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DOKTORSKA DISERTACIJA

Elektroporacija kot naravni mehanizem horizontalnega prenosa genov pri prokariotih

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DOCTORAL DISSERTATION

Electroporation as a natural mechanism of horizontal gene transfer in prokaryotes

Mentor: prof. Tadej Kotnik, Ph. D.

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Igor Marjanovič

KAZALO

POVZETEK

Več vej znanosti si še vedno prizadeva ugotoviti, kako je življenje nastalo. Čeprav so si teorije nastanka življenja na Zemlji v podrobnostih lahko močno različne, so si v grobem enotne, da se živa bitja skozi naravno selekcijo prilagajajo okolju, v katerem se nahajajo. Temu procesu prilagajanja skozi čas pa pravimo evolucija. Vemo, da so bila enocelična bitja prisotna pred večceličnimi, celice brez jedra (prokarioti) pa pred celicami z jedrom (evkarioti).

Do devetdesetih let prejšnjega stoletja je veljalo tudi prepričanje, da so bile najpomembnejši vir inovacij v evoluciji mutacije, ki so se z delitvijo celice (vertikalnim prenosom genov) prenašale na njene potomke. Ta teorija se je porušila, ko so znanstveniki pričeli sorodnost organizmov vrednotiti glede na sorodnost njihovih genomov (temu postopku pravimo filogenetska analiza) in pri tem ugotovili, da pri sledenju sorodnosti različnih genov pridejo do različnih sorodnostnih struktur (filogenetskih dreves). Študije genomov so pokazale tudi, da nekateri organizmi vsebujejo gen, ki ga njihovi bližji sorodniki nimajo, najdemo pa ga (enakega ali zelo podobnega) pri nekaterih evolucijsko zelo oddaljenih organizmih. Iz teh ugotovitev izhaja, da organizmi v splošnem prevzemajo gene ne le od celice, iz katere izvirajo, temveč tudi iz okolice oziroma od drugih organizmov. Ta proces imenujemo horizontalni prenos genov (HGT). Rezultati filogenetskih študij kažejo, da je bil HGT skozi evolucijo in je še danes pomemben vir inovacij, ki so omogočile hitrejši in bolj raznolik razvoj zgodnjega življenja.

V znanstveni literaturi zasledimo tri mehanizme HGT: naravno kompetenco, konjugacijo in transdukcijo. Vsi našteti mehanizmi so biološki in temeljijo na proteinih z ozko specifičnim delovanjem, iz česar sledi, da so tudi sami nastali šele v določeni fazi evolucije, zato se zastavlja vprašanje, ali za HGT obstaja tudi kak preprostejši, denimo povsem fizikalen mehanizem, ki je lahko deloval že vse od nastanka življenja. Eden najobetavnejših tovrstnih mehanizmov je elektroporacija.

Elektroporacija je pojav, ki omogoča vnos tujega materiala tako v prokariotske kot v evkariotske celice. Kot laboratorijska metoda je bila razvita pred štirimi desetletji, temelji pa na kratkotrajni izpostavitvi celice električnemu polju dovolj visoke jakosti, ki ga običajno ustvarimo z dovajanjem napetostnih pulzov na par elektrod, med katerima se nahajajo celice. Posledica izpostavitve celice takšnim pulzom je povečanje prepustnosti celične membrane, ki omogoči vnos najrazličnejših snovi, tudi DNA, iz okolice v celico, lahko pa tudi iztekanje snovi iz celice. Če iztekanje ni premočno in celica po končani izpostavitvi pulzom preživi, govorimo o reverzibilni, sicer pa o ireverzibilni elektroporaciji.

V naravi se ob udaru nevihtne strele v habitat prokariotskih organizmov v bližini točke udara ustvari električno polje, zadostno za povzročitev elektroporacije – zelo blizu točke udara so prisotni pogoji za ireverzibilno elektroporacijo in s tem iztekanje DNA, nekoliko dlje od te točke pa pogoji za reverzibilno elektroporacijo in s tem vnos DNA.

Za preučevanje elektroporacije kot naravnega mehanizma HGT je potrebno opraviti biološke poskuse, kjer se v kontroliranih laboratorijskih pogojih čim bolj približamo naravnim

razmeram ob udaru strele. V ta namen je bilo potrebno razviti napravo, ki nam take poskuse omogoča.

Po razčlenitvi zgoraj opisanih spoznanj in motivacije te disertacije sledi opis načrtovanja, izdelave in testiranja modularnega sistema za izpostavitev emulacijam električne strele (sistem »Znanstveni Emulator eVolucijskih Strel« – ZEVS) in pripadajočega visokonapetostnega generatorja (generator ZEVS). Sistem ZEVS nam omogoča, da biološke vzorce (celice ali tkiva) v kontroliranem okolju (točno določena dolžina obloka razelektritve, merjenje časovnega poteka električnega toka, ki teče skozi vzorec, snemanje poteka poskusov s hitro kamero) izpostavimo elektrostatični razelektritvi z nastavljivo amplitudo električnega toka (do nekaj sto amperov). To predstavlja ponovljivo emulacijo elektrostatične razelektritve, kakršna poteka pri naravni streli. Ta sistem raziskovalcem omogoča, da uporabijo poljubni generator elektrostatičnih razelektritev z ustrezno ozemljitveno elektrodo in prilagodljivo dolžino obloka. Modularna zasnova sistema omogoča hitro montažo in demontažo, kot tudi preprosto in temeljito čiščenje.

Pri razvoju sistema smo si pomagali z računalniškim modeliranjem, kjer smo pred izdelavo prvega prototipa načrtovali vse komponente, virtualno sestavili sistem in njegove dimenzije iterativno določili z numeričnimi izračuni porazdelitve električnega toka in jakosti električnega polja, temelječimi na metodi končnih elementov. Sistem smo zasnovali tako, da se ga da hitro sestaviti in razstaviti, kar omogoča preprosto transportiranje in izvajanje poskusov v različnih laboratorijih. Pozorni smo bili tudi na to, da je mogoče sistem enostavno in temeljito očistiti, kar bistveno zmanjša tveganje kontaminacije, hkrati pa omogoča ponovljivost poskusov. Pri izbiri materiala smo za dele, ki zahtevajo električno neprevodnost, uporabili polietilen, kjer je bila poleg neprevodnosti sestavnega dela potrebna tudi njegova prozornost, pa pleksi steklo. Elektrode smo prvotno izdelali iz bakra, a se je izkazalo, da razelektritve povzročajo njihovo korozijo, zato smo baker v nadaljevanju razvoja nadomestili z nerjavečim jeklom, ki se je izkazalo za ustrezno odporno proti razelektritveni koroziji. Pri ozemljitveni elektrodi, ki je v neposrednim stikom z biološkim vzorcem, pa je nerjaveče jeklo ustreznejše tudi zato, ker se v primerjavi z bakrom precej manj elektrolitsko raztaplja in tako manj kontaminira vzorec.

