the protein can be treated with a variety of specific and non-specific proteases to break it down into the peptide fragments of interest. The right selection of proteolytic enzymes was a crucial parameter to achieve digestion with maximum protein coverage. To maximize coverage of sequences, proteolysis is usually performed using a number of specific proteases such as trypsin, Asp-N, and/or Glu-C. An acceptable degree of protein coverage ranges from 80% to 90%. In addition, reducing agents such as dithiothreitol (DTT) and tris(2-carboxyethyl) phosphine (TCEP) can be used to digest strongly disulphide-bound proteins. Then, the covalent and irreversible labeled peptides were identified and analyzed by MS. The formation of peptides and their stable oxidized products obtained by protease digestion allows their separation in a reversed phase gradient and their quantification by MS. Separation in reverse-phase chromatography was possible thanks to the higher polarity of the modified peptides, due to the oxygen adducts, which was eluted earlier than the unmodified peptides. In fact, the proteolytic peptides and their oxidized products shown a very similar structure with a discrepancy of +16 or +32 atomic mass units (addition of one oxygen or two, respectively), which makes them very comparable and the ionization and detection difficult.

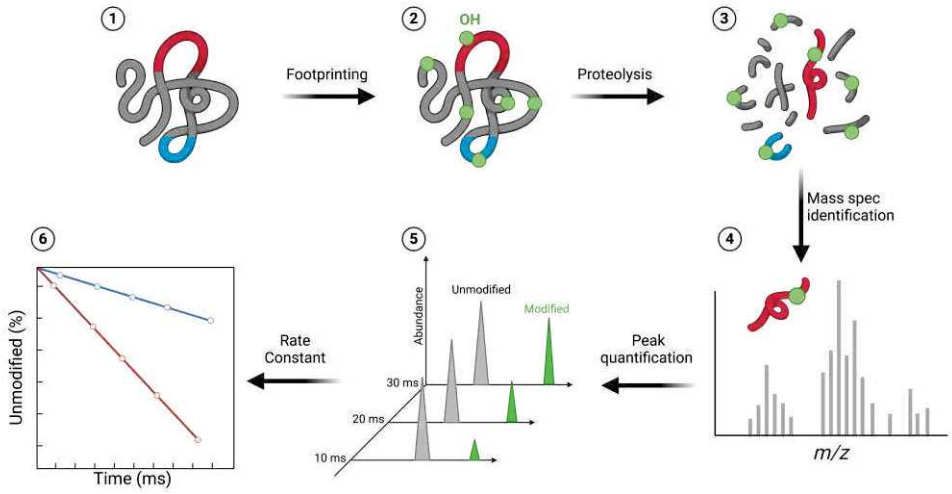


***Figure 38**: Schematic of an HRF experiment for rate determination. (1) Illustration of a generic protein, where residues on a protein in red are exposed to solvent and more prone to HRF, whereas other residues, in blue, are buried and less exposed due to tight packing and contact formation. (2) Covalent-labeling of protein sites by hydroxyl radicals (green dot) that are generated from Fenton chemistry. (3) The enzymatic digestion broken protein into a small peptide segments, cleaved by a specific protease. (4 and 5) Sequence and site of modified peptides are identified and the amount of modification is quantified, based on tandem mass spectroscopy analyses. (6) A characteristic footprinting rate is determined for each peptide/residue segment based on a slope of oxidation rate as a function of exposure time.(adapted from [90])*

3.6.5 HRF application to NHBA

Based on the previously internal data, we decided to apply a Fenton chemistry Hydroxyl Radical Footprinting (HRF) coupled to Mass Spectrometry analysis to compare the peculiar behavior of NHBA observed in IVRP experiment. In our experiments hydroxyl radical foot printing was applied to monitor the epitope binding site of the Alum formulate with recombinant proteins focusing on the residues known to be part of the epitope (from previous Hydrogen Deuterium exchange mass analysis (HDx-MS experiments).

Fenton chemistry was discovered by H. J. H. Fenton in 1890[114]. Fenton's observations showed the ability of ferrous iron(II) to catalytically promote the oxidation of tartaric acid by hydrogen peroxide. In Fenton reaction hydroxyl radicals are generated through the oxidation of Fe(II) to Fe(III) by H_2O_2. The Fenton system, reported in fig39, includes three essential components Fe(II)-EDTA, H_2O_2, and ascorbate. The reaction is monitored at multiple time points to determine the kinetics of modification of the amino acids susceptible to oxidation. The rate of amino acid residues modification will depend on their side chain reactivity ad on the solvent accessibility to different protein domains

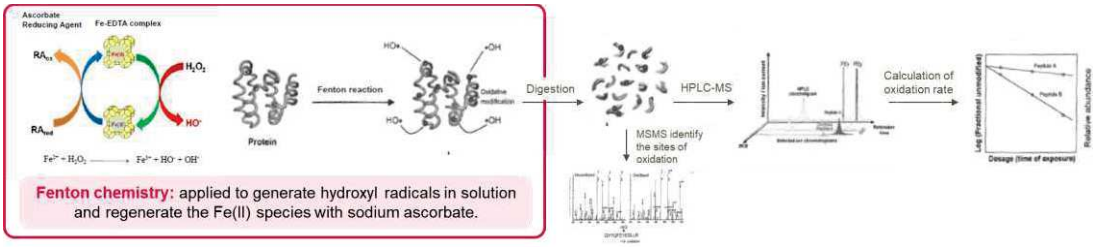


***Figure 39**: Schematic representation of Fenton system*

Fenton chemistry, over the years, has been very successful as a standard method for the structural analysis of nucleic acids. However, recent studies have reported the application of hydroxyl radical footprinting based on Fenton chemistry to

proteins[115]. Hettich and co-workers[116] applied the Fenton system to study the structure of apomyoglobin' in solution and analysed the products by LC-MS/MS with ES-FT-ICR-MS (Fourier transform ion resonance mass spectrometry). The results obtained from the footprinting were consistent with the structure of apomyoglobin by NMR. However, the presence of Fe2+ with EDTA and the high ascorbate concentrations required in the system, may influence the protein structure. This coupled with the ability of H_2O_2 to react directly with S-containing residues is one of the major limitations of the Fenton system.

3.6.6 Materials for HRF

$Fe(NH_3)_2(SO_4)_2$, tris(2-carboxyethyl)phosphine (TCEP), and sodium ascorbate were purchased from Sigma Aldrich. Na_2HPO_4, EDTA, and formic acid (FA) were purchased from Merck Millipore. Thiourea was purchased from Agilent Technologies. H_2O_2 was purchased from GE Healthcare. Oasis HLB 1 cc cartridges were purchased from Waters. All the reagents were stored in accordance with manufacturer recommendation and used without further purification.

