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Separation of three strains of polio virus by capillary zone electrophoresis and study of their interaction with aluminum oxyhydroxide.

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Abstract

The development of combination vaccines is essential to reduce the number of injections, shorten vaccination schedules and increase vaccination coverage. In these vaccine adjuvants can be used to modulate and enhance the immune response induced by the antigens. To support the development of combination vaccines, the study of antigen-adjuvant interactions in the final vaccine formulations is required as interaction competitions may take place between the different antigens. In the present work, a capillary zone electrophoresis (CZE) methodology was firstly optimized on six model proteins, namely bovine serum albumin, β -lactoglobulin, myoglobin, ribonuclease A, cytochrome C and lysozyme. A cationic dynamic coating (polybrene) and a zwitterionic amino acid (β -alanine) in the background electrolyte were used to reduce the phenomena of protein adsorption on the inner wall of the capillary and thus optimize the separation efficiency of the proteins/antigens. The developed methodology was then used to separate three stains from inactivated polio virus and to study their interaction with aluminum oxyhydroxide by simultaneously quantifying the three free antigens present in the formulation. The antigen-adjuvant interactions could be modulated by addition of phosphate ions playing the role of competitors for the poliovirus.

Keywords: Combination vaccines, capillary zone electrophoresis, virus, aluminum oxyhydroxide

1. Introduction

Three centuries after the first formal vaccination trials by Edward Jenner, vaccination remains one of the most impactful developments in medicine, lengthen life, in particular by reducing infant mortality^{1,2}. As the number of new vaccines increased, the immunization schedule became more and more challenging³. Combination vaccines are a powerful tool to limit the burden associated with multiple vaccine injections⁴. Combination vaccines are vaccines that are designed to protect against two or more diseases or against one disease caused by different strains or serotypes of the same organism. Reducing the number of injections by combining multiple antigens of one or several pathogens into a single syringe allows to reduce vaccination visits and saves time for patients and doctors. The simplification of vaccination schedules allows to increase vaccine coverage, which decreases the risk of epidemic outbreaks. Combination vaccines also allow to reduce health costs, as a large proportion of vaccination costs arises from maintaining cold chains, storage, transport and salaries of medical staff⁵. As the number of untreated medical needs remains high and as new infectious diseases are periodically emerging, the development of new vaccines, as well as the optimization of existing vaccines, represent a significant medical need requiring new combination vaccines.

Vaccine antigens alone have sometimes insufficient immunostimulatory capabilities. Adjuvants are substances that are frequently added into vaccine formulations to modulate and enhance the immune response induced by the antigen^{6,7}. In 1926, Glenny et al. showed the potential of aluminum salts to increase the production of antibodies⁸. Since then, aluminum salts have been the adjuvant of choice in more than 100 licensed vaccines, incorporated into billions of doses^{9,10}. Aluminum adjuvants will continue to be used in future vaccines as they are known to be effective with many of the various antigens and present good track record of safety¹¹. The strength of the interaction between antigens and adjuvants impacts the immune response¹²⁻¹⁴. A

strong antigen-adjuvant interaction is not always desired as the immunogenicity can be reduced when the antigen is too strongly adsorbed on the adjuvant¹⁵⁻¹⁷. In the case of combination vaccines, interaction competitions may take place between the different antigens with the adjuvant. This is why interactions studies between antigens and adjuvant are important for the development of combination vaccines and have to be performed on the final formulations. The quantification of the free antigens is desired for the study of antigen-adjuvant interactions. However, accurate simultaneous quantification of multiple antigens in combination vaccines is crucial and challenging¹⁸.

