DANIELA REIS JOAQUIM DE FREITAS
(ORGANIZADORA)

AGENDA GLOBAL DE PESQUISA EM CIÊNCIAS BIOLÓGICAS

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Telefone: +55 (42) 3323-5493
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APRESENTAÇÃO

A pesquisa não pode parar. Isto é um fato. E o livro "Agenda global de Pesquisa em Ciências Biológicas" é a prova de que o Brasil é profícuo quando se trata de pesquisa. Esta obra é composta por trabalhos científicos produzidos em diversas partes do país na forma de artigos originais e de revisão, que abordam desde o cultivo, triagem e citocompatibilidade de células-tronco mesenquimais expostas à nanotubos funcionalizados de carbono multicamadas até o controle de qualidade microbiológica do sururu (*Mytella falcata*) produzido no Rio de Janeiro, ou a análise temporal da disseminação de vegetação exótica em dunas do litoral do Rio Grande do Sul, ou o desenvolvimento do turismo e as mulheres erveiras da Amazônia. Todas estas pesquisas possuem campo dentro das Ciências Biológicas, mas fazem interface com meio Ambiente, Engenharia, Ciências da Saúde, Antropologia, Tecnologia de alimentos, entre outras áreas.

Ao longo de 13 capítulos serão discutidas diferentes temáticas, com embasamento teórico-científico adequado, atualizado e serão revistos conceitos importantes. Este livro é principalmente voltado para os estudantes e profissionais que desejam se aprofundar mais na pesquisa na grande área das Ciências Biológicas, com uma leitura rápida, dinâmica e cheia de possibilidades de aprendizado.

Assim como todas as publicações da Atena Editora, esta obra passou pela revisão de um Comitê de pesquisadores com mestrado e doutorado em programas de pós-graduação renomados no Brasil. Portanto, apresentamos ao leitor um trabalho de qualidade, atualizado e devidamente revisado por pares.

Boa leitura.

Daniela Reis Joaquim de Freitas

SUMÁRIO

CAPÍTULO 5

QUALITY CONTROL OF ANTIVIRAL VACCINES WITH THE LITESIZER

Data de aceite: 25/10/2021

Nathalie Etchart
Anton Paar GmbH

Eduardo C. Araújo
Anton Paar Brasil

Talita Cardeal
Anton Paar Brasil

ABSTRACT: The particle size of vaccines has a considerable influence on their half-life *in vivo*, as well as on their uptake by antigen-presenting cells. The surface charge of particles is also suspected of influencing the same parameters. Here we use DLS and ELS to characterize respectively the particle size and zeta potential of two inactivated antiviral vaccines.

KEYWORDS: DLS, ELS, particle size, zeta potential, pharmaceuticals, inactivated virus, vaccine adjuvants, aluminum salt, cold chain

CONTROLE DE QUALIDADE DE VACINAS ANTIVIRAIS UTILIZANDO DLS/ELS

RESUMO: O tamanho das partículas das vacinas tem uma influência considerável em sua meia-vida in vivo, bem como em sua absorção pelas células apresentadoras de antígenos. A carga superficial das partículas também é suspeita de influenciar os mesmos parâmetros. No artigo, utiliza-se DLS e ELS para caracterizar, respectivamente, o tamanho de partícula e o potencial zeta de duas vacinas antivirais inativadas.

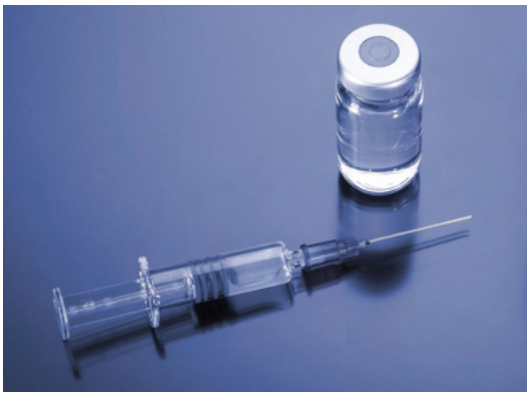
PALAVRAS - CHAVE: Vacina, DLS, ELS, tamanho de partículas, potencial zeta.

BACKGROUND

The particle size of vaccines has a considerable influence on their half-life *in vivo*, as well as on their uptake by antigen-presenting cells. The surface charge of particles is also suspected of influencing the same parameters. Here we use DLS and ELS to characterize respectively the particle size and zeta potential of two inactivated antiviral vaccines.