3.6.7 Mass spectra acquisition

Mass spectra were acquired in resolution mode (m/z 300-1600) on a Thermo Fisher Scientific Q Exactive Plus mass spectrometer equipped with a Heated Electrospray Ionization source (HESI-II). MS data was acquired in positive mode using a data-dependent acquisition (DDA) dynamically choosing the five most abundant precursor ions (Top 5) from the survey scan at 70,000 resolution. Fragmentation for peptides identification was obtained by Higher-energy collisional dissociation (HCD) at 17000 resolution and normalized collision energy (NCE) 26 eV. Automatic Gain Control (AGC) was set at 3E+6 for precursor ions and at 1E+5 for MS/MS acquisition, the isolation of precursor ions was performed with a 3 m/z window and isolation offset of 1 m/z. Maximum injection time was set 200 msec for precursor ions acquisition and at 150 msec for MS/MS acquisition. Mass accuracy was ensured by monitoring environmental contaminant Polysiloxane at 445.120025 m/z during the analysis.

3.6.8 UPLC chromatographic method

Chromatographic separation was performed using a C18-reversed phase column Acquity UPLC peptide CSH C18 130Å, 1.7µm 1 x 150 mmC, with a 60 min linear

gradient of 28–85% buffer B (0.1% (v/v) formic acid (FA) in ACN) at a flow rate of 50 µL/min and 50 °C column temperature, on a Acquity I-Class UPLC (Waters).

3.6.9 MS data interpretation

The mass spectrometric raw data were analyzed with the PEAKS software ver. 8.0 (Bioinformatics Solutions Inc.) for de novo sequencing, database matching identification, post translational modifications (PTM), and peptide precursor area calculation. Peptide scoring for identification was based on a database search with an initial allowed mass deviation of the precursor ion of up to 15 ppm. The allowed fragment mass deviation was 0.05 Da. NHBA protein peptides identification from MS/MS spectra was performed against Neisseria meningitidis NZ-05/33 protein database (NC_017518.1; 2,222 protein entries) combined with NHBA-NUbp fusion protein sequence (derived from N. meningitidis strain MC58). False positive results were statistically filtered out with a decoy database false discovery rate (FDR) set at 0.1%. Trypsin combined with GluC double enzymatic digestion was set as reference cleavage rule for the database searching tool. A maximum of five missed cleavages and maximum 5 modifications per peptide were set as variable during database search. Oxidation of Methionine, Tryptophan and Histidine, together with N-terminal pyroGlu, Gln/Asn deamidation, Arginine oxidation to glutamic semialdehyde, Proline oxidation to pyroglutamic acid, Tryptophan oxidation to oxonolattone, and tyrosine oxidation to 2-aminotyrosine were set as variable modifications. Peptide precursor areas were used for quantification with a mass tolerance of 20 ppm, a retention time shift tolerance of 2 min. Final result processing considered only methionine residues oxidation.

3.6.10 Methionine residue processing and result calculation

Among amino acids, methionine are the best residues to monitor with HFR to study protein structure. Indeed, methionine are the residues most sensitive to oxidation reactions, guaranteeing minimal oxidative stress for the conformational characterization of proteins. For this work, the previously internal developed method was used. To consider the analysis valid, the amount of oxidized residue must be linearly proportional to the time of the oxidation reaction. For this, the reliability of the

oxidation response of each residue is verified. Among the 14 methionine residues contained in the NHBA sequence, we focused our attention on only three of them (80; 100;105), based on the knowledge previously internal data reported in Alessandro Vadi's PhD thesis. In detail, we chose these three residues for two main reasons: they corresponded to the required linearity criterion (as demonstrated inby internal data in the contest of phD thesis); secondly, they were known to be part of the epitope recognized by the monoclonal antibody used in the IVRP assay (from previous hydrogen-deuterium exchange mass analysis (HDx-MS) experiments). The results were reported as percentages of individual unoxidized methionine residues. The percentage ratio was calculated between the total amount of unoxidized peptides, and the total amount of oxidized residues as shown in the formula below:

$$Met_x\ Unox\% = \frac{\sum Met_x\ unoxidized\ peptides\ area}{\sum Met_x peptides\ area}$$

Fenton chemistry was performed for the oxidation of amino acid residues at five different times: 0, 2, 4, 6 and 8 minutes. For each time point, the reaction was stopped by the addition of a thiourea solution. The unoxidized percentage of methionine residues was calculated for each time point and plotted over the five times for all four different formulas at different alum concentrations. The results were reported as slopes of the oxidation rate, since this is the only value affected by solvent accessibility. Slope values are obtained by the linear regressions calculation of the unoxidized percentages.

3.6.11 HRF sample preparation protocols:

Hydroxyl radical footprinting (HRF) protocol was applied at same time at the four DS NHBA formulate at different Alum concentration (0.5-1-2-3 mg/ml). For each was performed the experimental workflow shown in a schematic procedure in figure 40.

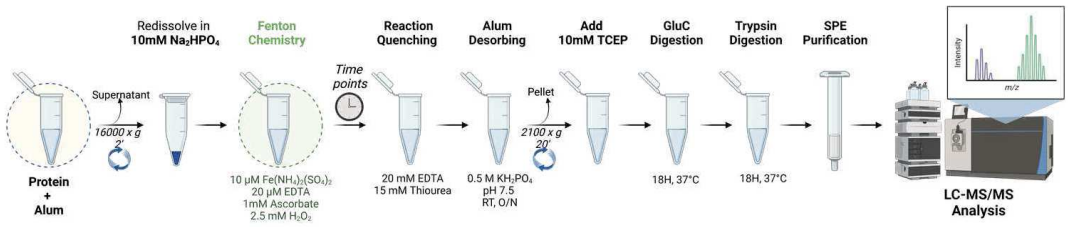


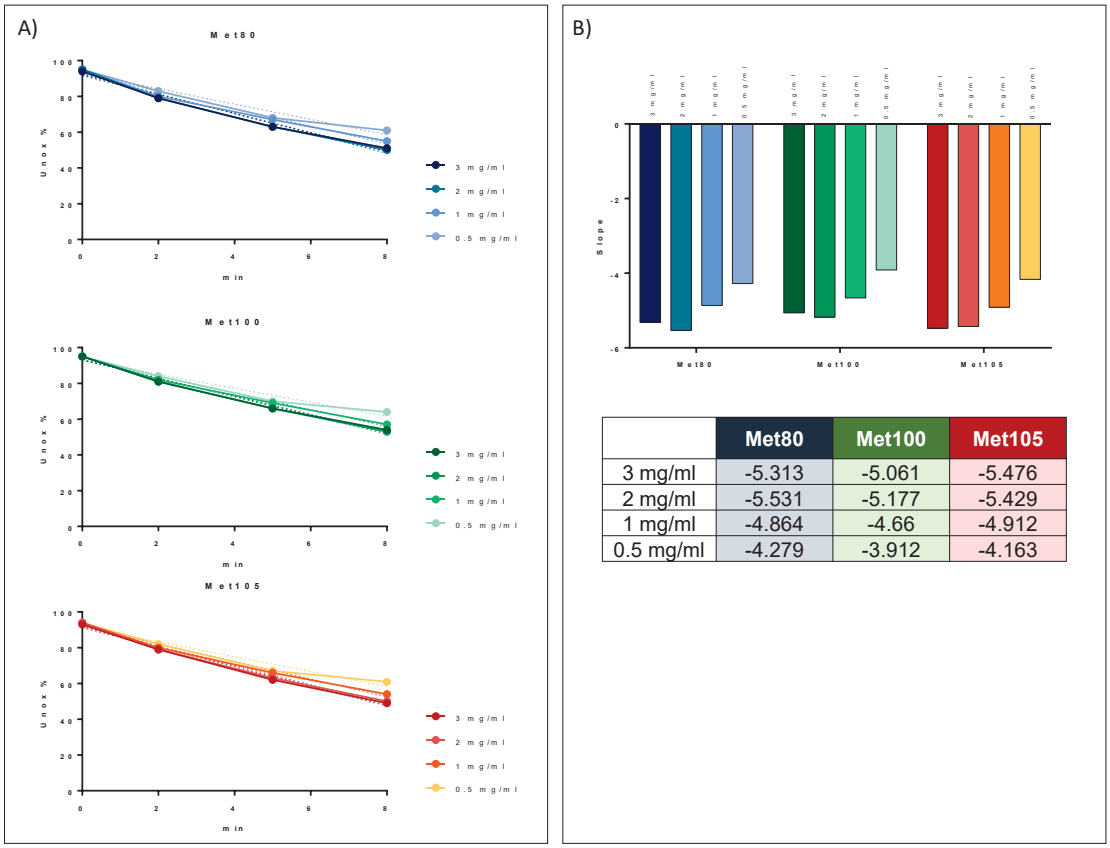
***Figure 40**: HRF schematic protocol workflow for Alum-compound*