Capillary zone electrophoresis (CZE) is a technique largely used for the separation of proteins; it is simple, fast, automatic and requires only a few nanoliters of samples, with a relatively low analytical costs¹⁹⁻²¹. Despite these advantages the use of CZE for the analysis of vaccine formulations is still limited²²⁻²⁵. An important issue concerning protein analysis by CZE is the possible adsorption onto the negatively charged surface of bare fused silica capillaries. Indeed, it is well known that adsorption severely degrades separation performances due to peak broadening²⁶. In order to reduce protein adsorption, a common approach is to use coated capillaries. Capillary coatings can be categorized as permanent coatings (covalently linked) and semi-permanent (physically adsorbed) coatings. Due to their ease of use, neutral semi-permanent coatings, such as hydroxypropylmethylcellulose (HPMC)²⁷⁻²⁹, hydroxypropylcellulose (HPC)³⁰⁻³², ultratrolTM LN³³, and polyethylene oxide (PEO)³⁴, or cationic coatings, such as polybrene (PB)^{35,36} and polydiallyldimethylammonium chloride (PDADMAC)³⁷ are often used. Using zwitterionic additives such as ϵ -aminocaproic acid (EACA) in the background electrolyte (BGE) was also reported to improve separation efficiency for the separation of monoclonal antibodies (mAb)³⁸⁻⁴¹. EACA being neutral between pH 4.43 and 10.75⁴², high concentrations of EACA in BGE allow to reduce protein adsorption without

impacting too much the conductivity (and Joule heating) in this pH range. Moreover, the low UV absorption of EACA has little impact on the detection sensitivity of proteins at 214 nm.

In this work, a CZE method, initially optimized for the separation of six model proteins (bovine serum albumin (BSA), β -lactoglobulin (BLG), myoglobin (MB), ribonuclease A (RNase A), cytochrome C (Cyt C) and lysozyme (LYZ)) was successfully applied to separate three inactivated poliomyelitis vaccine (IPV) strains, enabling the characterization of their interactions with aluminum oxyhydroxide. The impact of phosphate ions on the antigen / adjuvant interaction was also discussed.

2. Experimental section

2.1. Chemicals and Materials. Aluminium oxyhydroxide (AlOOH) and three inactivated poliomyelitis vaccine strains (IPV1, IPV2, IPV3) were provided by Sanofi Pasteur (Marcy-l'Étoile, France). Bovine serum albumin (BSA), β -lactoglobulin from bovine milk (BLG), myoglobin from equine skeletal muscle (MB), ribonuclease A from bovine pancreas (RNase A), cytochrome C from bovine heart (Cyt C), lysozyme from chicken egg white (LYZ), ϵ -aminocaproic acid (6-aminohexanoic acid, EACA), β -alanine (3-aminopropionic acid, β -ala), tris(hydroxymethyl)aminomethane (TRIS), phosphate buffered saline (PBS), and polybrene (hexadimethrine bromide, PB) were purchased from Sigma-Aldrich (Merk, Darmstadt, Germany). Formic acid (HCOOH) was purchased from VWR (Fontenay-sous-bois, France). Bare fused silica capillaries were purchased from Molex Polymicro Technologies (Phoenix, USA). Deionized water was further purified on a Milli-Q system from Millipore (Molsheim, France).

2.2. Sample preparation. Stock solutions of BSA, BLG, MB, RNase A, Cyt C and LYZ were prepared at 4 g/L in ultrapure water. Protein mixture at 0.67 g/L final concentration for each protein was then obtained by mixing an equivalent volume of each of these stock solutions.

Protein mixture at 0.33 g/L for each protein was also prepared in the same way in TRIS buffer (10 mM TRIS-HCl, 157 mM NaCl, pH 7.4). ALOOH solution was prepared at 1 g/L in TRIS buffer and also in PBS buffer (10 mM PBS, 157 mM NaCl, pH 7.4). A mixture of the three inactivated poliovirus strains (IPV123) at 0.1 g/L for IPV1 and IPV3 and 0.2 g/L for IPV2 was prepared in TRIS buffer and also in PBS buffer.

2.3. Capillary coating. Bare fused silica capillaries of 50 μm i.d. \times 50 cm or 75 cm total length were used. The capillaries were coated with polybrene using the following successive flushes: ultrapure water (1 min), 0.5 % (w/w) polybrene in water (7 min), ultrapure water (1 min), background electrolyte (1 min). The coated capillaries were systematically rinsed with a 0.5 M NaOH - 2 M NaCl mixture (7 min) and then coated again using the previous protocol before use and between two successive runs.