RESULTS

A tick-borne encephalitis (TBE) vaccine displays a monomodal particle size distribution in the lower micrometer range, corresponding to the expected size of the aluminum salt adjuvant. A cell-based influenza vaccine, in contrast, is shown to contain both split viruses (ca. 30 nm) and larger aggregates (ca. 250 nm). Zeta potential measurements indicate that both vaccines consist of weakly anionic particles. Interestingly, we demonstrate that simulated cold chain disruptions (heat treatment, freeze-thawing) induce significant changes in the particle size distribution of both vaccines.



1 | INTRODUCTION

Vaccines often constitute the only line of defense against viral infections, as the range of antiviral drugs currently available and treatment success are limited. The situation is different for bacterial infections, where antibiotics are an efficient therapeutic intervention.

Antiviral vaccines can consist of live-attenuated viral particles, which produce a low-noise infection in the recipient. While this strategy is able to mimic an infection by the pathogen very closely, and generally triggers a very robust immune response, it has the potential for serious side effects in immuno- compromised individuals.

Thus, a majority of antiviral vaccines now consist in formulations which do not have the potential for replication in the host. These range from chemically inactivated whole or split viruses, to recombinant proteins or virus-like particles produced by genetic engineering. While these vaccines have a better safety profile, they also tend to trigger weaker immune responses than their live-attenuated counterparts.

Hence, many of them are administered together with so-called vaccine adjuvants, which increase the efficacy and longevity of the immune response.

The oldest and still most popular such adjuvant is aluminum salt (e.g., aluminum hydroxide or aluminum hydroxyphosphate). Its immuno-stimulatory properties are believed to be linked both to its capacity to adsorb and retain antigens for long periods at the site of injection, and to its ability to trigger the local release of pro-inflammatory mediators (1).

The particle size of a vaccine has a significant impact on its immunogenicity. With few exceptions, viruses are nanoparticles ranging in size from 15 to 300 nm. Upon injection, particles in this size range are efficiently taken up by dendritic cells, a class of sentinel cells uniquely endowed with the ability to induce both antibody- and killer cell-mediated immunity (2). In contrast, particles in the micrometer range, such as aluminum salt particles (1), are preferentially taken up by monocytes and macrophages, which predominantly induce an antibody-mediated immune response.

Bearing in mind that the distribution of the different classes of sentinel cells in the

body is strongly tissue- specific, a vaccine's particle size should therefore be tailored:

- Based on the type of immune response required to counter the pathogen, and
- Depending on the vaccine's delivery route (3).

Dynamic light scattering (DLS) is a fast and non- invasive measurement method which elucidates the size distribution of particles in the lower nanometer to lower micrometer size range. This makes DLS a method of choice for the quality control of antiviral vaccines (3).

Here we demonstrate the ability of the Litesizer to determine the particle size of two antiviral vaccines, an aluminum salt-adjuvanted, inactivated tick-borne encephalitis (TBE) vaccine, and a non-adjuvanted, inactivated and split influenza vaccine. We simulated cold chain disruptions by comparing samples stored in optimal conditions to samples that were heat-treated or submitted to a freeze-thaw cycle.

In addition, ELS measurements were performed to assess the zeta potential of the vaccine particles, giving additional clues on the stability of the preparations.

2 I EXPERIMENTAL SETUP

2.1 Samples

Two antiviral vaccines were purchased from a local pharmacy:

- TBE vaccine (FSME-Immun®, Pfizer, Austria)

Inactivated vaccine based on the Neudörfl strain of TBE. The virus is propagated in chicken embryo fibroblasts and inactivated by formaldehyde. Aluminum hydroxide is used as adjuvant, and human serum albumin as stabilizer. One dose consists of 2.4 μg of viral antigen in 0.5 mL of diluent (4).

- Influenza vaccine (Flucelvax®, Seqirus, USA) Quadrivalent influenza vaccine containing 2 strains of influenza A viruses and 2 strains of influenza B viruses, according to the WHO guidelines for the 2019-2020 northern hemisphere influenza season. The viruses are propagated in the continuous MDCK cell line, inactivated by b- propiolactone, detergent-disrupted and purified. One dose is formulated to contain 15 μg of viral hemagglutinin (HA) from every viral strain, or 60 μg total HA, in 0.5 mL (5).