V prvih serijah preizkusov sistema ZEVS smo kot generator razelektritev uporabili komercialni električni paralizator (taser), ki je generiral razelektritveni tok s trajanjem nekaj sto nanosekund, nato pa smo načrtovali in zgradili visokonapetostni električni generator, ki temelji na krmiljeni 5 kV razelektritvi kondenzatorja s kapacitivnostjo 1 µF (generator ZEVS) in je po časovnem poteku generiranega razelektritvenega toka precej bolj podoben dejanskim nevihtnim strelam (dvig toka z ničle do maksimuma v približno 5 µs, nato pa eksponentno upadanje s časovno konstanto približno 75 µs).

Prve biološke poskuse smo opravili na bakterijah *Escherichia coli*, nasajenih na agarju v petrijevkah z notranjim premerom 86 mm, razelektritve pa smo generirali z električnim paralizatorjem. Petrijevke z agarjem in nasajenimi bakterijami smo vstavili v sistem ZEVS in preko konične elektrode v središče petrijevke dovedli 10 zaporednih razelektritev. Tok vsake razelektritve je imel največjo vrednost pri ~100 A, dvižni čas od nič do največje vrednosti

~0.1 µs in čas upada do polovične vrednosti ~0.3 µs. Dolžina obloka pri vsaki razelektritvi je bila ~15 mm. Pri poskusih smo v krožnem področju do radija 5 mm od središča petrijevke dobili območje skoraj popolnoma brez kolonij *E. coli*. Izračunana jakost električnega polja na tej radialni razdalji je bila ~8 kV/cm. Opisani eksperimentalni rezultati in izračuni skupaj povedo, da je bilo osrednje območje brez živih bakterij zaradi njihove ireverzibilne elektroporacije.

Drug sklop bioloških poskusov smo opravili na ovarijskih celicah kitajskega hrčka (celicah CHO), ki so evkariotske. Tudi pri teh poskusih smo za generator razelektritev še uporabljali električni paralizator. Celice CHO smo nasadili v petrijevke z notranjim premerom 52 mm. Preden smo petrijevke izpostavili razelektritvam, smo iz njih odstranili gojišče in nato dodali 1.5 ml svežega gojišča, ki je vsebovalo 4 µg/ml plazmidne DNA pEGFP-N1, z izražanjem katere nastaja zeleno fluorescirajoči protein (GFP). Nato smo petrijevke zaporedoma vstavljali v sistem ZEVS in vsaki dovedli 10 elektrostatičnih razelektritev. Električni tok vsake razelektritve je imel največjo vrednost pri ~14 A, dvižni čas od nič do največje vrednosti ~0.5 µs in čas upada do polovične vrednosti ~1.5 µs. Dolžina obloka pri vsaki razelektritvi je bila ~7 mm. V krožnem pasu na razdalji od 3 do 15 mm od središča petrijevke smo zaznali fluorescenco GFP, ki je odražala vnos in izražanje pEGFP-N1, torej je bilo to območje reverzibilne elektroporacije. Z izračunom smo ocenili jakost električnega polja 15 mm od središča petrijevke na 1.11 kV/cm, 3 mm od središča pa na 5.54 kV/cm, kar nakazuje, da so bile celice v osrednjem območju, kjer nismo zaznali fluorescence GFP, mrtve zaradi ireverzibilne elektroporacije, v zunanjem območju, kjer prav tako ni bilo zaznavne fluorescence GFP, pa niso bile elektroporirane, zato ni prišlo do vnosa DNA.

Tretji sklop poskusov pa je bila ireverzibilna elektroporacija spor bakterije Bacillus pumilus, nasajenih na agarju v petrijevkah, pri teh poskusih pa smo kot napetostni generator uporabili tako električni paralizator kot generator ZEVS, ki smo ga takrat že razvili. Z obema generatorjema smo dosegli ponovljivo inaktivacijo spor. Pri poskusih s paralizatorjem smo z 20 razelektritvami dobili inaktivacijo na 0.65% celotne petrijevke, medtem ko je območje inakativacije pri uporabi generatorja ZEVS pokrivalo 7% celotne petrijevke pri eni razelektritvi, 27% pri desetih in 55% pri petdesetih razelektritvah.

Opravljeni poskusi so pokazali, da je sistem ZEVS primeren za preučevanje vplivov elektrostatičnih razelektritev na prokariotske in evkariotske celice ter da z njim lahko povzročimo tako ireverzibilno elektroporacijo, katere posledica je lahko tudi iztekanje DNA, kot reverzibilno elektroporacijo, ki privede do vnosa DNA in njeno izražanje.

Poskusa ireverzibilne elektroporacije na celicah *E. coli* in genske transfekcije na celicah CHO nakazujeta, da bi bila elektroporacija dejansko lahko četrti mehanizem prenosa HGT v naravi, vendar pa bo za zanesljivejši in kvantitativno relevanten odgovor potrebno s sistemom ZEVS opraviti dodatne poskuse na organizmih, katerih naravno okolje je dosegljivo nevihtnim strelam (denimo na bakterijah, ki naseljujejo površinske morske in sladke vode). Poleg tega pa bo potrebno namesto modelnih laboratorijskih molekul DNA, kot so tiste z genom za GFP ali odpornostjo na antibiotike, uporabiti naravno DNA brez modifikacij, s katerimi umetno povečamo njihovo stabilnost ter verjetnost vnosa in izražanja.

ABSTRACT

Multiple scientific disciplines are still trying to determine how life began. Although competing theories on the origins of life on Earth differ in many aspects, they all agree that the genetic makeup of organisms is adapted to the environment in which they live by the forces of natural selection; this process is known as evolution. We know that single-cell organisms existed before multi-cell organisms and that cells without a nucleus (prokaryotes) existed before cells with a nucleus (eukaryotes).

Up until the 1990s, it was widely assumed that the prevailing source of innovations in evolution are mutations occurring during cell division and thus transferred to daughter cells (vertical gene transfer). This theory collapsed when scientists began to analyze the relatedness of organisms by looking at the similarities of their genomes (a process called phylogenetic analysis). They discovered that tracking the similarities of different genes can lead to different branching diagrams of relatedness (phylogenetic trees). Genome studies have also shown that some organisms contain a gene that is absent in their close relatives, but present in identical or only slightly altered form in some evolutionarily very distant organisms. These findings implied that the genetic material is not only inherited from the parent cells, but can also originate from the surroundings and from other organisms. This process is known as horizontal gene transfer (HGT). The results of phylogenetic studies show that HGT has been an important source of innovation for evolution that enabled a faster and more diverse development of early life.

