Nanosecond pulsed electric field (nsPEF) and vaccines: a novel technique for the inactivation of SARS-CoV-2 and other viruses?

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\textbf{ABSTRACT}

Since the beginning of 2020, worldwide attention has been focused on SARS-CoV-2, the second strain of the severe acute respiratory syndrome virus. Although advances in vaccine technology have been made, particularly considering the advent of mRNA vaccines, up to date, no single antigen design can ensure optimal immune response. Therefore, new technologies must be tested as to their ability to further improve vaccines. Nanosecond Pulsed Electric Field (nsPEF) is one such method showing great promise in different biomedical and industrial fields, including the fight against COVID-19. Of note, available research shows that nsPEF directly damages the cell’s DNA, so it is critical to determine if this technology could be able to fragment either viral DNA or RNA so as to be used as a novel technology to produce inactivated pathogenic agents that may, in turn, be used for the production of vaccines. Considering the available evidence, we propose that nsPEF may be used to produce inactivated SARS-CoV-2 viruses that may in turn be used to produce novel vaccines, as another tool to address the current COVID-19 pandemic.

\textbf{KEY MESSAGES}

- Viral inactivation by using pulsed electric fields in the nanosecond frequency.
- DNA fragmentation by a Nanosecond Pulsed Electric Field (nsPEF).
- Opportunity to apply new technologies in vaccine development.

\textbf{Introduction}

Nanosecond Pulsed Electric Field (nsPEF) is a novel technology first developed in 1995 \cite{1}, exhibiting an explosive research growth since \textasciitilde 2005 \cite{2}. This technique consists of the delivery of a series of pulses composed of high amplitude electric fields (\textasciitilde 1–300 kV/cm) in the nano and sub-nanoseconds timescale into biological tissues or cells. Its primary effect on cells in the formation of membrane nanopores and the activation of ionic channels \cite{3-14}. The main cellular consequence of nsPEF is the increment in the cytoplasmic concentration of Ca\textsuperscript{2+} \cite{15-18}, triggering signalling cascades ending either on apoptosis \cite{19-25}, or cell proliferation \cite{26-28} and differentiation \cite{29}. In contrast to other forms of electrostimulation, such as electroporation, nsPEF uses timescales similar to that of the charging time of cellular membranes (\textasciitilde 100 ns in mammalian cells). This turns nsPEF capable of affecting inner organelles \cite{15,30-33}, including the nucleolus \cite{30}, making nsPEF a unique tool to manipulate cells. As different cell types have different membrane charging times and different conductivity in their surrounding media, the effects of nsPEF could be cell-type tailored. This characteristic has been exploited by researchers to propose a broad spectrum of nsPEF applications such as: neuron \cite{7,11,34-37} and myocyte activation \cite{38-41}, wound healing \cite{6,42-44}, phenotype manipulation \cite{29}, modulation of gene expression \cite{45-50}, the antiparasitic effect \cite{51-53}, increment of the immune response \cite{54-59}, cell proliferation \cite{26-29}, improvement in fermentation \cite{60,61} and sterilisation for the food industry \cite{62-64}, seed germination \cite{65-67} and, most importantly, cancer treatment \cite{2}.

\textbf{nsPEF-induced damage in cell’s DNA}

As of May 2022, there is only one study proposing the use of nsPEF as an alternative technology to fight against the COVID-19 pandemic \cite{68}. Given the
improvement of the immune response after a nsPEF stimulus [54–59], Alawadhi et al. [68] proposed that the delivery of a nsPEF pulse during the application of a SARS-CoV-2 vaccine may increase the title of antibodies, therefore improving the SARS-CoV-2 host immunity. Despite an interesting approach, it is worth mentioning that several studies link the DNA damage caused by nsPEF to cell apoptosis. In fact, DNA damage can be described as a secondary effect of nsPEF application [3,19,69]. Notably, Chen and co-workers showed a strong nsPEF effect on the HL-60 cell nucleus [70]. They observed the quenching of acridine orange (a DNA-intercalating dye) fluorescence after the application of a nsPEF protocol, suggesting that nsPEF directly alters DNA conformation. Stacey et al. [71] registered nsPEF-derived DNA damage by performing a comet assay to evaluate cell survival. To determine if DNA damage was a direct effect of nsPEF, Jurkat cells were lysed and their DNA extracted right after the application of a nsPEF protocol. Their results were interpreted as DNA damage resulting directly from nsPEF exposure [71]. In a continuation study, Stacey et al. [72] collected cells under alkaline conditions to unwind DNA, allowing detection of double- and single-stranded breaks. In their report (see Figure 1) they show a clear DNA fragmentation of nsPEF exposed cells vs the control condition. This direct damage to DNA caused by nsPEF can be understood because DNA is a heavily-charged and polyelectrolyte macromolecule so the proximity of its linear/folded structures to the nuclear membrane might render it susceptible to nsPEF effects. Other studies also support that nsPEF and other closely-related technologies, such as intense burst sinusoidal electric field (IBSEF), can directly cause DNA damage [72–74]. Furthermore, nsPEF exposed cells show a DNA electrophoresis migration profile similar to that observed in gamma-irradiated K562 erythroid cells [75].