4 mL of each alum formulation was centrifuged at 16000g for 2 minutes and the supernatant was removed. Pellet was redissolved in an 500µl of Na_2HPO_4 and split in 4 aliquots of 100µl each. Fenton chemistry was performed by adding in order: 10µM $Fe(NH_4)_2(SO_4)_2$ with 20µM EDTA, 1mM sodium ascorbate and 2.5 mM hydrogen peroxide were mixed with 100 µl of the samples solution previously obtained. For aliquots of each sample were produced in order to replicate the reaction at 0, 2, 6 and 8 min time points (0 min treated samples were added with $Fe(NH_4)_2(SO_4)_2$, EDTA and Ascorbate only). After reaction was completed, 20 µl of stop solution 30mM (EDTA and 15mM thiourea) were added to the samples. The desorption process was then carried out by adding 0.5 M KH_2PO_4 pH 7.5 to each sample and left stirring overnight at room temperature. Sample was centrifugate at 2100g per 20 min and the supernatant was recovered and pellet was discharged. addition of 15 mm TCEP as stabilizer for oxidized residues after Fenton chemistry reaction. Double enzymatic digestion was performed by adding GluC and Trypsin directly into the prepared samples (two aliquots for each enzyme) and then incubated at 37°C for 18h. Digestion was stopped adding 5 µl of Formic acid 100% to each sample. Sample purification was obtained with SPE OASIS (Waters) treatment: activation (ACN), conditioning (1% FA + 1 mM DTT), sample loading, sample wash (1% FA + 1 mM DTT), and elution (60% ACN + 0.1% FA). Samples were evaporated to dryness in SPE-Dry and resuspended in 0.1% FA.

4.3 Results and Discussion

As previously reported, the ability of HRF to discriminate different domains of NHBA proteins based on complementary structural results obtained by NMR and HDx[46, 117] was demonstrated in the previous internal data . An ex novo treatment was developed to be applied on the Alum adjuvant and new MS data processing. In addition, a variable number of methionine residues (9 or 10) distributed along the NHBA-NUbp sequence were monitored and correlated to their propensity to be modified by hydroxyl radicals with solvent accessibility. Following analysis via HFR of NHBA samples with and without alum present, greater solvent accessibility of the amino acid residues in the sample without alum was found, due to the presence of the adjuvant in the already adsorbed formulation. This was corroborated by the results obtained on residues (such as 50 and 293) by comparing the difference in the slope values obtained in the alum and non-alum samples, this means the adsorption of aluminium had an affect on the solvent accessibility of these residues. Furthermore, the experimental data demonstrated that Fenton-type reactions on the adjuvanted product due to the presence of aluminium does not interfere with the production of hydroxyl radicals.

Based on these data, the four monovalent-NHBA formulations with different amounts of alum were tested. The final objective was to verify the feasibility of HRF as an orthogonal technique to confirm the peculiar behavior observed by NHBA in the IVRP test.



	Met80	Met100	Met105
3 mg/ml	-5.313	-5.061	-5.476
2 mg/ml	-5.531	-5.177	-5.429
1 mg/ml	-4.864	-4.66	-4.912
0.5 mg/ml	-4.279	-3.912	-4.163

***Figure 41**: Comparison of NHBA formulations at different alum concentrations (3.0-2.0-1.0-0.5 mg/ml). A) Details of the oxidation rate of the three methionine residues 80, 100 and 105 are shown at the top as linear regression plots. B) The slopes of the oxidation rate of the methionine residues are represented in barrel plots in the lower part, where the numerical values of the linear slopes for the three methionine residues are shown. A higher value corresponds to greater accessibility to the solvent.*

For this analysis, three methionine residues were monitored and reported: 80, 100, 105. The decision to monitor these residues was derived from previous HRF protein mapping experiments, which showed greater accessibility than others, such as 579 and 406, which are in regions of high structure and less accessible to solvents. Furthermore, HDX studies have shown that they are part of the conformational epitope recognized by the mAb used in the IVRP assay.

The results are shown in Figure 41 as slopes of the oxidation rate in three different modes. On the left, the slopes are plotted as linear regression lines for each residue monitored in the four monovalent-NHBA formulations at different alum concentrations (3.0-2.0-1.0-0,5 mg/ml). On the x-axis is reported the oxidation time, while on the y-axis the percentage of unoxidized for each sample tested. On the right-hand side, the slopes of the oxidation rate were illustrated as a barrel diagram distributed along the

alum concentrations (on the x-axis) for each methionine residues. At the bottom, the Table shows the numerical values of the linear slopes obtained, broken down for each methionine and Alum concentration in the formulation.

Also in our case, the presence of alum did not interfere with the production of hydroxyl radicals and then the suitability of the test for the purpose. As can be seen, the slopes of the oxidation rate met the linear regression criteria for all three residues. The results, shown in Figure 41, show greater oxidation rate of the residues at higher alum concentrations. This means a major solvent exposure compared to samples formulated with a low alum concentration. This can be confirmed by the difference between the slope values of the samples at 3 mg/ml alum (e.g. -5,061 for Met100) and those at 0.5 mg/ml alum (e.g. -3,912 for Met100).

The results obtained are in line with the IVRP observations. Indeed, as described in paragh 3.4, the relative potency decreases in formulations at a low alum concentration. This means that in the presence of less alum, binding between mAb and its specific binding site is more difficult. This indicates a low availability of the epitope, due to two possible factors: either a structural change in the protein or lower accessibility due to steric clutter at the binding site. The data generated by HRF confirm that there is no evident structural change in the protein. The lower oxidation of the residues in the presence of smal amounts of Alum actually shows that there is less accessibility to the epitope confirming a possible clutter making the binding between mAb and epitope difficult to reach

In conclusion, the possibility of applying the HRF mass technique as an orthogonal technique was demonstrated in the case of further investigation of possible anomalies found on adjuvanted systems. Several attempts were made to apply the same protocol to the finalformulate compound in the presence of the three antigens formulated at different alum concentrations. Unfortunately, no significant results were obtained. In most cases, adequate coverage of the protein sequences was not obtained, probably due to inadequate enzymatic digestion. Automated methods to speed up reaction times were employed, but even there without any particular success as there was interference with the instrumentation used to perform the Fenton chemistry and Alum. However, this remains a future prospect to be investigated and improved.