The scientific literature recognizes three mechanisms of HGT: natural competence, conjugation and transduction. All of the stated mechanisms are biological and are based on proteins, each with a highly specific function, which implies that these mechanisms are themselves products of evolution and had thus only occurred during a certain stage of the evolutionary history. Consequently, we are left with the question whether there exists a mechanism, perhaps based on simpler physical principles, that could have acted ever since the dawn of life. One of the most promising such mechanisms is electroporation.

Electroporation is a phenomenon that enables the entry of exogenous matter into prokaryotic as well as eukaryotic cells. As a laboratory method it was developed four decades ago and is based on short-term exposure of cells to a sufficiently strong electric field. The field is usually created by delivering voltage pulses to a pair of electrodes between which the cells are positioned. The result of exposure to such pulses is increased permeability of the cell plasma membrane, which enables the entry of a wide range of molecules, including DNA, from the environment to the cell, as well as release of such molecules from the cell into the environment. If the outflow from the cell is not too strong and the cell survives the exposure to the pulses, this phenomenon is termed reversible electroporation, otherwise it is known as irreversible electroporation.

In natural habitats hit by a lightning stroke, the electric field in the ground near the lightning's point of entry is sufficient for electroporation; very close to that point the conditions are those for irreversible electroporation and hence release of DNA, while in the

adjacent region in the downward and outward direction the conditions for reversible electroporation are met, and hence for uptake of DNA.

To assess electroporation as a natural mechanism of HGT, it is necessary to conduct biological experiments, where in controlled laboratory conditions we strive to come as close as possible to emulating natural conditions of lightning striking the ground. For this purpose, we needed to develop a setup allowing such experiments.

The analysis of the abovementioned findings and motivations for this dissertation are followed by the description of design, construction and testing of a modular system for lightning exposures (Scientific Emulator of Evolutionary Lightning, with the acronym ZEVS in Slovene) and the corresponding high-voltage generator. The ZEVS system allows to expose biological samples (cells or tissues) in a controlled environment (precisely determined length of the discharge arc, monitoring the time course and amplitude of the electric current flowing through the sample, filming the experiments with a high-speed camera) to electrostatic discharges with adjustable amplitude of electric current (up to several hundred amperes). This provides a reproducible emulation of electrostatic discharges that occur in natural lightning strokes. The system allows the researchers to use an arbitrary generator of electrostatic discharges with an adequate receiving (ground) electrode and an adjustable arc length. The modular design of the system enables quick assembly and disassembly, as well as simple and thorough cleaning.

For the development of the system, we used computer modeling, where we designed and analyzed the entire system virtually before buildng the first actual prototype. The dimensions of the system were determined iteratively using numerical calculations of the distribution of electric current and field based on the finite elements method. The system was designed such that it was easy to assemble and disassemble, facilitating transport and thus allowing to conduct experiments in different laboratories. We also paid attention to allow for the system to be cleaned simply and thoroughly, which substantially decreases the risk of contamination, while allowing for the reproducibility of experiments. As a material for components that are required to be nonconductive, we chose polyethylene. For components where non-conductivity as well as transparency was required, we used Plexiglas. Electrodes were initially made of copper, but we discovered that the electric discharges caused substantial corrosion of such electrodes, so we later replaced copper with stainless steel, which turned out to be sufficiently resistant to corrosion caused by electric discharges. For the ground electrode, which is in direct contact with the biological sample, the choice of stainless steel proved additionally advantageous as it is less susceptible to electrolytic dissolution and thus results in a much weaker contamination of the biological sample by the metal ions.

In the first experimental trials of the ZEVS system, we modified a commercial electric Taser and used it as the electric discharge generator, yielding a discharge current that lasted several hundred nanoseconds. Later, we designed and constructed a high-voltage electric generator that delivers arcs by a controlled 5 kV discharge of a 1 µF capacitor (the ZEVS generator). Compared to the Taser, the ZEVS generator discharge current was much closer

in its time course to an actual lightning stroke (zero-to-peak time of ~5 μs followed by exponential decay from the peak with a time constant of ~75 μs, corresponding to a peak-to-half time of ~100 μs).

The first biological experiments were conducted on *Escherichia coli* bacteria planted on agar in petri dishes having inner diameter of 86 mm, with discharges generated by the Taser. Petri dishes with agar and the plated bacteria were inserted into the ZEVS system, the discharge was delivered from the conical electrode, entering vertically downwards into the center of the petri dish, and we supplied 10 consecutive such discharges. The current of each discharge had the peak value of ~100A, zero-to-peak time of ~0.1 μs, and peak-to-half time of ~0.3 μs. The length of the arc of each discharge was ~15 mm. The experiments produced a circular region of radius of 4 mm from the center of the petri dish in which there were almost no detectable colonies *E. coli*. The calculated electric field strength at that radial distance was ~8 kV/cm. The acquired results together with these calculations imply that the region devoid of viable bacteria was due to their irreversible electroporation.

The second set of biological experiments was conducted on Chinese Hamster Ovary (CHO) cells, which are eukaryotic, again using the Taser to generate the discharges. CHO cells were plated in petri dishes having inner diameter of 52 mm. Before exposing the petri dishes to the discharges, we removed the original culture medium and then added 1.5 ml of a fresh culture medium containing 4 μg/ml plasmid DNA pEGFP-N1 that contains a gene encoding the green fluorescent protein (GFP). We then placed the petri dishes into the ZEVS system and exposed each dish to 10 electrostatic discharges. The electric current of each discharge had a peak value of ~14 A, zero-to-peak time of ~0.5 μs and peak-to-half time of ~1.5 μs. The length of the electric arc in each discharge was ~7mm. On the area spanning radially from 3 to 15 mm from the center of the petri dish, we detected GFP fluorescence, reflecting uptake of pEGFP-N1 and its expression, and thus corresponding to the area of reversible electroporation. By calculattion, we estimated the electric field strength at 15 mm from the center of the petri dish as 1.11 kV/cm, and at 3 mm as 5.54 kV/cm. This suggests that the central region with no gene expression was subject to irreversible electroporation and thus cell death, while in the outer region in which there was also no detectable expression the cells were not electroporated, and thus there was no DNA uptake.

The third set of experiments was irreversible electroporation on bacterial spores of Bacillus pumilus planted on agar in petri dishes. For these experiments, we used the Taser generator, as well as the ZEVS generator that we had already developed at that stage. With both discharge generators we achieved reproducible inactivation of the spores. With experiments utilizing the Taser, we achieved inactivation in 0.65% of the entire petri dish after delivering 20 electric discharges. Using the ZEVS generator, the area of inactivation was 7% using one discharge, 27% after 10 discharges, and 55% after 50 discharges.

The conducted experiments have shown that the ZEVS system is suitable for studying the effects of discharges on both prokaryotic and eukaryotic cells, and that with it we can achieve irreversible electroporation that causes leakage of DNA, as well as reversible electroporation that results in uptake and expression of DNA.