2.2 Sample Treatment

Before treatment, the vaccines were stored in their original conditioning at +4 °C, according to manufacturer recommendations. Each sample was then split in 3 sub-samples in sterile microtubes, which were stored as follows:

- One sample was further stored at the optimal temperature of +4 °C (“Untreated” sample).
- One sample was stored in a dry oven set at 50 °C for 24 hrs., then returned to +4 °C (“Heat-treated” sample).
- One sample was frozen at -18 °C for 24 hrs., then thawed at +4 °C (“Freeze--thawed” sample).

All samples were tested in parallel, between 24 and 28 hrs. after collection from the original conditioning.

2.3 DLS Measurements

For DLS measurements, the TBE vaccine samples were diluted 1/10 in sterile saline solution (0.9 % NaCl). The influenza vaccine samples were diluted 1/5 in sterile saline. These dilutions were established as optimal for DLS measurements in a preliminary experiment (data not shown).

The measurements were performed on an Anton Paar Litesizer 500 instrument. Compared to disposable (polystyrene) cuvettes, quartz cuvettes display superior optical qualities and reduced protein adsorption, and were thus selected here.

Measurement were performed at 25 °C, in the 175° measurement angle (back angle), and using a manual quality mode (12 runs, 10 seconds per run). The optical filter and the focus position were selected automatically by the instrument.

Repetition series consisted in a minimum of 5 and a maximum of 8 consecutive measurements. Results were averaged over the whole measurement.

Statistical significance was established by standard t- test (two-sample, assuming unequal variance).

Differences between data sets were considered significant when the returned *P* value was < 0.05.

2.4 ELS measurements

Samples were analyzed in native, undiluted form, using the Univette and the low-volume accessory (sample volume: 50 μL).

Zeta potential measurements were performed on a Litesizer 500 instrument at 25 °C, and using the Protein mode, which introduces small breaks between measurement runs and therefore limits Joule heating. The voltage was adjusted automatically by the instrument. The quality was set to the manual mode, with 200 runs per measurement.

Repetition series consisting in 5 consecutive measurements were performed.

3 | RESULTS AND DISCUSSION

3.1 Particle Size Measurement of TBE Vaccine Samples

As shown in Figure 1, the intensity-weighted particle size distribution of an optimally-stored TBE vaccine (“Untreated”, green curve) displays a single peak culminating between 2 and 3 μm. This is in accordance with the known particle size of commercial aluminum hydroxide used as vaccine adjuvant, which is in the lower micrometer range (1).

Furthermore, the particle size distribution of both mean curves (Figure 1) and of all individual measurements (not shown) is entirely devoid of peaks in the 0-to-1000 nm size range. This indicates that viral particles are not detectable as free-floating entities, and are likely overwhelmingly adsorbed onto the adjuvant particles.

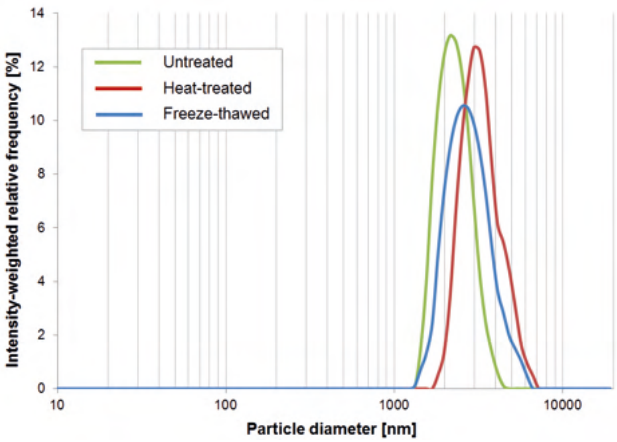


Figure 1: Intensity-weighted particle size distributions of TBE vaccine samples, either untreated (green), heat-treated (red), or freeze-thawed (blue). Curves represent the averages from 5 to 8 consecutive measurements.

The particle size distributions of both freeze-thawed and heat-treated vaccine samples display monomodal distributions of slightly enlarged sizes (Figure 1). This is confirmed by the hydrodynamic diameter (HDD) results obtained for these samples (Table 1, Figure 2). Indeed, the mean HDD of the freeze-thawed sample is slightly but significantly increased compared to that of the untreated sample, from 3030 to 3582 nm ($P < 0.05$). The heat-treated sample displays a more pronounced increase in HDD, from 3030 to 4030 nm ($P < 0.01$).