Chapter IV.
CONCLUSION AND DISCUSSION

The aim of this work was to apply different analytical tools for the characterization of aluminium adsorbed antigens. To achieve this goal, three well-known recombinant proteins described in the literature and a model adjuvant formulation were selected : Neisseria adhesin A (NadA); Neisserial Heparin Binding Antigen (NHBA); and factor H-binding protein (fHbp) as antigens, and aluminium hydroxide (Alum) as adjuvant system, one of the most common adjuvant-salts used in approved vaccines.

Aluminum-containing compounds are the most widely used adjuvants in vaccines, due to their excellent safety profile, low cost and good adjuvanticity with various antigen classes. In comparison with the large body of literature studies that describe new antigens and/or adjuvants and their immunological profiles, there are comparatively few studies regarding physico-chemical characterization of antigen-adjuvant interactions. Indeed, one of the main analytical challenges in this context is the determination of the purity and quantification of the antigens in the final product of adjuvanted vaccines. This is due to the high degree of complexity regarding the characterization of vaccine formulations, as the combination of low antigen doses and colloidal systems poses many analytical difficulties. The analytical tools commonly applied to monitor proteins (e.g. electrophoresis, Western blot, and liquid chromatography) cannot be used directly on the vaccine product due to the interference of the adjuvant compound. Therefore, to characterize the antigen in adjuvanted vaccines, it is necessary to recover them completely from adjuvants surface through desorption. The high concentration of salts and surfactants used in the desorption procedure, can result in incomplete recovery of the antigen, with the loss of some chemical and physical properties that compromise its integrity. Furthermore, the desorption procedure involves the use of centrifugation to recover the desorbed antigens. This causes the release of the antigen into the aqueous phase prior to analysis, in which some components of the desorption buffer may remain in the sample and potentially cause interference with the assay (e.g. surfactants impact HPLC methods

and histidine buffers are not suitable for colorimetric assays). In addition, the understanding of the physico-chemical proprieties of the two components in the formulation (adjuvant-antigen) is crucial, and critical parameters (such as the degree of antigen desorption and the colloidal stability) must to be monitored since they could have an impact on the efficacy, safety and shelf-life of the final product. For this reason, there is a great need for techniques that are able to directly quantify adsorbed antigens.

In the first part of this work, CE techniques were exploited to develop an analytical tool able to quantify antigens without a desorption from aluminium.

In the second part, two analytical tools were applied to study the behaviour of recombinant proteins in the presence of aluminium, and to assess the impact of aluminium hydroxide on immunogenicity and antigen structure.

IVRP-Luminex technology was used to investigate the influence of aluminium concentration on interactions between antigen and aluminium,as it is able to work directly on formulations without being affected by the presence of aluminium.

A mass spectrometry HRF technique was used as an orthogonal method to monitor the epitope binding site of the alum adjuvant with recombinant proteins, focusing on the residues known to be part of the epitope (from previous hydrogen deuterium exchange mass analysis (HDx-MS) experiments). The combination of physiochemical characterization and immunological studies was applied for the characterization of antigen-adjuvant interaction.

5.1 Chapter II- Method for direct quantification of antigens in adjuvanted vaccine

In chapter II capillary electrophoresis (CE) was exploited in an innovative way in order to develop an assay to simultaneously characterize multiple antigens adsorbed on adjuvant in the final formulation of adjuvanted vaccines. The approach to analyze antigens directly without physical separation from the adjuvant has been explored with the potential to be widely applied to monitor the quantity and stability of antigenic components in formulations containing aluminum salts. The assay was developed for

the simultaneous characterization of three recombinant proteins : Neisseria adhesin A (NadA), Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp) formulated with aluminum hydroxide (AH) as an adjuvant system. The first attempt was performed to assess the ability of the assay to detect and quantify the antigenic components in a single run. A screening of different BGE buffers was initially performed to define which buffer caused the least interference during the run. The pH of BGE was also tuned on the basis of the isoelectric point of the analytes to ensure that they were fully charged and able to migrate within the capillary. The three recombinant proteins, fHbp; NadA and NHBA, were analyzed without aluminium present. Good resolution was obtained using a buffer of TRIS acetate at 50 mM with 15 mM of SDS at pH 8.0. The addition of aluminium. or the antigen-aluminium complex, led to poor reproducibility under the same conditions. As reported in section 2.6.3, one important factor is related to the sedimentation of aluminium, which can cause inhomogeneity in the final sample. Another issue of the analysis was that multiple peaks were observed in sample containing aluminium with adsorbed proteins, which was presumably due to different aluminium populations: the peak distribution differed from run to run, leading to non-reproducible quantification. Different investigations on the buffer molarity were performed to increase assay resolution and to ensure a precise and robust quantification. Nevertheless, no improvements regarding the aluminium profile were observed. Furthermore, the physical-chemical characteristics of the adjuvanted components had a negative impact on the robustness of the separation, principally due to the presence of multiple sharp peaks in the aluminium-containing samples, which rendered it difficult to associate a specific peak with specific antigen or antigen-alum complex.

The maincause of the multiple peaks was identified as the inhomogeneity of sampling caused by the sedimentation of aluminium within the autosampler vial. Regular CE methods involved conditioning of the capillary prior to sample injection, and during this time the aluminium-compounds samples begin their sedimentation process. This process leads to several aluminium populations with differing charges and sizes, thus creating inhomogeneous samples. The different profiles obtained after injection of the same samples over time, confirmed this hypothesis. To avoid the issue, different strategies were proposed and tested.

Sedimentation is a slow process that occurs over time. Initial attempts to avoid sample inhomogeneity consisted of letting the aluminium settle in the vial before prior to sample injection.

Changes in the method were next investigated. The sample was injected immediately prior to capillary reconditioning to reduce the waiting time in the autosampler vial during capillary rinsing, reducing the time for sedimentation. At the end of the run, the MeOH wash was replaced with HCl to reduce possible interactions between the capillary walls and samples. Neither of these actions resolved the problem of the multiple sharp peaks in the aluminium-contianing samples.

In conclusion, the results showed that this technique is able to separate, detect and quantify the antigenic components in samples with complex matrices as vaccine adjuvant formulations, and that it can probably be used for the quantification of non-adsorbed proteins in vaccine samples without desorbing. It was used to detect differences in quantification after the adsorption phase. Nevetheless, under these conditions, the presence of aluminium, does not allow for the quantification of antigens adsorbed on aluminium, of for identification of the single antigen-aluminium populations within a multivalent vaccine.