2.4. CE. Experiments were carried out on a 1600 CE Agilent system (Santa Clara, USA) (Figure 1 and 2), on a PA800 CE system from SCIEX (Framingham, USA) (Figure 2) and on a 7100 CE Agilent system (Santa Clara, USA) (Figure 4 and 5). The systems were equipped with diode array detectors (DAD). Capillaries dimensions and injections conditions are stated in the figures caption.

3. Results and discussion

3.1. Optimization of the separation of multiple proteins/antigens by CZE

The first step of this work was to develop a CZE method allowing to simultaneously analyze multiple antigens. A mixture of six model proteins (BSA, BLG, MB, RNase A, Cyt C and LYZ) in water was first used as model antigen mixture. Table 1 gathers the isoelectric points (pI), molar

mass and effective electrophoretic mobility (μ_{ep}) of the different studied proteins. A dynamic polybrene cationic coating was used to prevent protein adsorption onto the capillary surface, leading to improved separation efficiencies and better migration time repeatabilities compared to fused silica capillaries. Analyses were carried out at pH below 4.7 in order to keep all the proteins positively charged and to avoid electrostatic interactions with the cationic capillary coating. Separations were performed in counter electroosmotic mode: proteins were detected in their increasing order of effective mobility. The choice of the BGE constituents was also a key parameter to optimize for improved separation efficiencies. As shown in Figure 1A, the baseline resolution of the different proteins was poor at pH 2.6 in 50 mM HCOOH BGE. In contrast, Figure 1B displays a significantly improved separation of the same protein mixture analyzed at pH 4.6 in 175 mM HCOOH BGE containing 400 mM EACA. Indeed, EACA is known to reduce protein adsorption by competitive adsorption of the zwitterion EACA aminoacid onto the capillary wall^{38,43}. In the literature, EACA was often used at pH 4.6-6.0⁴⁰, however at pH 4.6 the acidic group of EACA (pK_a : 4.4 (-COOH), 10.8 (-NH₂))⁴² becomes partially protonated leading to increased conductivity of the BGE (current intensity \sim -70 μ A at -20 kV) and lower proportion of zwitterionic forms. Alternatively, to work at pH 4.6, we propose to use β -alanine (pK_a : 3.6 (-COOH), 10.2 (-NH₂))⁴⁴ instead of EACA, since its pI (6.9) is lower of about one pH unit compared to EACA. As shown in Figure 1C, the separation of the six proteins at pH 4.6 in 50 mM HCOOH with 400 mM β -ala was baseline resolved in a shorter time (within 10 min) than for EACA, due to lower conductivity (lower ionic strength, generated current of -19 μ A at -20 kV). Indeed, it well known that the electroosmotic mobility increases faster with decreasing ionic strength compared to protein electrophoretic effective mobility^{45,46}. It can be observed that the resolution of MB (peak 3) and RNase A (peak 4), was better in HCOOH - β -ala BGE than in HCOOH - EACA BGE. As a first conclusion, the use of β -ala allows to perform electrophoretic

separation at lower pH than EACA, with reasonable BGE conductivity and modest Joule heating.

Consequently, HCOOH – β -ala based electrolytes were retained for the following experiments.

Table 1. Isoelectric point (pI), molar mass (M) and effective electrophoretic mobility (μ_{ep}) of the model proteins. Experimental conditions for μ_{ep} determination: PB-coated capillary, 50 cm total length (41.5 cm to the detector) \times 50 μ m i.d. BGE: HCOOH 50 mM + β -Ala 400 mM pH 4.6. Hydrodynamic injection: 42 mbar, 6 s. Applied voltage: –20 kV (from inlet). UV detection: 214 nm. Temperature: 25 °C. Proteins concentration: 0.67 mg/mL in water.