Experiments of irreversible electroporation in *E. coli* and of gene uptake in CHO cells suggest that electroporation could act as the fourth natural mechanism of HGT. To arrive at a reliable and quantitatively relevant answer, however, it is necessary to conduct further experiments on organisms whose natural environment is accessible to lightning strokes (e.g. bacteria populating the top layers of seawater and freshwater habitats). Furthermore, for reliable conclusions it is important to use natural DNA, devoid of artificial modifications often present in commercially available DNA with the aim to increase its stability and/or the efficiency of uptake and expression.

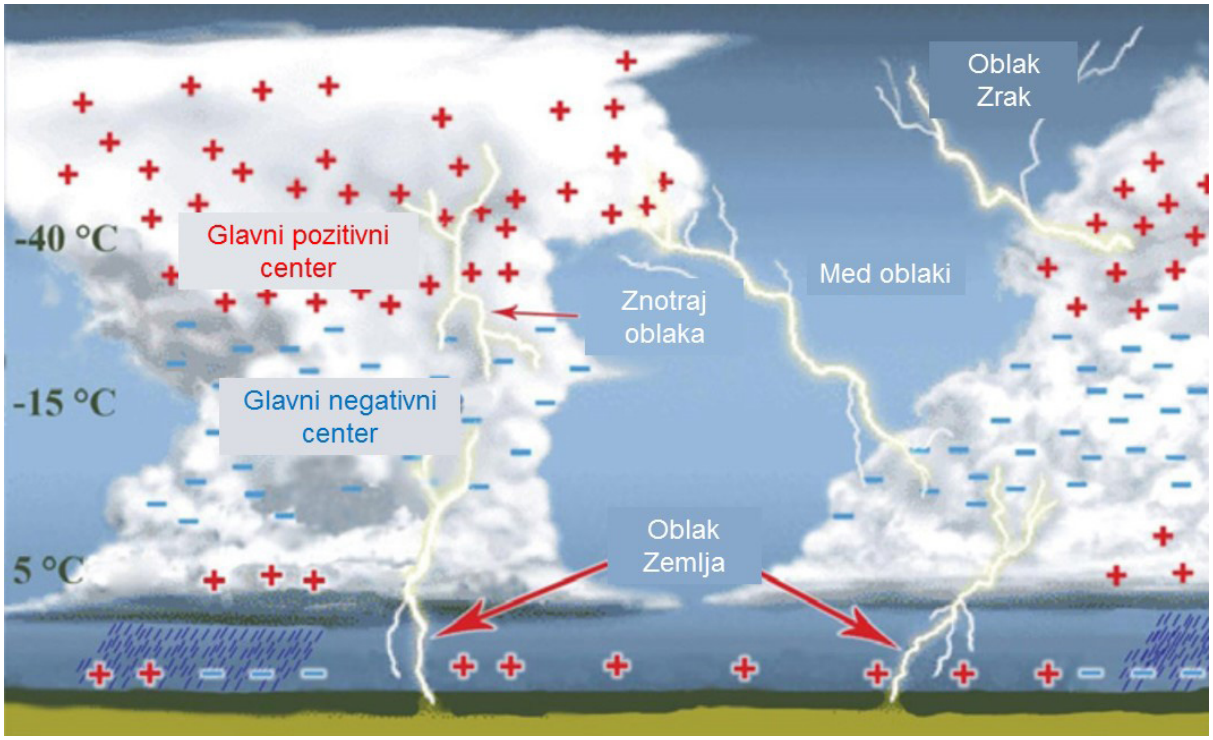
1 UVOD

1.1 Strela

Beseda strela označuje naravni pojav elektrostatične razelektritve, ki je posledica nenadne izenačitve električnega potenciala med dvema različno električno nabitima območjema preko ioniziranega zraka. Ta različno nabita območja so lahko znotraj nevihtnega oblaka, med dvema nevihtnima oblakoma, med oblakom in drugim območjem v zraku, ali pa med nevihtnim oblakom in površino Zemlje, nad katero se ta oblak nahaja. Električno nabita območja med seboj izenačijo potencial preko vzpostavitve visokoprevodnega ioniziranega kanala v zraku. Ker skozi ta prevoden kanal v kratkem času steče velika količina električnega naboja, se kanal zelo hitro segreje, kar zaznamo vidno kot svetlobni blisk, temu pa sledi še tlačni val, ki ga zaznamo kot zvočni pok in mu pravimo grmenje.

V vsakem danem trenutku se na Zemlji odvija v povprečju 2000 neviht. Skupno pri teh nevihtah vsako sekundo sprosti v povprečju med 30 do 100 strel, kar pomeni več kot 8 milijonov strel na dan (Dwyer in Uman, 2014).

Na Slika 1 je shematsko prikazana tipična porazdelitev električno nabitih delcev v oblaku. Shema tudi prikazuje različne tipe razelektritev in sicer razelektritev med oblakom in tlemi (CG strela - »Cloud to Ground«), znotraj oblaka (IC strela – »Intracloud«), med oblaki (CC strela – »Cloud to Cloud«) in med oblakom in zrakom (CA strela – »Cloud to Air«). Na sliki sta označena pozitivno in negativno električno nabita centra. Glavni pozitivni center se nahaja v zgornjem delu oblaka, medtem ko se negativni center nahaja v spodnjem delu oblaka, pod negativnim centrom pa se lahko nahaja še en, manjši pozitivni center. Dejanska porazdelitev nabojev je lahko tudi precej bolj kompleksna od tiste na Sliki 1 in se lahko od oblaka do oblaka močno razlikuje. Včasih je lahko porazdelitev celo obrnjena, tako da se pozitivni center nahaja na dnu oblaka, negativni pa na vrhu, a je to redkost (MacGorman in Rust, 1998). Le 25% vseh strel je med oblakom in tlemi (CG strela).