Parameter	Untreated	Heat-treated	Freeze-thawed
HDD [nm]	3030	4030	3582
Standard deviation [nm]	157	511	277
Rel. standard deviation [%]	5.18	12.68	7.73

Table 1: Hydrodynamic diameter (HDD) results for untreated, heat- treated or freeze-thawed TBE vaccine samples. Averages from 5 to 8 consecutive measurements.

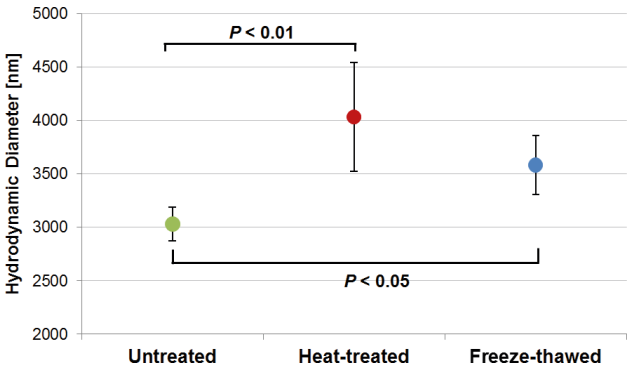


Figure 2: Hydrodynamic diameter results for untreated, heat- treated and freeze-thawed TBE vaccine samples. Statistical significance between the different data sets, as determined by standard t-test, is shown on the relevant brackets.

Of note, the standard deviation of the HDD for the heat-treated sample is much larger than that of the untreated sample (Figure 2). This sub-optimal reproducibility suggests that the Litesizer might be detecting very large aggregates in the heat-treated sample, which cannot be accurately represented as they are above the Litesizer detection limit of 10 μm.

Taken together, these data indicate that the Litesizer is able to measure the particle size of an aluminum salt-adjuvanted viral vaccine. The detected particles are of the size expected for aluminum hydroxide.

Interestingly, a significant increase in HDD is observed for both heat-treated and freeze-thawed vaccine samples, indicating that major disruptions in the cold chain promote adjuvant aggregation.

3.2 Particle Size Measurement of Influenza Vaccine Samples

The expected size of the live influenza virus is around 100 nm, but inactivation and detergent splitting has been shown to reduce the size of the primary particles in vaccine preparations (6).

The particle size distribution of the untreated influenza vaccine sample shows a bimodal distribution, with a major peak culminating around 250 nm and a minor peak culminating around 30 nm (Figure 3, green curve). Thus, our results suggest that the

vaccine under study is composed of split viral particles of diameter around 30 nm, and of aggregates of larger sizes.

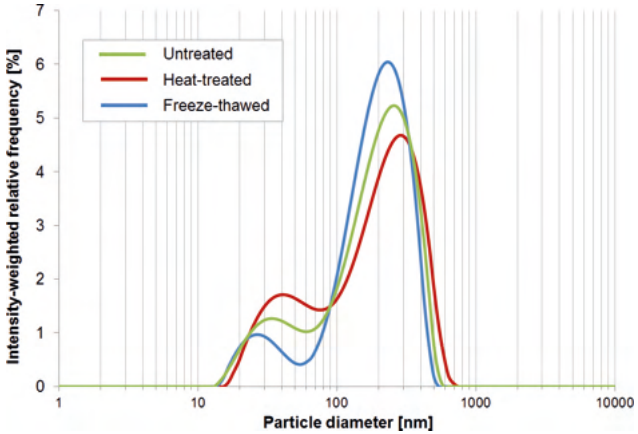


Figure 3: Intensity-weighted particle size distribution of influenza vaccine samples, either untreated (green), heat-treated (red), or freeze-thawed (blue). Curves represent the averages from 6 consecutive measurements.

Because large particles contribute much more strongly to the DLS signal than smaller ones, the fact that large particles visually dominate the distribution does not necessarily indicate that most of the material is in aggregated form. In fact, when the particle size distribution was switched from an intensity-weighted to a volume-based or a number-based one, the aggregate peak disappeared and only the minor peak (ca. 30 nm) was visible (data not shown). This indicates that the primary particles are much more numerous than the aggregates. However, since the DLS method is intrinsically intensity-weighted, and since the characterization of aggregates is highly relevant to the quality control of vaccines, we performed result analysis exclusively on the intensity- weighted data.

Figure 3 also reveals that both heat-treated and freeze-thawed preparations retain a bimodal profile, but that subtle differences in the size distribution arise from treatment. The heat-treated sample shows a major peak of decreased magnitude, and a minor peak of increased magnitude.