Is electroporation an alternative to induce direct DNA damage in cells?

Significant damage to DNA can also be achieved by electroporation (EP) resulting in the activation of apoptotic pathways leading to DNA fragmentation. For instance, irreversible electroporation (IRE) induces DNA fragmentation by apoptosis as determined by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay [76–78]. Importantly, no available evidence in the literature suggests that EP may induce DNA damage directly but not EP? This is particularly appealing due to the fact that the electric fields applied in both nsPEF and EP are similar (0.1–100kV/cm) [79]. A theoretical analysis could shed some light on the controversy surrounding this question. If the cell is considered, for the sake of simplicity, like solid metal and conducting sphere, we know that when an external electric field is applied to it, electrons contained in the sphere should migrate to the anode. After a defined amount of time, this continuous migration of electrons should result in an asymmetric charge distribution—creating a self-induced electric field around the sphere (the reaction field) that could nullify the external electric field—resulting in zero electric fields inside the sphere. This is similar to what may happen in cells due to nsPEF application; however, instead of electrons moving around creating an equilibrium in charge distribution, a much longer time, far behind the time-scale of nsPEF application is needed, to nullify the applied electric field. The characteristic time it takes for the external electric field in cells to dissipate is in the order of microseconds or even milliseconds [80]. However, when nsPEF is applied, the pulse duration is in the nanosecond or even sub-nanosecond scale. Hence, during the application of nsPEF, internal charges will continue to move by the influence of the external electric field, continuously perturbing the internal structure and dynamics of the cell.

Conformational changes in proteins due to nsPEF

Besides the formation of nanopores in membranes [4,6,8], extensive available evidence suggests that the application of nsPEF protocols may also affect the
structure of voltage gated (VG) ion channels such as VG Calcium Channels \[7,9,10,12–14\] and VG Sodium Channels \[5,7,11\]. Whether nsPEF induces directly the gating of ion channels or their gating is the result of the charge imbalance produced by the movement of ions as a result of the external electric field, is still a matter of debate \[81\]. Thus, the conformational changes occurring in these channels may either be a direct result of the applied electric field or be the consequence of the internal ion imbalance. Interestingly, available evidence gathered from Molecular Dynamics (MD) simulation suggests that conformational changes resulting from nsPEF occur directly in the voltage sensing structure of the human VG calcium channel \[82\]. On top of that, Beebe et al. \[83\] showed that a single nsPEF pulse (the same nsPEF protocol used to elicit DNA damage), decreased the activity of the C-subunit of the cAMP-dependent protein kinase (cAMP-dPKA), during 15 min after nsPEF application. Although the actual mechanisms of nsPEF-induced inactivation remain to be determined, it should happen through reversible conformational changes in a fraction of the cAMP-dPKA proteins: since 15 min after the pulse, the basal activity was recovered. Moreover, particularly interesting for our hypothesis, pulsed electric field technology has been evaluated to determine its ability for viral inactivation. Mizuno el at. \[84\], used wine vesicular disease virus (SVDV) and equine herpes virus-1 (EHV-1) to determine the effect of high voltage pulsed electric fields. Of note, both viruses were successfully inactivated. Notably, the shape of the protein shell of SVDV remained unaltered while its RNA completely disappeared. Despite apparent damage to the envelope around EHV-1 being detected, the authors suggested that it may be related to either the used medium or some other differences in the applied protocol. It is worth mentioning that MD studies (mimicking a nsPEF protocol consisting of two pulses lasting for 8 ns of 1,000 kV/cm and 10,000 kV/cm) suggested an irreversible conformational change resulting in the disruption of the myoglobin secondary structures without fragmentation \[85,86\]. However, these MD studies used a much higher electric field compared to that of the 60 kV/cm necessary to produce direct DNA fragmentation in cells.