To increase the efficiency of the technique, a solution must be used in order to avoid the problem of sedimentation, or to slow it down over time. One possible solution could be the addition of chemical stabilizers (acting as anticoagulants) to the samples. These could cause changes to the chemical and physical characteristics of the compounds, however, by altering antigen-adjuvant interactions. An alternative, which would perhaps not alter the characteristics of the final product, would be to keep the samples in constant agitation until the point of injection. This would lead to greater polydispersion of the aluminium, with less chance of creating aggregates, and thus settling, over time. This configuration is not currently possible , but it certainly remains an open point for future studies.

5.2 Chapter III- Impact of difference Alum concentration on the response and antigen structure

In chapter III, experiments were performed to investigate two analytical tools to better understand the protein-aluminium interaction: an immuno-based technique and mass spectrometry. While the immune response of aluminium is well documented, its effect on antigen stability remains under discussion. Protein stability could be affected by structural changes, such as protein unfolding, which occurs after adsorption on aluminum adjuvant particles. These effects are time-dependent, but it is unclear whether they are reversible or irreversible, and whether there is a detrimental effect on immunogenicity[77, 118]. In the second part of this work, the identification of an analytical tool capable of more better understanding the behaviour of recombinant proteins in the presence of aluminium was targeted. The presence of the aluminium, and its effect on the structure or immunogenicity of the adsorbed antigens, was also investigated.

To study the aluminium-antigen interaction, multivalent (NHBA; recombinant fHbp and NadA proteins) and monovalent (NHBA antigen only) formulations were tested at different alum concentrations. In order to assess whether the presence of aluminium at different concentrations might have an impact on antigen immunogenicity, a multivalent IVRP-Luminex instrument was used . The ability of this assay to work directly on formulations without being influenced by the presence of aluminium, and the possibility of handling multiple analytes in a single assay, made possible the use of the vaccine model directly without antigen desorption. A small quantity of material was required, reducing time and number of experiments. Multivalent and monovalent formulations at 3.0 mg/ml were selected as reference, while formulations at different aluminium concentrations (2.0-1.0-0.5 mg/ml) were used as tests. In each formulation, the quantity of antigen was kept constant (0.1 mg/ml) for each recombinant protein. The IVRP results showed the peculiar behaviour of the NHBA antigen in both multivalent and monovalent formulations. A lower relative potency was observed as the concentration of aluminium decreased. As reported in section 3.4 , it was clear that the lower quantity of aluminium reduces the ability of mAb's to bind antigen specific epitope. The results obtained over the time (2 and 4 months) suggested that the aging effect was not time dependent. Furthermore, the percentage of adsorbed antigen did not

influence the assay, as both free and adsorbed antigena were identified in the early phase of the assay. This suggests that, at lower aluminium concentrations, the surface area available for antigen adsorption is decreased. Under these conditions, proteins were able to change their structure to maximize adsorption. In addition, the decreased surface area means that proteins are adsorb closer together, causing a masking of the sites involved in antibody binding. This effect was most evident in NHBA antigens, possibly due to the presence of the epitope in the Arg-rich region located in the flexible loop between the beta-barrel of the C-terminus and the N-terminus region. [86, 87] It is possible that this induced a 'rearrangement' of the protein to maximize the possibility ofadhesion. To fully understand this peculiar behaviour, it was decided to prepare monovalent formulations (with NHBA as a unique antigen) in which the antigen concentration was kept constant (0.1 mg/ml) whilst varying the concentration of Alum (3.0-2-1-0.5 mg/ml). To have more control within the analytical panel, a fresh multivalent formulation at 3.0 mg/ml of Alum and 0.1 mg/ml for each recombinant protein (NHBA; fHbp; NadA) was also prepared. When the formulations at 3.0 mg/ml were compared, no difference was observed. When the alum concentration decreases in the monovalent, however, the same behaviour was observed as in the multivalent formulation. Interestingly, since no other proteins were present to compete for surface availability, the effect in the monovalent protein was less pronounced.

To test this hypothesis, hydroxyl radical foot printing was explored. Hydroxyl radical footprinting (HRF) is a labeling approach to probe the solvent accessibility of residues within folded proteins through their covalent modifications. In a Fenton-type reaction, hydroxyl radicals are generated through the oxidation of Fe(II) to Fe(III) by H_2O_2. The Fenton system, (fig 39) includes three essential components: Fe(II)-EDTA, H_2O_2, and ascorbate. The reaction was monitored at multiple time points to determine the kinetics of modification of the amino acids susceptible to oxidation. The rate of modification of amino acid depended on their side chain reactivity, and on the solvent accessibility to different protein domains. The extent of the modification of the amino acid side chains in proteins in solution depends both on its ability to react with hydroxyl radicals, and on its steric accessibility to the solvent. Methionine is one of the most sensitive residues towards oxidation,and its use guarantees the minimum oxidative stress for the conformational characterization of proteins to be determined. Each residue monitored by HRF required verification of the reliability of the response to oxidation; this

acceptance criterion was satisfied when the quantity of oxidized residues is linearly proportional to the oxidation time.

It is possible to use this technique to perform the modification of the amino acid residue without desorption. This allows a 'printing' of the antigen adsorbed on the aluminium. It was decided to monitor three methionine residues already studied and know to be part of the epitope recognized by the antibody (IVRP). the level of oxidation at 5 different time points 0-2-4-6-8 minutes of the Met 80-100-105 residues was recorded. The results obtained on the NHBA monovalent formulations confirmed what was seen in the immunoassay experiments. The slope obtainedshowed that compounds with higher aluminium concentration were more oxidized than the samples containing lower concentrations of aluminium. This means that at lower Alum concentration the residues are less exposed to the solvent and therefore more masked. Consequently, in terms of immunoassay, this results in a less accessibility of antibody to the epitope.

Unfortunately, performed experiments on multivalent formulations were unsuccessful, as overly complex matrices resulted in insufficient coverage of the protein sequences after enzymatic digestion. An attempt was made to implement the method with multivalent formulates using semi-automated systems (to reduce as much as possible the exposure time of the protein sequences to the oxidizing agents, so as not to induce structural changes), but evein in this case, interference with aluminium was encountered.