Interestingly, the freeze-thawed sample displays an opposite behavior, with a major peak of increased magnitude and a minor peak of decreased magnitude. This suggests that freeze-thawing tends to increase the aggregation of split virus particles, while heat treatment reduces it.

In addition to the HDD, which is calculated over the whole distribution, the Litesizer software calculates the mean diameter of particles contained in up to 3 individual peaks in the distribution. Furthermore, the area under the curve is calculated for every peak (Table 2), indicating the relative contribution of the particles in the relevant peaks to the DLS signal.

Parameter	Untreated	Heat-treated	Freeze-thawed
HDD ± SD [nm]	156 ± 2.2	151 ± 4.4	164 ± 3.3
Peak 1 by Intensity ± SD [nm]	231 ± 13.6	260 ± 25.5	218 ± 9.3
Peak 2 by Intensity ± SD [nm]	34.2 ± 3.8	42.3 ± 5.4	29.4 ± 2.6
Area under curve - Peak 1 ± SD [%]	**83.7 ± 2.6**	**76.1 ± 3.9**	**89.2 ± 0.9**
Area under curve - Peak 2 ± SD [%]	**16.3 ± 2.6**	**23.9 ± 3.9**	**10.8 ± 0.9**

Table 2: Hydrodynamic diameter (in nm), peak by intensity (in nm) and area under the curve – Peak (in %) for untreated, heat-treated and freeze-thawed influenza vaccine samples.

Results from the area under the curve for peak 1 (aggregates) and peak 2 (primary particles) confirm the differences between the samples observed in the particle size distribution. As shown in Figure 4, the contribution of primary particles (peak 2) to the DLS signal increases from 16 to 24 % when the sample is heat-treated ($P < 0.01$). Conversely, the primary particles only account for 11 % of the DLS signal in the freeze-thawed sample ($P < 0.001$).

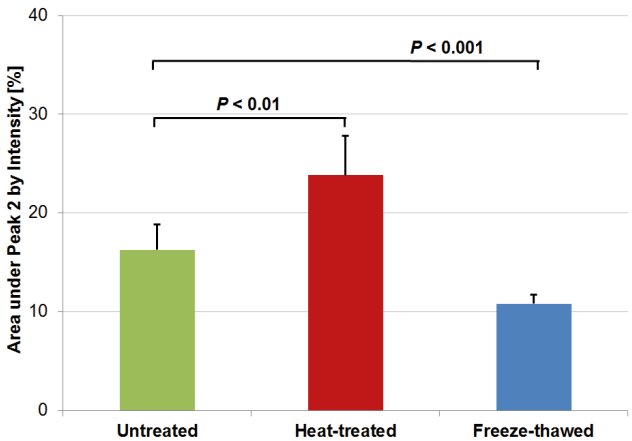


Figure 4: Area under the curve for peak 2 (primary particles), by intensity, in %, for untreated, heat-treated and freeze-thawed influenza vaccine samples. Statistical significance between the different data sets, as determined by standard t-test, is shown on the relevant brackets.

These results indicate that subtle but significant differences in the relative proportion of primary particles and aggregates can be efficiently detected by DLS. Our observations also suggest that heat treatment and freeze-thawing have opposite effects on the aggregation behavior of inactivated influenza particles, with freeze-thawing promoting aggregation while

heat treatment leads to de-aggregation.

3.3 Zeta Potential Measurement of TBE and Influenza Vaccine Samples

The surface charge of particles, as measured by their zeta potential, is commonly used as indicator of colloidal stability. The larger the absolute zeta potential, the greater the repulsive forces between the particles are, and the more stable the preparation will be. But, in the case of particles destined for *in vivo* administration, it is also demonstrated that zeta potential influences particle uptake by specific cells.

On exosomes, negative charges have been shown to limit protein opsonization. This, in turn, slows down their clearance by the reticulo-endothelial system and increases their lifetime in vivo (7). Other reports indicate that cationic nanoparticles are preferentially taken up by dendritic cells, and result in increased local immune responses, compared to their anionic counterparts (8). In all, this suggests that the measurement of zeta potential is highly relevant to the quality control of vaccine preparations.

Here, the zeta potential of TBE samples (untreated, heat-treated or freeze-thawed) was measured with the Univette and its low-volume accessory, which enabled measurements with as little as 50 μL of sample.