	BSA	LGB	MB	RNase A	Cyt C	LYZ
M (kDa)	66.5	18.4	17.6	13.7	12.3	14.3
pI	4.7 – 5.6 ⁴⁷	5.1 – 5.5 ⁴⁷	6.5 – 7.2 ⁴⁸	8.9 – 9.6 ⁴⁷	9 – 10 ⁴⁹	10.7 ⁵⁰
μ_{ep} ($10^{-9} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$)	2.2	5.8	10.1	10.7	13.9	14.8

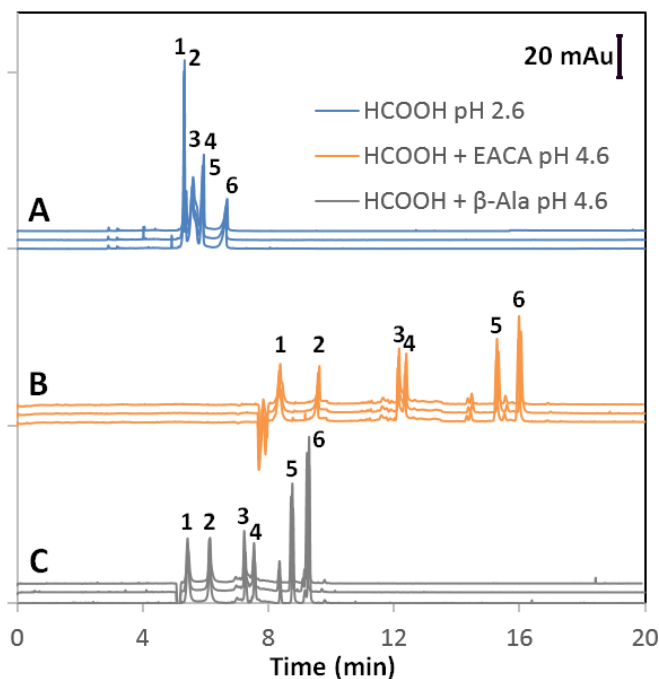


Figure 1: Influence of the addition of EACA or β -Ala on protein separation using a PB-coated capillary. Electropherograms obtained in: 50 mM HCOOH, pH 2.6 (A), 175 mM HCOOH + 400 mM EACA, pH 4.6 (B), 50 mM HCOOH + 400 mM β -Ala, pH 4.6 (C). Experimental conditions: PB-coated capillary, 50 cm total length (40 cm to the detector) \times 50 μ m i.d. Hydrodynamic injection: 42 mbar, 6 s.

Applied voltage: -20 kV (from inlet). UV detection: 214 nm. Temperature: 25 °C. Proteins concentration: 0.67 mg/mL in water. Peak identification: BSA (1), LGB (2), MB (3), RNase A (4), Cyt C (5), LYZ (6).

The net charge of the proteins, and therefore their electrophoretic mobility, directly depends on the pH of the BGE and can change the selectivity of the separation. Mixtures of 400 mM β -Ala with different concentrations of HCOOH at pH below or equal to 4.7 have been tested (see Figure 2). At pH 4.7 the intensity of peaks 1 and 2 was low, most likely due to some protein adsorption. At pH lower than 4.6 , the separation of peaks 3 and 4, and of peaks 5 and 6 decreased, leading to overlapping peaks. The pH was therefore set at 4.6 , considered as the optimal value in the following.