**The application of nsPEF protocols to inactivate SARS-CoV-2**

A thorough revision of the literature suggests that a single nsPEF pulse of 60 kV/cm with a duration of 60 ns is actually capable of fragmenting the DNA shielded by the cell nucleus \[71\]. It is then possible that by modulating different parameters of the nsPEF protocol (i.e. electric field intensity, number of pulses and their duration), a specific combination of them...
may fragment SARS-CoV-2 RNA, without causing irreversible conformational changes on the envelop and spike viral proteins. As the SARS-CoV-2 virus has a non-segmented positive single-stranded long RNA, with 26–32 kb [87,88], it is expected to be more susceptible to nsPEF-derived single-stranded breaks as the energy required to produce double-stranded breaks is much higher. Therefore, in our opinion, the application of a specific nsPEF protocol into a solution containing SARS-CoV-2 viral particles may result in RNA fragmentation and a completely inert viral particle (Figure 2). Importantly, if the elicited conformational changes occurring in viral proteins are indeed reversible, it will overcome one of the main problems of current vaccines using inactivated viruses. Traditional methods to produce inactivated viruses use heat, detergents and other chemicals. These are aggressive techniques that may produce drastic changes in the conformation of proteins, particularly in the case of spike proteins in SARS-CoV-2. These conformational changes may occlude or even create new epitopes that can hinder the immune response. Of note, Liu et al. used state-of-the-art cryo-electron microscopy technologies to characterize the architecture of inactivated SARS-CoV-2 viruses [89]. The authors found that the viral spikes are mostly in a postfusion state, a conformation that is not desirable for vaccine development because, in vivo, neutralising antibodies will not recognise the prefusion conformation of the SARS-CoV-2 spike (S) protein. Additionally, there is no evidence of whether the rest of the SARS-CoV-2 proteins retain their conformation after inactivation by current methods. Thus, maintaining the native conformation is particularly interesting as the majority of RNA-based vaccines only encode for the target protein antigen of the SARS-CoV-2 virus. In fact, four of the most used vaccines worldwide [90] are RNA-based encoding for the S protein [91]. The usage of an inactivated virus having its proteins in its biologically relevant conformation may elicit a more relevant immune response. Therefore, other SARS-CoV-2 proteins exposed in the capsid besides the S protein such as the envelope (E), nucleocapsid (N) and membrane glycoprotein (M) may also play a role in the enhancement of an appropriate immune response (Figure 3) [92].

As was described in the introduction, one of the primary effects of nsPEF is the induction of nanopores in cell membranes [4,6,8]. Although these nanopores could also appear in the SARS-CoV-2 membrane after the application of a nsPEF protocol they would spontaneously reseal after the given time. Evidence for this resealing of the pores has been reported in EP. Saulis et al. [93] observed the complete resealing of membrane pores occurring in human red blood cells, 20–30 min after the application of an EP electric pulse of 4 kV/cm of 2 μs. This transient formation of pores has also been reported in other cell types [94–96]. In the case of nanopores, the resealing process is much faster. Recent results conclude that nanopore resealing occurs in a couple of minutes [97–103].

It is important to mention that other kinds of electromagnetic waves capable to damage nucleic acids, such as x-Ray [104] or gamma-Radiation [105] based on highly unspecific ionising radiations are not suitable for viral inactivation, since their photons are also absorbed by proteins. Thus, these radiations cannot produce inactivated SARS-CoV-2 viruses suitable to be used for a vaccine because their application will elicit irreversible conformational changes in the SARS-CoV-2 proteins. Therefore, the advantage of nsPEF is that it may be a suitable technique than can be used for the production of highly immunogenic and inactivated SARS-CoV-2 viruses.

Despite seeming an obvious alternative, we believe that nsPEF has not been taken into account perhaps because it is a new field that most molecular biologists are unfamiliar with. Additionally, this technique may be of particular interest to the industry considering that nsPEF is an inexpensive and accessible technology. In fact, it is perfectly feasible to build an in-house nsPEF device. An excellent review remarking on this point can be read at [106].

Conclusions

A larger body of research is needed to capitalise on the basic knowledge accumulated in the nsPEF field in order to translate it into real demonstrable applications. This paper proposes one of such new applications. In the light of the continuous appearance of new SARS-CoV-2 variants, evaluating nsPEF to generate inactivated SARS-CoV-2 that may, in turn, be used for the production of novel vaccines, is an urgent task. It is expected that, after the application of a suitable nsPEF protocol an inactivated virus harbouring intact proteins and fragmented RNA, should be produced. Thus, resulting inactivated viruses could be used to develop a novel SARS-CoV-2 vaccine where inert viruses expose their proteins in the same conformation as the original viruses. This will ensure that the viral epitopes will remain intact boosting the immune response. This new approach to vaccine development may be not only important to fight against the
COVID-19 pandemic, but also to develop vaccines suitable to better address future pandemics.

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**Author contributions**

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