REFERENCE

1. HogenEsch, H., D.T. O'Hagan, and C.B. Fox, *Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want.* NPJ Vaccines, 2018. **3**: p. 51.
2. Montomoli, E., et al., *Current adjuvants and new perspectives in vaccine formulation.* Expert Rev Vaccines, 2011. **10**(7): p. 1053-61.
3. Wack, A. and R. Rappuoli, *Vaccinology at the beginning of the 21st century.* Curr Opin Immunol, 2005. **17**(4): p. 411-8.
4. Griffin, J.F., *A strategic approach to vaccine development: animal models, monitoring vaccine efficacy, formulation and delivery.* Adv Drug Deliv Rev, 2002. **54**(6): p. 851-61.
5. Glenny, A.T., et al., *Immunological notes. XVII–XXIV.* The Journal of Pathology and Bacteriology, 1926. **29**(1): p. 31-40.
6. Serruto, D., et al., *The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.* Vaccine, 2012. **30 Suppl 2**(0 2): p. B87-97.
7. Malito, E., et al., *Structure of the meningococcal vaccine antigen NadA and epitope mapping of a bactericidal antibody.* Proc Natl Acad Sci U S A, 2014. **111**(48): p. 17128-33.
8. Maritan, M., et al., *Crystal structures of human Fabs targeting the Bexsero meningococcal vaccine antigen NHBA.* Acta Crystallogr F Struct Biol Commun, 2017. **73**(Pt 6): p. 305-314.
9. Burton, D.R., *What Are the Most Powerful Immunogen Design Vaccine Strategies? Reverse Vaccinology 2.0 Shows Great Promise.* Cold Spring Harb Perspect Biol, 2017. **9**(11).
10. Delany, I., R. Rappuoli, and E. De Gregorio, *Vaccines for the 21st century.* EMBO Mol Med, 2014. **6**(6): p. 708-20.
11. Saleh, A., et al., *Vaccine Development Throughout History.* Cureus, 2021. **13**(7): p. e16635.
12. Rappuoli, R.a.L.V., *I vaccini dell'era globale* october 2021: Zanichelli
13. Skibinski, D.A., et al., *Combination vaccines.* J Glob Infect Dis, 2011. **3**(1): p. 63-72.
14. Tan, L.K., G.M. Carlone, and R. Borrow, *Advances in the development of vaccines against Neisseria meningitidis.* N Engl J Med, 2010. **362**(16): p. 1511-20.
15. Mora, M., et al., *Reverse vaccinology.* Drug Discov Today, 2003. **8**(10): p. 459-64.
16. Rappuoli, R., *Reverse vaccinology.* Curr Opin Microbiol, 2000. **3**(5): p. 445-50.
17. Rappuoli, R., et al., *Reverse vaccinology 2.0: Human immunology instructs vaccine antigen design.* J Exp Med, 2016. **213**(4): p. 469-81.
18. Nandy, A. and S.C. Basak, *Bioinformatics in Design of Antiviral Vaccines*, in *Encyclopedia of Biomedical Engineering*, R. Narayan, Editor. 2019, Elsevier: Oxford. p. 280-290.
19. Brito, L.A. and D.T. O'Hagan, *Designing and building the next generation of improved vaccine adjuvants.* J Control Release, 2014. **190**: p. 563-79.
20. O'Hagan, D.T. and E. De Gregorio, *The path to a successful vaccine adjuvant--'the long and winding road'.* Drug Discov Today, 2009. **14**(11-12): p. 541-51.
21. Dey, A.K. and I.K. Srivastava, *Novel adjuvants and delivery systems for enhancing immune responses induced by immunogens.* Expert Rev Vaccines, 2011. **10**(2): p. 227-51.

22. O'Hagan, D.T., et al., *The history of MF59(®) adjuvant: a phoenix that arose from the ashes.* Expert Rev Vaccines, 2013. **12**(1): p. 13-30.
23. Apostólico Jde, S., et al., *Adjuvants: Classification, Modus Operandi, and Licensing.* J Immunol Res, 2016. **2016**: p. 1459394.
24. Didierlaurent, A.M., et al., *AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity.* J Immunol, 2009. **183**(10): p. 6186-97.
25. Kundi, M., *New hepatitis B vaccine formulated with an improved adjuvant system.* Expert Rev Vaccines, 2007. **6**(2): p. 133-40.
26. Harper, D.M., et al., *Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial.* Lancet, 2004. **364**(9447): p. 1757-65.
27. Singh, M. and D. O'Hagan, *Advances in vaccine adjuvants.* Nature Biotechnology, 1999. **17**(11): p. 1075-1081.
28. Fox, C.B., et al., *Working together: interactions between vaccine antigens and adjuvants.* Ther Adv Vaccines, 2013. **1**(1): p. 7-20.
29. Dey, A.K., P. Malyala, and M. Singh, *Physicochemical and functional characterization of vaccine antigens and adjuvants.* Expert Rev Vaccines, 2014. **13**(5): p. 671-85.
30. Hogenesch, H., *Mechanism of immunopotentiation and safety of aluminum adjuvants.* Front Immunol, 2012. **3**: p. 406.
31. Green, M.D. and N.H. Al-Humadi, *Chapter 27 - Preclinical Toxicology of Vaccines11Disclaimer: The findings and conclusions in this chapter have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy*, in *A Comprehensive Guide to Toxicology in Nonclinical Drug Development (Second Edition)*, A.S. Faqi, Editor. 2017, Academic Press: Boston. p. 709-735.
32. Holt, L.B., *Quantitative studies in diphtheria prophylaxis: the second response.* Br J Exp Pathol, 1950. **31**(2): p. 233-41.
33. Levesque, P.M. and U. de Alwis, *Mechanism of adsorption of three recombinant Streptococcus pneumoniae (Sp) vaccine antigens by an aluminum adjuvant.* Hum Vaccin, 2005. **1**(2): p. 70-3.
34. Hem, S.L. and H. Hogenesch, *Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation.* Expert Rev Vaccines, 2007. **6**(5): p. 685-98.
35. Noe, S.M., et al., *Mechanism of immunopotentiation by aluminum-containing adjuvants elucidated by the relationship between antigen retention at the inoculation site and the immune response.* Vaccine, 2010. **28**(20): p. 3588-94.
36. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. **287**(5459): p. 1816-20.
37. Pizza, M. and R. Rappuoli, *Neisseria meningitidis: pathogenesis and immunity.* Current Opinion in Microbiology, 2015. **23**: p. 68-72.
38. Tettelin, H., et al., *Complete genome sequence of Neisseria meningitidis serogroup B strain MC58.* Science, 2000. **287**(5459): p. 1809-15.
39. Masignani, V., et al., *Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870.* J Exp Med, 2003. **197**(6): p. 789-99.
40. Cantini, F., et al., *Solution Structure of the Factor H-binding Protein, a Survival Factor and Protective Antigen of Neisseria meningitidis*.* Journal of Biological Chemistry, 2009. **284**(14): p. 9022-9026.
41. Cendron, L., et al., *Structure of the uncomplexed Neisseria meningitidis factor H-binding protein fHbp (rLP2086).* Acta Crystallogr Sect F Struct Biol Cryst Commun, 2011. **67**(Pt 5): p. 531-5.