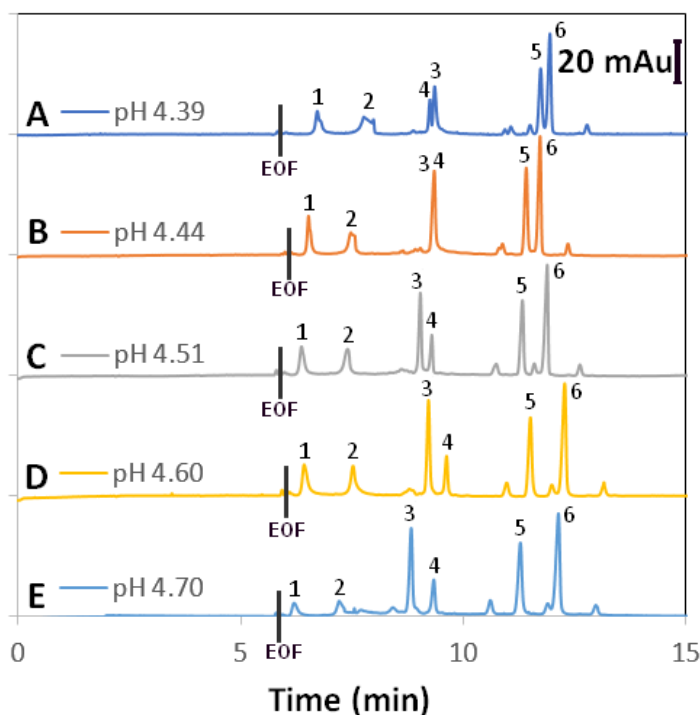
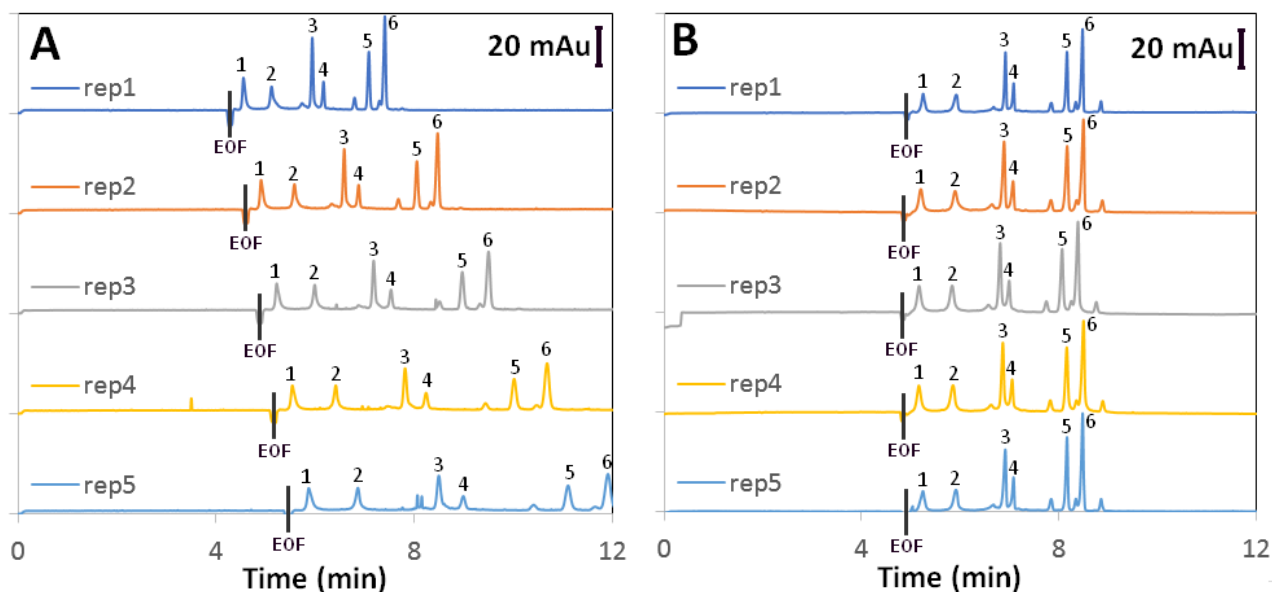


Figure 2: Influence of the pH of the BGE on the separation of six model proteins. Electropherograms obtained in: β -Ala 400 mM + HCOOH: 80 mM (A), 70 mM (B), 60 mM (C), 50 mM (D), 40 mM (E). Experimental conditions: PB-coated capillary, 51.6 cm total length (41.5 cm to the detector) \times 50 μ m i.d. capillary. Other experimental conditions as in Figure 1. Peak identification: BSA (1), LGB (2), MB (3), RNase A (4), Cyt C (5), LYZ (6).

Intra-capillary repeatability was investigated in Figure 3 using a PB coated capillary and two different rinsing conditions between successive runs. As shown in Figure 3A, when rinsing the capillary for 1 min with water and 4 min with BGE, migration times increased, and separation efficiency decreased with the repetitions. In contrast, when the rinsing protocol include a rinsing step with a mixture of NaOH 1M and NaCl 2M⁵¹ followed with a PB coating of the capillary, the intra-capillary repeatability improved (from 14 % to 0.5 % for the RSD on migration times, $n=5$



repetitions).