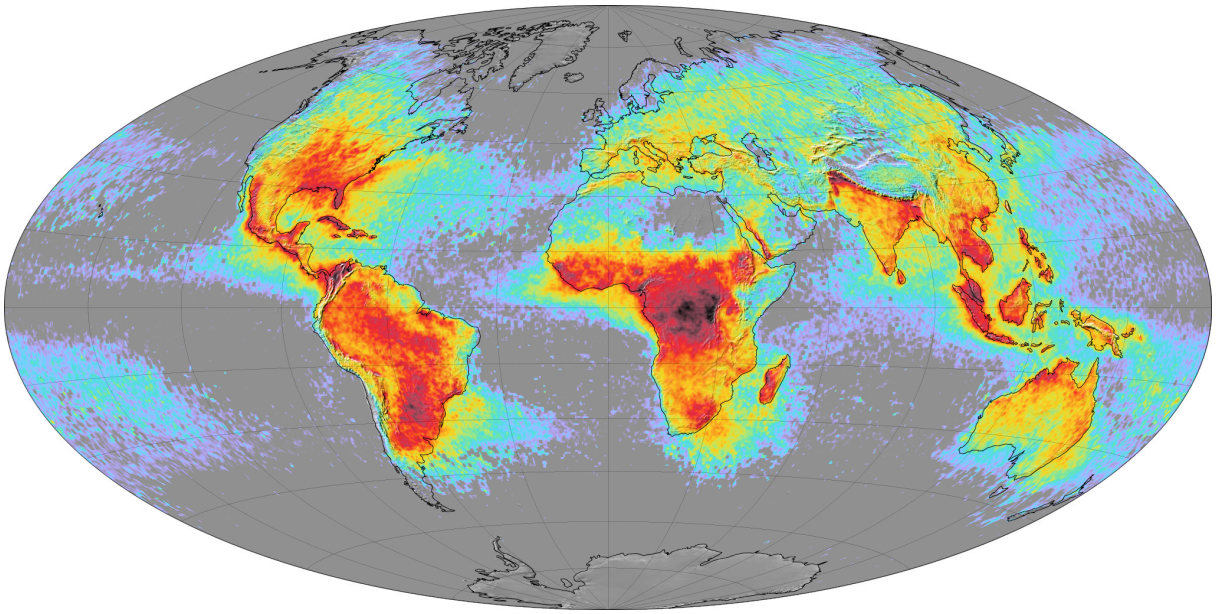


Slika 1: Električno nabiti centri v oblakih in tipi elektrostatičnih razelektritev. Povzeto po Dwyer in Uman, 2014.

2

Ker je težko predvideti, med katerima točkama bo prišlo do razelektritve, je preučevanje strel z neposrednimi meritvami zelo zahtevno; že pri strelah tipa CG je to težko in zahteva dolgoletne meritve na mestih, kjer so ti udari sorazmerno pogosti (npr. na vrhovih gora ali strelovodih visokih stavb), pri strelah tipov IC in CC pa so takšne meritve skorajda neizvedljive, saj bi jih morali opravljati z balonom in pri tem uganiti, med katerim dvema točkama v oblaku oziroma med oblakoma bo potekala razelektritev.

Lastnosti strele so odvisne tudi od lastnosti okolja – nadmorske višine, geografske širine, vetrnih tokov, relativne vlažnosti zraka, bližine hladnih oziroma toplih zračnih mas. Udari strel niso enakomerno porazdeljeni po Zemlji. Približno 70% vseh strel tipa CG udari v kopno, najpogostejše so v tropskem pasu, kjer prihaja do najpogostejših in najmočnejših mešanj hladnih in toplih zračnih mas, hkrati pa je vsebnost vlage v zraku zaradi njegove višje povprečne temperature lahko visoka, kar olajša nastajanje nevihtnih oblakov (Slika 2). Najredkeje pa se pogoji za strele vzpostavijo na severnem in južnem polu Zemlje, kjer se zračne mase gibljejo počasneje, zrak pa vsebuje najmanj vlage (Oliver, 2005).



Slika 2: Porazdelitev strel na Zemlji. Povzeto po http://geology.com/articles/lightning-map.shtml.

Najbolj pogost naravni pojav, ki vodi do strel, so nevihtni oblaki. Do strel lahko pride tudi v drugih primerih, kjer prihaja do trenja med različno toplimi in različno vlažnimi plastmi zraka, še posebej, če so v zraku prisotni električno nabiti delci – v tornadih, prašnih nevihtah, gozdnih požarih, še posebej izrazito pa med vulkanskimi izbruhi (Oliver, 2005).

S stališča te disertacije so pomembne predvsem strele tipa CG, torej takšne med oblakom in tlemi, na površini oziroma tik pod površino katere se tako danes kot v preteklosti nahaja velika večina habitatov organizmov. Zato se bomo od tu naprej osredotočili le na strele tipa CG, ki so v naslednjih podpoglavjih opisane podrobneje.

1.1.1 Kratek zgodovinski pregled

Znanstveno preučevanje strel se je začelo v 18. stoletju, med pionirji tovrstnih raziskav pa je bil Benjamin Franklin. Leta 1749 je opisal podobnosti med nevihtno strelo in laboratorijskimi razelektritvami (iskrami), ki jih je ustvarjal z že dolgo znano metodo drgnjenja dveh različnih materialov (npr. volne in voska), nato pa še s štiri leta prej izumljenim prototipom električnega kondenzatorja – leidensko steklenico (ime je dobila po mestu Leiden, kjer je živel njen izumitelj Pieter van Musschenbroek). Leta 1752 je opravil tudi znameniti eksperiment z letečim zmajem, s katerim je potrdil, da so strele v resnici električni pojav – med nevihto je spustil zmaja proti nevihtnemu oblaku, prevodno vrvico z zmaja pa je pripel na leidensko steklenico, ki se je nato naelektrila. Franklin je bil tudi prvi, ki je izmeril, da je spodnji del oblaka najpogosteje nabit negativno (Dwyer in Uman, 2014; Cohen, 1996). Po Franklinu je preučevanje strel zamrlo in se nadaljevalo šele v začetku 20. stoletja, ko se mu je posvetil meteorolog in fizik Charles T. R. Wilson (Wilson, 1921, 1916), ki je pozneje za izum meglične celice za zaznavanje vesoljskih delcev prejel Nobelovo nagrado. Pred letom 1970 je bila glavna motivacija za preučevanje strel zaščita daljnovodov in transformatorskih postaj (Dwyer in Uman, 2014), z razmahom zračnega prometa in elektronskih naprav pa sta

vse bolj v ospredje prihajala tudi tveganost in škoda, ki so jo strele lahko v zraku povzročile letalom in helikopterjem, na tleh pa vse večjemu številu domačih elektronskih naprav. Hkrati je razvoj računalništva omogočil tudi bolj napredno zajemanje in analizo podatkov. Leta 1975 so se začeli tudi prvi poskusi umetnega proženja strel med nevihto s pomočjo raket, ki so jih usmerili v nevihtni oblak, za seboj pa so vlekle tanko bakreno žico. Po žici je nato prišlo do razelektritve, kar je pomenilo, da je strela udarila na pričakovano lokacijo na Zemlji, to pa je raziskovalcem omogočilo snemanje razelektritve s hitro kamero in merjenje ključnih električnih parametrov, kot sta amplituda električnega toka in njegov časovni potek v razelektritvi (Rakov, 2013). V zadnjih tridesetih letih so pri preučevanju neviht odkrili tudi vrsto spremljajočih pojavov, kot so rezelektritve nad vrhovi oblakov in nastanek elektromagnetnega valovanja zelo kratkih valovnih dolžin in visokih energij, kot so gama in rentgenski žarki (Dwyer s sod., 2012; McCarthy in Parks, 1985).