42. Schneider, M.C., et al., *Neisseria meningitidis recruits factor H using protein mimicry of host carbohydrates.* Nature, 2009. **458**(7240): p. 890-893.
43. Giuliani, M.M., et al., *The region comprising amino acids 100 to 255 of Neisseria meningitidis lipoprotein GNA 1870 elicits bactericidal antibodies.* Infect Immun, 2005. **73**(2): p. 1151-60.
44. Serruto, D., et al., *Neisseria meningitidis GNA2132, a heparin-binding protein that induces protective immunity in humans.* Proceedings of the National Academy of Sciences, 2010. **107**(8): p. 3770-3775.
45. Sahu, A. and M.K. Panoburn, *Identification of multiple sites of interaction between heparin and the complement system.* Molecular Immunology, 1993. **30**(7): p. 679-684.
46. Esposito, V., et al., *Structure of the C-terminal domain of Neisseria heparin binding antigen (NHBA), one of the main antigens of a novel vaccine against Neisseria meningitidis.* J Biol Chem, 2011. **286**(48): p. 41767-41775.
47. Esposito, S., et al., *A phase 2 randomized controlled trial of a multicomponent meningococcal serogroup B vaccine, 4CMenB, in infants (II).* Hum Vaccin Immunother, 2014. **10**(7): p. 2005-14.
48. Comanducci, M., et al., *NadA, a novel vaccine candidate of Neisseria meningitidis.* J Exp Med, 2002. **195**(11): p. 1445-54.
49. Hoiczyk, E., et al., *Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins.* Embo j, 2000. **19**(22): p. 5989-99.
50. Hamborg, M. and C. Foged, *Characterizing the Association Between Antigens and Adjuvants*, in *Subunit Vaccine Delivery*, C. Foged, et al., Editors. 2015, Springer New York: New York, NY. p. 413-426.
51. Clapp, T., et al., *Vaccines with aluminum-containing adjuvants: optimizing vaccine efficacy and thermal stability.* J Pharm Sci, 2011. **100**(2): p. 388-401.
52. D'Souza, A.J., et al., *Rapid deamidation of recombinant protective antigen when adsorbed on aluminum hydroxide gel correlates with reduced potency of vaccine.* J Pharm Sci, 2013. **102**(2): p. 454-61.
53. Estey, T., et al., *Evaluation of chemical degradation of a trivalent recombinant protein vaccine against botulinum neurotoxin by LysC peptide mapping and MALDI-TOF mass spectrometry.* J Pharm Sci, 2009. **98**(9): p. 2994-3012.
54. Heiger, D., *High performance capillary electrophoresis*. 2000: Agilent Technologies.
55. Gao, Z. and W. Zhong, *Recent (2018–2020) development in capillary electrophoresis.* Analytical and Bioanalytical Chemistry, 2022. **414**(1): p. 115-130.
56. Petersen, J.R., et al., *Capillary electrophoresis and its application in the clinical laboratory.* Clin Chim Acta, 2003. **330**(1-2): p. 1-30.
57. Harstad, R.K., et al., *Capillary Electrophoresis.* Anal Chem, 2016. **88**(1): p. 299-319.
58. Chetwynd, A.J., et al., *Current Application of Capillary Electrophoresis in Nanomaterial Characterisation and Its Potential to Characterise the Protein and Small Molecule Corona.* Nanomaterials (Basel), 2018. **8**(2).
59. D.Altria, K., *Capillary Electrophoresis Guidebook- Principles, Operation, and Applications*, ed. M.i.M. Biology. 1995: Humana Press.
60. Brown, W.A., *Principles and Practice*. 1997: Academic Press; 1997.
61. Liu, F.K., *Using micellar electrokinetic chromatography for the highly efficient preconcentration and separation of gold nanoparticles.* J Chromatogr A, 2009. **1216**(12): p. 2554-9.
62. Liu, F.-K. and G.-T. Wei, *Adding sodium dodecylsulfate to the running electrolyte enhances the separation of gold nanoparticles by capillary electrophoresis.* Analytica Chimica Acta, 2004. **510**(1): p. 77-83.
63. Lin, K.H., T.C. Chu, and F.K. Liu, *On-line enhancement and separation of nanoparticles using capillary electrophoresis.* J Chromatogr A, 2007. **1161**(1-2): p. 314-21.

64. Vessely, C., et al., *Stability of a trivalent recombinant protein vaccine formulation against botulinum neurotoxin during storage in aqueous solution.* J Pharm Sci, 2009. **98**(9): p. 2970-93.
65. Kim, H.R., et al., *Analysis of plasma protein adsorption onto PEGylated nanoparticles by complementary methods: 2-DE, CE and Protein Lab-on-chip system.* Electrophoresis, 2007. **28**(13): p. 2252-61.
66. Okamoto, Y., F. Kitagawa, and K. Otsuka, *Separation of cationic polymer particles and characterization of avidin-immobilized particles by capillary electrophoresis.* Electrophoresis, 2006. **27**(5-6): p. 1031-40.
67. Galisteo, F. and W. Norde, *Protein Adsorption at the Agl-Water Interface.* Journal of Colloid and Interface Science, 1995. **172**(2): p. 502-509.
68. Clausi, A.L., et al., *Inhibition of aggregation of aluminum hydroxide adjuvant during freezing and drying.* J Pharm Sci, 2008. **97**(6): p. 2049-61.
69. Agarwal, S., et al., *Effect of Aluminum Adjuvant and Preservatives on Structural Integrity and Physicochemical Stability Profiles of Three Recombinant Subunit Rotavirus Vaccine Antigens.* Journal of Pharmaceutical Sciences, 2020. **109**(1): p. 476-487.
70. Jully, V., et al., *Mechanisms of Antigen Adsorption Onto an Aluminum-Hydroxide Adjuvant Evaluated by High-Throughput Screening.* J Pharm Sci, 2016. **105**(6): p. 1829-1836.
71. Schmitt-Kopplin, P., *Capillary electrophoresis. Methods and protocols. Preface.* Methods Mol Biol, 2008. **384**: p. vii-ix.
72. Rabe, M., D. Verdes, and S. Seeger, *Understanding protein adsorption phenomena at solid surfaces.* Advances in colloid and interface science, 2011. **162 1-2**: p. 87-106.
73. Dong, A., et al., *Secondary structures of proteins adsorbed onto aluminum hydroxide: infrared spectroscopic analysis of proteins from low solution concentrations.* Anal Biochem, 2006. **351**(2): p. 282-9.
74. Norde, W., *Adsorption of proteins from solution at the solid-liquid interface.* Advances in Colloid and Interface Science, 1986. **25**: p. 267-340.
75. Manning, M.C., et al., *Stability of protein pharmaceuticals: an update.* Pharm Res, 2010. **27**(4): p. 544-75.
76. Wittayanukulluk, A., et al., *Effect of microenvironment pH of aluminum hydroxide adjuvant on the chemical stability of adsorbed antigen.* Vaccine, 2004. **22**(9-10): p. 1172-1176.
77. Hem, S.L., et al., *Preformulation studies—The next advance in aluminum adjuvant-containing vaccines.* Vaccine, 2010. **28**(31): p. 4868-4870.
78. Zheng, Y., et al., *Structural changes of protein antigens due to adsorption onto and release from aluminium hydroxide using FTIR–ATR.* Spectroscopy, 2007. **21**: p. 354051.
79. Shank-Retzlaff, M., et al., *Correlation between mouse potency and in vitro relative potency for human papillomavirus Type 16 virus-like particles and Gardasil vaccine samples.* Hum Vaccin, 2005. **1**(5): p. 191-7.
80. Cook, D.B., et al., *Multiplexing protein and gene level measurements on a single Luminex platform.* Methods, 2019. **158**: p. 27-32.
81. Marquette, C.A., B.P. Corgier, and L.J. Blum, *Recent advances in multiplex immunoassays.* Bioanalysis, 2012. **4**(8): p. 927-36.
82. Rahmoune, H. and P.C. Guest, *Application of Multiplex Biomarker Approaches to Accelerate Drug Discovery and Development.* Methods Mol Biol, 2017. **1546**: p. 3-17.
83. van Gageldonk, P.G., et al., *Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus.* J Immunol Methods, 2008. **335**(1-2): p. 79-89.
84. Europe.www.combistat.eu, C.v.E.-C.o.