Figure 3: Influence of rinsing conditions between successive runs on the intra-capillary repeatability. Experimental conditions: PB coated capillary, 50 cm total length (41.5 cm to the detector) \times 50 μ m i.d. capillary. Rinsing steps: 1 min H₂O, 4 min BGE (A), 1 min H₂O, 7 min NaOH 0.5 M + NaCl 2 M, 1 min H₂O, 7 min PB, 1 min H₂O, 4 min BGE (B). BGE: HCOOH 50 mM + β -Ala 400 mM pH 4.6. Other experimental conditions as in Figure 1. Peak identification: BSA (1), LGB (2), MB (3), RNase A (4), Cyt C (5), LYZ (6).

3.2. Monitoring multiple protein interaction with aluminum oxyhydroxide by CE

The interactions between the six model proteins with aluminum oxyhydroxide were studied by injecting the protein mixture (initial concentration of 0.17 g/L for each protein) after two

hours of contact with aluminum oxyhydroxide (0.5 g/L) in TRIS buffer at pH 7.4, and after centrifugation (see experimental part for more details). Figure 4A compares the electrophoretic separation of the protein's solution before (upper trace) and after (lower trace) interaction with AlOOH. In the latter case, the supernatant resulted from the centrifugation of the protein-AlOOH mixtures was injected. It is worth noting that centrifugation prevents the capillary from clogging with AlOOH. Figure 4B compares the protein peak area normalized to the area of the TRIS ion (noted T on the electropherogram) before and after interaction with AlOOH. The electropherogram of the supernatant shows that BSA (1) and LGB (2) peaks were not detected (Figure 4A), demonstrating a complete adsorption of these proteins on AlOOH. On the contrary, MB (3), RNase A (4), Cyt C (5) and LYZ (6) proteins did not interact with AlOOH since the peak area did not significantly change before /after the AlOOH addition (see peak area ratio in Figure 4B). These results are in agreement with electrostatic interactions. Indeed, AlOOH ($pI \sim 11^{52,53}$) is positively charged at pH 7.4, while BSA and LGB are negatively charged ($pI < 5.6$), MB is almost neutral, and RNase A, Cyt C and LYZ are positively charged ($pI > 8.9$, see Table 1).