1.1.2 Naelektritev in razelektritev

Proces naelektritve v nevihtnem oblaku še ni v celoti pojasnjen (Rakov, 2013; Saunders, 1993), vemo pa, da ta proces poteka, če ima zrak v oblaku temperaturo pod lediščem (najpogosteje med −10 °C in −20 °C), in da do naelektritve prihaja zaradi trkov med sodro ter drobnimi ledenimi kristalčki (lahko pa tudi podhlajenimi drobnimi kapljicami) v oblaku. Sodro tvorijo kroglice zmrznjene vode premera nekaj milimetrov, katerih masa in velikost sta manjši kot pri toči (tako se sodra lahko nekaj časa zadržuje v oblaku, toča pa pade na tla), a večji kot pri drobnih ledenih kristalčkih in podhlajenih kapljicah v oblaku, ki so tako lahki, da jih lahko zračni tokovi dvigajo proti vrhu oblaka. Ko sodra pridobiva na velikosti, se postopoma giblje proti dnu oblaka, na svoji poti pa trka ob kristalčke, ki se dvigujejo z zrakom in se tako pomikajo v nasprotni smeri. Ob trkih se naboji med temi delci prenašajo tako, da se ledeni kristalčki naelektri pozitivno in tako prenaša pozitivni naboj proti vrhu oblaka, sodra pa se naelektri negativno, zaradi česar je pri dnu oblaka vse večji negativen naboj. Tako v oblaku postopoma pride do močne naelektritve, z nekaj deset coulombipozitivnega naboja v zgornjih delih oblaka in podobno količino negativnega naboja pri dnu oblaka (Dwyer in Uman, 2014; Rakov, 2013; Saunders, 1993).

Strele, ki ustvarijo razelektritev med oblakom in tlemi (strele tipa CG), delimo glede na začetno lokacijo in smer razelektritve, kot tudi glede na polariteto električnih nabojev. Do elektrostatične razelektritve v grobem pride v dveh korakih. Najprej se postopoma, po segmentih zraka med oblakom in Zemljo, izgradi električno visoko prevoden kanal ioniziranega zraka (*vodilni kanal*), ko ta poveže oblak in tla, pa steče skozenj močan električni tok med oblakom in tlemi (*povratni udar*).

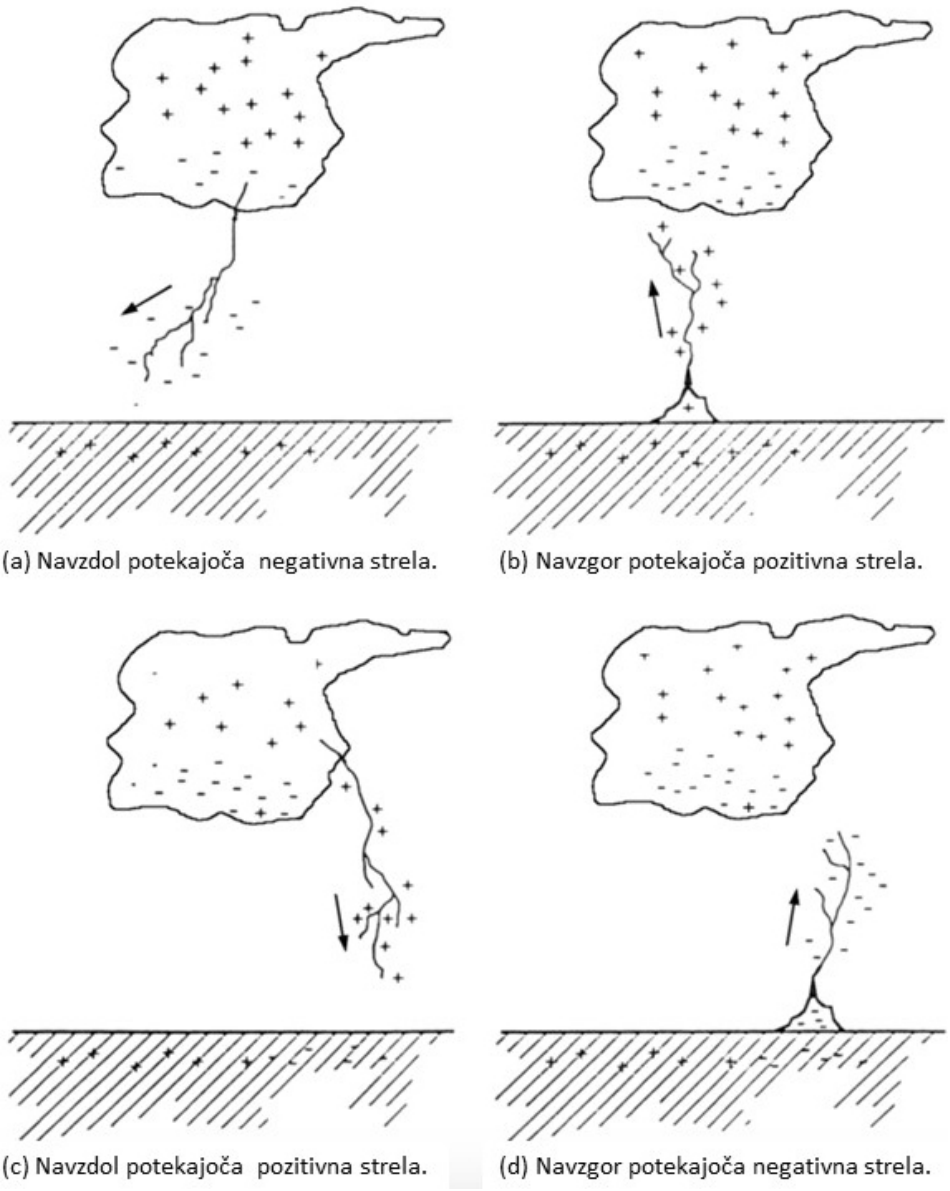
Vzpostavitev prevodnega kanala je zapleten postopek in še danes ni v celoti pojasnjen (Berkopec, 2012; Rakov in Uman, 2007). Izziv predstavlja predvsem dejstvo, da fizikalne zakonitosti povedo, da lahko do razelektritve med oblakom in tlemi pride le, če jakost električnega polja v zraku med njima preseže vrednost, pri kateri pride do dielektrične porušitve zraka. Ta vrednost je odvisna od vlažnosti in tlaka zraka, na nadmorski višini 0 m pri suhem zraku znaša E_b = 2.6 x 10^6 V/m, v vlažnem zraku ob padavinah pa upade na

vrednost med 1.0 in 1.4 x 10^6 V/m (Solomon s sod., 2001). Največja jakost električnega polja v zraku tik pred udarom strele (E_{max}) bi morala torej dosegati vsaj porušitveno jakost (veljati bi torej moralo $E_{max} \geq E_b$). Ko so poskušali to jakost izmeriti, pa največja izmerjena vrednost ni presegla niti 10% E_b (Solomon s sod., 2001; Stolzenburg in Marshall, 2008). Predlaganih je bilo nekaj teorij, kako bi lahko kljub tako nizki izmerjeni vrednosti E_{max} ta vrednost dejansko (vsaj lokalno in za kratek čas) presegala E_b; med širše sprejetimi sta dve, od katerih se ena naslanja na lokalno ojačeno električno polje v bližini naelektrenih vodnih kapljic in kristalčkov, druga pa na ionizirajoče delovanje visokoenergijskih elektronov, ki priletijo iz vesolja in jih električno polje v oblaku še dodatno pospeši (Rakov, 2013; Berkopec, 2012; Solomon s sod., 2001). Podrobnejša obravnava omenjenih teorij presega okvir te disertacije.