As shown in Figure 5 and Table 3, the zeta potential of the TBE vaccine particles can be efficiently measured with the Litesizer, with good repeatability. The mean zeta potential appears weakly negative (ca.- 12 mV), and is not noticeably modified by heat treatment nor by freeze-thawing.

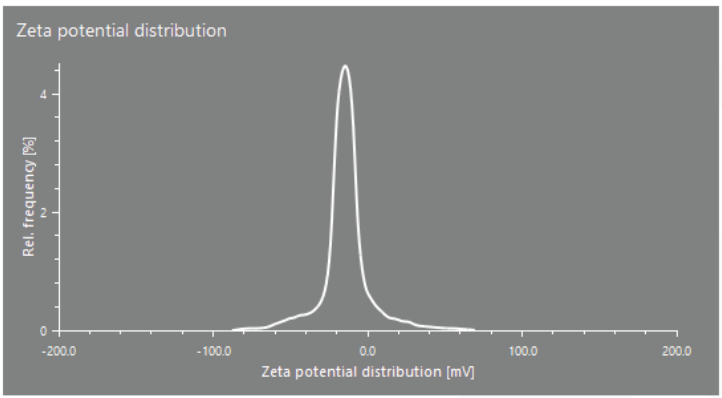


Figure 5: Representative Zeta potential distribution for an untreated TBE vaccine sample. Measurement performed with the Univette and the small volume accessory (sample volume = 50 µL).

Parameter	Untreated	Heat-treated	Freeze-thawed
Mean ZP	- 12.2 mV	- 11.5 mV	- 12 mV
Standard Deviation	1.3 mV	0.3 mV	0.2 mV
Rel. standard deviation	10.6 %	2.6 %	1.4 %

Table 3: Zeta potential results for untreated, heat-treated and freeze-thawed TBE vaccine samples. Mean results from 5 consecutive measurements.

The influenza vaccine sample also displays weakly negative (ca. - 6 mV) zeta potential values, which are also not significantly modified by heat treatment nor by freeze-thawing (Figure 6, Table 4).

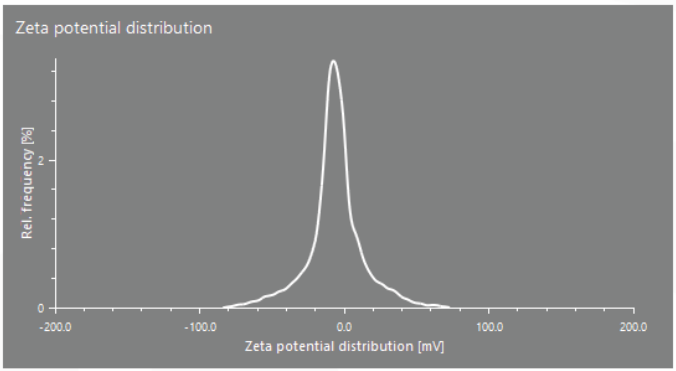


Figure 6: Representative Zeta potential distribution for an untreated influenza vaccine sample. Measurement performed with the Univette and the small volume accessory (sample volume = 50 μL).

Parameter	Untreated	Heat-treated	Freeze-thawed
Mean ZP	- 6.6 mV	- 6.0 mV	- 5.7 mV
Standard Deviation	0.9 mV	0.8 mV	1.3 mV
Rel. standard deviation	13.7 %	12.5 %	22.4 %

Table 4: Zeta potential results for untreated, heat-treated and freeze-thawed influenza vaccine samples. Mean results from 5 consecutive measurements.

As a rule of thumb, colloids are considered stable when absolute zeta potential values are over 30 mV. One can therefore speculate that the low zeta potential magnitude observed here for both vaccines accounts for their tendency to aggregate in response to cold chain disruptions.

4 | CONCLUSION

Both the size and the zeta potential of particles are relevant for the shelf life and immunogenicity of vaccine preparations. Here we demonstrate that the Litesizer is a quick and effective tool for the characterization of antiviral vaccines.

The TBE vaccine, which is adjuvanted with aluminum salt, is shown to consist of particles in the lower micrometer range, as expected from the literature. In the influenza vaccine, which is devoid of adjuvant, both split viruses and larger aggregates are observed. For both vaccines, simulated cold chain disruptions (heat treatment or freeze-thawing) are shown to trigger subtle but significant changes in the particle size distribution.

Zeta potential measurements were also successfully performed, demonstrating that both vaccines consist of weakly anionic particles. Cold chain disruptions do not appear to significantly modify the surface charge of the vaccine particles.

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