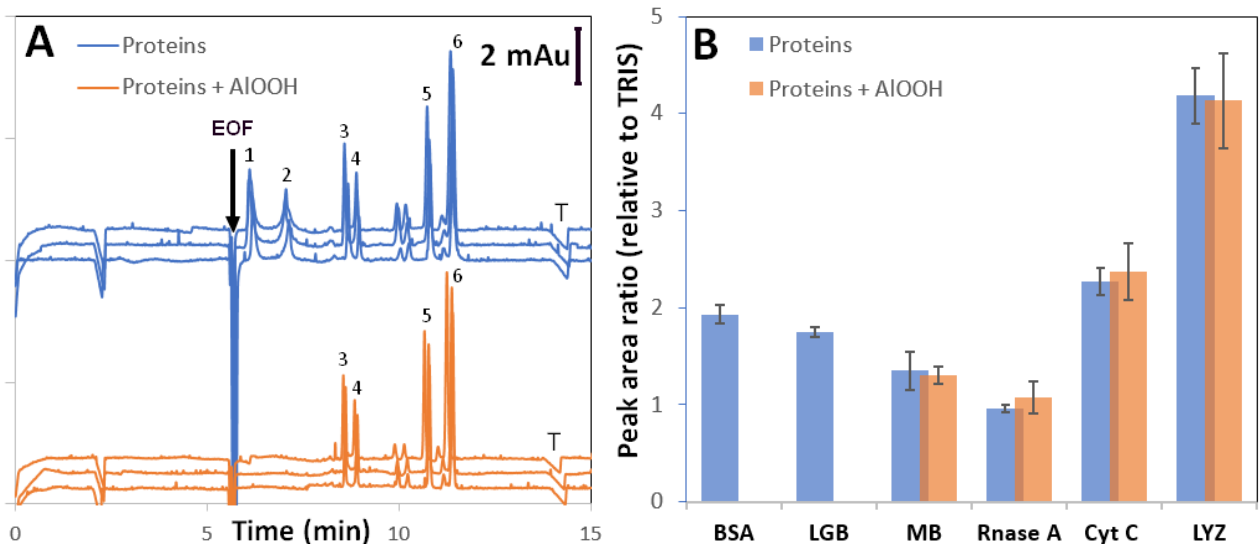


Figure 4: Monitoring multiple protein interactions with AlOOH. Electropherograms before and after addition of AlOOH (**A**) and the corresponding histograms of the normalized protein peak area relative TRIS (**B**). Experimental conditions: PB-coated capillary, 50 cm total length (41.5 cm to the detector) × 50 µm i.d. BGE: HCOOH 50 mM + β-Ala 400 mM pH 4.6. Hydrodynamic injection: 42 mbar, 6 s. Applied voltage: -20 kV (from inlet). UV detection: 214 nm. Temperature: 25 °C. Proteins concentration: 0.17 mg/mL in TRIS buffer. Peak identification: BSA (1), LGB (2), MB (3), RNase A (4), Cyt C (5), LYZ (6), TRIS ion (T).

3.3. Application to monitoring of interaction between inactivated poliovirus strains and aluminum oxyhydroxide by CE

The optimized method was next applied to the separation of three inactivated poliovirus strains (IPV1, IPV2 and IPV3), which are made up of four different proteins (VP1, VP2, VP3 and VP4). Keeping all the experimental parameters as previously defined for the separation of the six model proteins, the separation between polio strains 1 and 2 was not baseline resolved, as shown in Figure 5A (at -20 kV). Reducing the applied voltage to -10 kV led to a better separation of the three strains (Figure 5A) in good agreement with recent results showing that reducing the separation voltage increased the separation efficiency of proteins even in the presence of very weak residual adsorption⁵⁴. The capillary was also lengthened from 50 cm (41.5 cm to the detector) up to 75 cm (66.5 cm to the detector) to get baseline resolution between IPV1 and IPV2, as shown in Figure 5B.

The interaction between the three IPV strain and AlOOH was then investigated. The IPV-AlOOH mixing protocol was the same the one used for the model proteins. In the TRIS buffer (10 mM TRIS, 157 mM NaCl, pH 7.4), the polio strains were not detected after their contact with AlOOH, which can be explained by the fact that the three antigens are negatively charged at pH 7.4 (pI varying in the 6.1-6.4 range) and thus interacted with AlOOH. In contrast, when conducting the experiment in the PBS buffer (10 mM PBS, 157 mM NaCl, pH 7.4), the three IPV

could be detected meaning that the interaction with AlOOH was suppressed in presence of phosphate ions which are known to strongly interact with AlOOH⁵⁵⁻⁵⁷. This example clearly demonstrates the interest of the developed method to study antigen / adjuvant interactions in vaccine formulations and the effect of excipients (nature of buffering ions, aminoacids, additives, preservatives...).