Slika 3 prikazuje različne podtipe strel tipa CG, glede na smer izgradnje vodilnega kanala in polariteto električnih nabojev v tem kanalu. Na sliki 3a je predstavljena najpogostejša razelektritev, pri kateri se izgradnja vodilnega kanala začne v spodnjem delu oblaka, zaradi česar je tudi zrak v kanalu ioniziran negativno, ko kanal doseže tla, pa se skozenj v tla prenese negativni naboj (pretežno elektroni); to razelektritev imenujemo *navzdol potekajoča negativna strela* in predstavlja približno 90% vseh strel tipa CG. Na sliki 3c je predstavljena *navzdol potekajoča pozitivna strela*, pri kateri se tvorba vodilnega kanala prav tako začne v oblaku, vendar v njegovih zgornjih delih, zaradi česar se tudi zrak v kanalu naelektri pozitivno, ko kanal doseže tla, pa se skozenj v tla prenese pozitivni naboj (dejanski nosilci električnega toka strele so tudi tukaj pretežno elektroni, ki tako potujejo po kanalu v nasprotni smeri – iz tal v oblak); ta razelektritev predstavlja približno 10% vseh strel tipa CG. Sliki 3b in 3d ponazarjata *navzgor potekajočo pozitivno* in *navzgor potekajočo negativno strelo*, pri katerih se začne kanal graditi v smeri od tal proti oblaku, kar pa se v naravi zgodi pri manj kot 1% vseh strel tipa CG (Dwyer in Uman, 2014).

K izvoru navzdol potekajoče negativne strele pripomore lokalna razelektritev med spodnjim delom negativno nabitega oblaka in majhnim pozitivno nabitim delom tal pod njim, kot kaže Slika 1. Lokalna razelektritev poskrbi za sprostitev elektronov s sodre in ostalih težjih delcev. Ker imajo elektroni majhno maso, so prosti zelo mobilni in se v električnem polju premikajo precej lažje in hitreje kot težji električno nabiti delci (naelektrene molekule zraka, kapljice in kristalčki ledu, zrnca sodre). Zato so prav elektroni glavni nosilci električnega toka, ki steče po vzpostavitvi kanala med oblakom in tlemi.

Izgradnja kanala je pogosto večstopenjska; v teh primerih govorimo o *koračnem vodilnem kanalu*, saj se elektroni po zraku proti tlom ne gibljejo zvezno, temveč v sunkovitih korakih, od katerih je tipično vsak dolg nekaj deset metrov, sledijo pa si z zamiki nekaj deset mikrosekund, ki pa s približevanjem tlom upada: koraki tik pod oblakom si sledijo z zamiki približno 50 µs, tisti tik nad tlemi pa z zamiki okoli 10 µs (Dwyer in Uman, 2014).



Slika 3: Različne vrste strel tipa CG, glede na izvor razelektritve. a) navzdol potekajoča negativna, b) navzgor potekajoča pozitivna, c) navzdol potekajoča pozitivna in d) navzgor potekajoča negativna strela. Povzeto po Dwyer in Uman, 2014.

V času, ko se koračni vodilni kanal navzdol potekajoče negativne strele še izgrajuje, se prosti elektroni z dna oblaka gibljejo vzdolž že izgrajenih segmentov proti tlom s hitrostjo približno 2×10^5 m/s (Dwyer in Uman, 2014; Rakov, 2013). Preden zadnji korak kanala doseže Zemljo, se vzdolž kanala nabere za nekaj coulombov prostih elektronov (pri kanalu dolžine 5 km in tipični naelektritvi med oblakom in tlemi 10^{-3} C/m je v kanalu naboj 5 C), skozenj pa v sunkih teče tok, ki v povprečju znaša med 100 in 200 A, v konicah ob izgradnji novega koraka kanala pa okoli 1000 A (Dwyer in Uman, 2014). Ta električni tok ob izgradnji vsakega koraka odda tudi pulze elektromagnetnega valovanja v več delih elektromagnetnega spektra, v radiofrekvenčnem področju in optičnem področju (vidna in ultravijolična svetloba), pa tudi v področju rentgenskih žarkov, katerih skupna energija znaša približno 200 keV (Dwyer in

Uman, 2014). Vidni premer kanala znaša med 1 m in 10 m, a večina električnega toka teče skozi njegov osrednji del, ki ima premer le nekaj centimetrov (Loeb, 1966).

Ko se negativno naelektren vodilni kanal korakoma približuje tlom, se na površini tal začno nabirati pozitivni naboji, njihova koncentracija pa s približevanjem korakov kanala tlom narašča. Ko je konica kanala že blizu tal, je koncentracija teh pozitivnih nabojev pogosto dovolj velika, da iz tal preskočijo v konico približujočega se kanala in prevodno pot med oblakom in tlemi tako sklenejo nekaj hitreje, kot bi se ta sklenila z izgradnjo vodilnega kanala v smeri navzdol. Ko kanal poveže oblak s tlemi, prosti elektroni iz zadnjega koraka kanala stečejo v tla skoraj z svetlobno hitrostjo, temu pa sledi povratni udar – skozi kanal stečejo v tla še vsi ostali prosti elektroni iz višjeležečih korakov kanala, njegove neposredne okolice in dna oblaka, ki se gibljejo s hitrostmi med 1/3 in 1/2 svetlobne hitrosti in ustvarijo močan električni tok s konico velikosti nekaj deset kiloamperov (Dwyer in Uman, 2014; Rakov, 2013).

Torej ko kanal poveže oblak s tlemi, se ustvari močan električni tok s konico, sunek svetlobe, ki ga zaznamo kot blisk, in tlačni val, ki ga zaznamo kot grom.