85. Colaprico, A., et al., *Adsorption onto aluminum hydroxide adjuvant protects antigens from degradation.* Vaccine, 2020. **38**(19): p. 3600-3609.
86. Domina, M., et al., *Epitope Mapping of a Monoclonal Antibody Directed against Neisserial Heparin Binding Antigen Using Next Generation Sequencing of Antigen-Specific Libraries.* PLOS ONE, 2016. **11**(8): p. e0160702.
87. Vacca, I., et al., *Neisserial Heparin Binding Antigen (NHBA) Contributes to the Adhesion of Neisseria meningitidis to Human Epithelial Cells.* PLoS One, 2016. **11**(10): p. e0162878.
88. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants.* Curr Opin Immunol, 2009. **21**(1): p. 23-9.
89. Ghimire, T.R., *The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm.* SpringerPlus, 2015. **4**(1): p. 181.
90. Jones, L.S., et al., *Effects of Adsorption to Aluminum Salt Adjuvants on the Structure and Stability of Model Protein Antigens*.* Journal of Biological Chemistry, 2005. **280**(14): p. 13406-13414.
91. Scheiblhofer, S., et al., *Influence of protein fold stability on immunogenicity and its implications for vaccine design.* Expert Rev Vaccines, 2017. **16**(5): p. 479-489.
92. Maste, M.C.L., W. Norde, and A. Visser, *Adsorption-Induced Conformational Changes in the Serine Proteinase Savinase: A Tryptophan Fluorescence and Circular Dichroism Study.* J Colloid Interface Sci, 1997. **196**(2): p. 224-230.
93. Liu, F., et al., *Kinetics and Mechanisms of Protein Adsorption and Conformational Change on Hematite Particles.* Environmental Science & Technology, 2019. **53**(17): p. 10157-10165.
94. Wei, Y., et al., *Adsorption-induced changes in ribonuclease A structure and enzymatic activity on solid surfaces.* Langmuir, 2014. **30**(49): p. 14849-58.
95. Harris, J.R., et al., *Alhydrogel® adjuvant, ultrasonic dispersion and protein binding: a TEM and analytical study.* Micron, 2012. **43**(2-3): p. 192-200.
96. Ausar, S.F., et al., *Application of extrinsic fluorescence spectroscopy for the high throughput formulation screening of aluminum-adjuvanted vaccines.* J Pharm Sci, 2011. **100**(2): p. 431-40.
97. M. Chance, *"Mass Spectrometry for protein-protein interactions and dynamics"*

Wiley

2008.
98. Tullius, T.D. and B.A. Dombroski, *Hydroxyl radical "footprinting": high-resolution information about DNA-protein contacts and application to lambda repressor and Cro protein.* Proc Natl Acad Sci U S A, 1986. **83**(15): p. 5469-73.
99. Chance, M.R., et al., *Examining the conformational dynamics of macromolecules with time-resolved synchrotron X-ray 'footprinting'.* Structure, 1997. **5**(7): p. 865-9.
100. Hanai, R. and J.C. Wang, *Protein footprinting by the combined use of reversible and irreversible lysine modifications.* Proc Natl Acad Sci U S A, 1994. **91**(25): p. 11904-8.
101. Garcia, N.K., et al., *Optimizing Hydroxyl Radical Footprinting Analysis of Biotherapeutics Using Internal Standard Dosimetry.* Journal of the American Society for Mass Spectrometry, 2020. **31**(7): p. 1563-1571.
102. Huang, R.Y.C. and G. Chen, *Higher order structure characterization of protein therapeutics by hydrogen/deuterium exchange mass spectrometry.* Analytical and Bioanalytical Chemistry, 2014. **406**(26): p. 6541-6558.
103. McKenzie-Coe, A., N.S. Montes, and L.M. Jones, *Hydroxyl Radical Protein Footprinting: A Mass Spectrometry-Based Structural Method for Studying the Higher Order Structure of Proteins.* Chemical Reviews, 2022. **122**(8): p. 7532-7561.

104. Horinishi, H., et al., *STATES OF AMINO ACID RESIDUES IN PROTEINS. 3. HISTIDINE RESIDUES IN INSULIN, LYSOZYME, ALBUMIN AND PROTEINASES AS DETERMINED WITH A NEW REAGENT OF DIAZO-I-H-TETRAZOLE.* Biochim Biophys Acta, 1964. **86**: p. 477-89.
105. Wang, L. and M.R. Chance, *Protein Footprinting Comes of Age: Mass Spectrometry for Biophysical Structure Assessment*.* Molecular & Cellular Proteomics, 2017. **16**(5): p. 706-716.
106. Xu, G. and M.R. Chance, *Hydroxyl Radical-Mediated Modification of Proteins as Probes for Structural Proteomics.* Chemical Reviews, 2007. **107**(8): p. 3514-3543.
107. Xu, G. and M.R. Chance, *Radiolytic Modification of Acidic Amino Acid Residues in Peptides: Probes for Examining Protein–Protein Interactions.* Analytical Chemistry, 2004. **76**(5): p. 1213-1221.
108. Xu, G., et al., *Secondary Reactions and Strategies To Improve Quantitative Protein Footprinting.* Analytical Chemistry, 2005. **77**(10): p. 3029-3037.
109. Guan, J.Q. and M.R. Chance, *Structural proteomics of macromolecular assemblies using oxidative footprinting and mass spectrometry.* Trends Biochem Sci, 2005. **30**(10): p. 583-92.
110. Xu, G., K. Takamoto, and M.R. Chance, *Radiolytic modification of basic amino acid residues in peptides: probes for examining protein-protein interactions.* Anal Chem, 2003. **75**(24): p. 6995-7007.
111. Huang, W., et al., *Quantitative mapping of protein structure by hydroxyl radical footprinting-mediated structural mass spectrometry: a protection factor analysis.* Biophys J, 2015. **108**(1): p. 107-15.
112. Maleknia, S.D. and K.M. Downard, *Advances in radical probe mass spectrometry for protein footprinting in chemical biology applications.* Chem Soc Rev, 2014. **43**(10): p. 3244-58.
113. Wang, L. and M.R. Chance, *Structural mass spectrometry of proteins using hydroxyl radical based protein footprinting.* Anal Chem, 2011. **83**(19): p. 7234-41.
114. Fenton, H.J.H., *LXXIII.—Oxidation of tartaric acid in presence of iron.* Journal of the Chemical Society, Transactions, 1894. **65**(0): p. 899-910.
115. Nehls, T., et al., *Fenton-Chemistry-Based Oxidative Modification of Proteins Reflects Their Conformation.* International Journal of Molecular Sciences, 2021. **22**(18): p. 9927.
116. Sharp, J.S., J.M. Becker, and R.L. Hettich, *Protein surface mapping by chemical oxidation: structural analysis by mass spectrometry.* Anal Biochem, 2003. **313**(2): p. 216-25.
117. Maritan, M., et al., *Structures of NHBA elucidate a broadly conserved epitope identified by a vaccine induced antibody.* PLoS One, 2018. **13**(8): p. e0201922.
118. Morefield, G.L., et al., *Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro.* Vaccine, 2005. **23**(13): p. 1588-95.