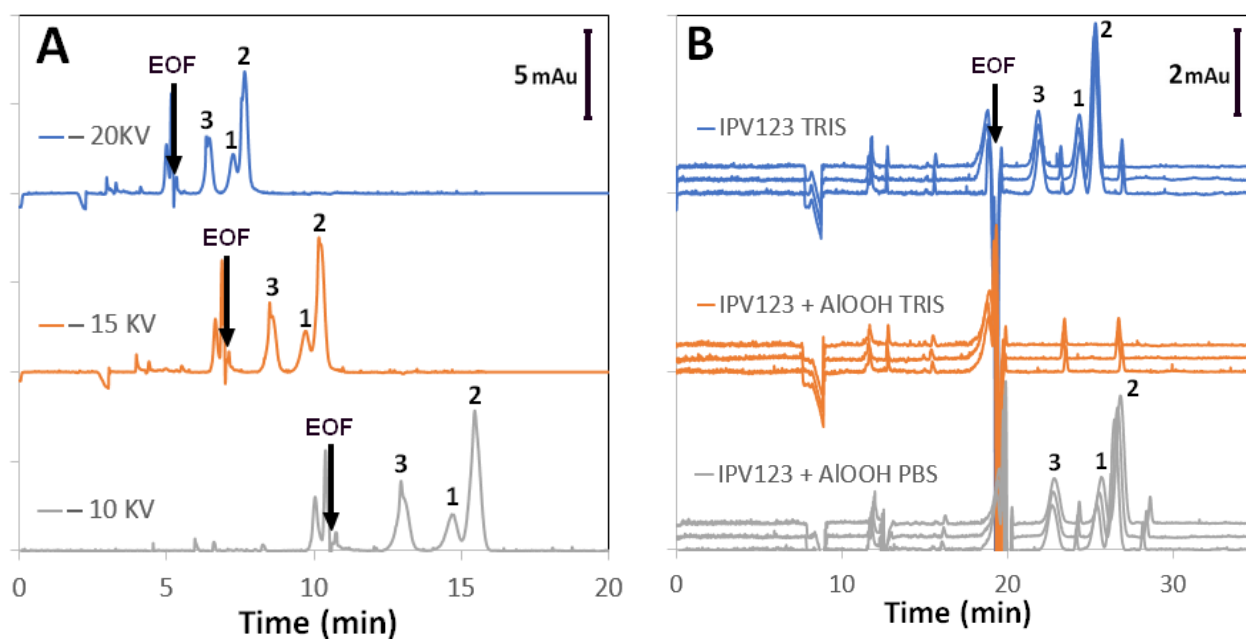


Figure 5: Electrophoretic separation of the three inactivated poliovaccine antigen (IPV). Electropherograms with different applied voltage (A) and before (upper trace) and after interacting with AlOOH (middle trace in TRIS buffer, lower trace in PBS buffer) (B). Experimental conditions: PB-coated capillary, 50 cm total length (41.5 cm to the detector) \times 50 μ m i.d. (A), 75 cm total length (66.5 cm to the detector) \times 50 μ m i.d. (B) Separation voltage: as indicated on the figure (A), -10 kV (B). Hydrodynamic injection: 42 mbar, 6 s. UV detection: 214 nm. Temperature: 25 $^{\circ}$ C. Peak identification: IPV3 (1), IPV1 (2), IPV2 (3).

4. Conclusions

This work illustrates the suitability of capillary zone electrophoresis (CZE) to study interactions between antigens and adjuvants in the case of vaccines containing mixtures of

several antigens. It has been shown that β -alanine can be advantageously used as an alternative to EACA in the background electrolyte to improve the protein separation at pH 4.4-4.7 range. β -alanine based BGE can be used for the analysis of proteins with lower pI compared to the use of EACA. A dynamic polybrene capillary coating was used to reduce protein adsorption on the capillary wall and to optimize separation efficiency. The developed methodology led to baseline separation between the three poliovirus strains without denaturation and allowed to individually study their interaction with aluminum oxyhydroxide adjuvant. The methodology gives a rapid and direct access to the percentage of free antigens in a combination vaccine formulation and can therefore be used to optimize vaccine formulations by monitoring the impact of the physico-chemical parameters on the interactions or to follow vaccine stability.

With regard to its limitations, CZE with UV detection has detection limits close to 0.01 g/L in antigen concentration. A fluorescent detector with fluorescent responding antigens would be required to lower the quantification limit. The development of new analytical methods to study combination vaccines goes hand in hand with the development of these vaccines, which are essential to shorten vaccination schedules and increase vaccination coverage. The study of interactions between antigens and adjuvants allow a better understanding of the adjuvant impact on the immunogenicity, which is useful for the development of new vaccines. The developed method can also be used to optimize combination vaccines formulations by monitoring the impact of the physicochemical parameters on the interaction and to follow vaccines stability.

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