Ko koračni vodilni kanal potuje proti tlom, se med zaporednimi koraki pogosto razveji. Ko ena izmed vej ustvari stik s tlemi, elektroni iz stranskih vej stečejo nazaj v osrednji kanal in skozenj v tla. Močan električni tok povratnega udara segreje zrak v kanalu na približno 30,000 °C, kar privede do sprostitve svetlobe (tako vidne kot ultravijolične), vidni del katere zaznamo kot blisk, in tlačnega vala, ki lahko doseže tudi več kot 10 atmosfer in ga zaznamo kot grom oziroma grmenje (Rakov, 2013).

Ko se povratni udar zaključi, električni tok preneha teči, vendar v približno 80% primerih navzdol potekajočih negativnih strel nato pride do dodatnih razelektritev, ki najpogosteje potekajo po kanalu, ki ga je ustvarila prvotna razelektritev; najpogosteje je zaporednih razelektritev med 3 in 5, sledijo pa si v zamikih, ki najpogosteje znašajo med 40 in 50 ms (Uman, 1987). V primerjavi s prvim povratnim udarom so dodatne razelektritve precej manjše po količini naboja, ki ga prenesejo iz oblaka v tla, in po električnem toku, pa tudi manj razvejane, saj že obstaja prevodna pot do tal. A kljub temu približno tretjina dodatnih razelektritev le deloma potuje po že ustvarjenem kanalu, nekaj korakov pred stikom s tlemi pa se razveji in vstopi v tla v drugi točki. Po koncu vsake razelektritve začne prevodnost kanala hitro upadati, zato so nadaljnje razelektritve po istem kanalu mogoče le, če od zadnje ni poteklo več kot približno 100 ms, sicer se kanal dokončno zapre (Dwyer in Uman, 2014).

Medianska maksimalna vrednost (amplituda) električnega toka pri prvem povratnem udaru navzdol potekajoče negativne strele znaša okoli 30 kA (pri naknadnih udarih med 10 in 15 kA), čas dviga toka z ničle na maksimalno vrednost nekaj mikrosekund (pri naknadnih udarih nekaj desetink mikrosekunde) (Dwyer in Uman, 2014; Kotnik, 2013a; Rakov, 2013; Chowdhuri s sod., 2005), količina prenešenega naboja iz oblaka v tla približno 30 C, prenos naboja pa je merjeno od trenutka, kot se vzpostavi prevodni kanal med oblakom in tlemi, končan v nekaj sto mikrosekundah (Dwyer in Uman, 2014; Rakov, 2013).

Poglejmo še najpomembnejše podatke za navzdol potekajoče pozitivne strele, kakršnih je, kot je bilo že omenjeno, približno desetina vseh strel tipa CG. Koračni vodilni kanal se pri teh razelektritvah začne na obrobju zgornjih delov oblaka, kjer se nahajajo pozitivni naboji, ali pa v ožjem delu dna oblaka, ki je lokalno naelektren pozitivno. Pri takšnih razelektritvah lahko amplituda električnega toka preseže tudi 250 kA, medtem ko najmočnejše navzdol potekajoče negativne strele dosežejo okoli 100 kA (Rakov, 2013; Chowdhuri s sod., 2005). Vendar to odseva predvsem večjo raznolikost pozitivnih strel v primerjavi z negativnimi, mediana amplitude električnega toka pa je le rahlo višja in znaša okoli 35 kA (Chowdhuri s sod., 2005). Pri pozitivnih strelah pride praviloma le do ene razelektritve, a je pri najmočnejših med njimi skupna količina prenesenega naboja kljub temu daleč višja od tiste, ki jo lahko v tla prenese zaporedje negativnih strel po istem kanalu (Dwyer in Uman, 2014; Chowdhuri s sod., 2005).

1.1.3 Uporabnost strel

Elektrostatične razelektritve se v industriji uporabljajo na različnih področjih, od svetil do obdelave različnih materialov, še posebej za varjenje, segrevanje (obločne peči) in za rezanje materialov s plazmo. Pred štirimi leti je bila opravljena raziskava, ki je pokazala, da lahko z nanosekundnimi električnimi pulzi, ki jih dovedemo v kožo z elektrostatičnimi razelektritvami, dosežemo večji vnos in posledično izražanje DNA v celicah kože, kot s klasičnim kontaktnim dovajanjem električnih pulzov (npr. prek ploščatih elektrod, pritisnjenih ob kožo) (Broderick s sod., 2011). Poleg tega pa je bilo v zadnjih letih objavljenih tudi nekaj raziskav o tem, ali bi lahko elektrostatične razelektritve, ki jih povzročajo strele, delovale kot morebitni naravni mehanizem prenosa DNA med organizmi in tako prispevale k njihovi raznolikosti v biološki evoluciji (Kotnik, 2013a; Demaneche s sod., 2001).

Učinki, ki jih elektrostatične razelektritve ustvarjajo v stiku z enostavnimi materiali, so dobro poznani. Večina tehnoloških procesov izkorišča hitro povečanje temperature, ki je posledica električnega toka ob razelektritvi, medtem ko pri svetilih emisija svetlobe temelji tudi na ionizaciji in kvantnem vzbujanju plina, skozi katerega teče električni tok. Ko električni tok, doveden z elektrostatično razelektritvijo, teče skozi snov, ustvarja lokalno električno polje, ki je najmočnejše tam, kjer je tokovna gostota najvišja, to je ponavadi v točki vstopa električnega toka v snov. Električni tok se nato razširi po snovi, zato se lokalna vrednost električnega polja proporcionalno porazdeli in je zato manjša. Če električni tok točkasto (skozi majhno površino) ne le vstopa v snov, temveč tudi izstopa iz nje, tokovna gostota in posledično lokalna vrednost električnega polja zopet naraste tudi v točki izstopa.

1.1.4 Vpliv elektrostatičnih razelektritev na živo snov

Pri elektrostatični razelektritvi v živi snovi so prisotni vsi osnovni fizikalni procesi kot v neživi snovi (električni tok, inducirano električno polje, dvig temperature, možna je tudi ionizacija, ki jo povzroči električni tok), poleg teh pa tudi vrsta biokemičnih in biofizikalnih pojavov. Električno polje do ~60 mV/cm trajanja do ~100 µs stimulira živčna in mišična vlakna, veliko močnejše električno polje (nekaj sto V/cm ali več) pa lahko tako v vzdražnih kot v

nevzdražnih celicah povzroči občuten dvig prepustnosti njihovih membran (elektroporacija) in/ali zlitje dveh ali več celic (elektrofuzija). Če celica po izpostavitvi električnemu polju preživi in si opomore, je elektroporacija reverzibilna, sicer pa ireverzibilna – ireverzibilna elektroporacija torej povzroči celično smrt. Električno polje večjih jakosti, ki traja dovolj dolgo, pa lahko povzroči še toplotne poškodbe celice in njenih molekul – denaturacijo proteinov in denaturacijo (taljenje) DNA.

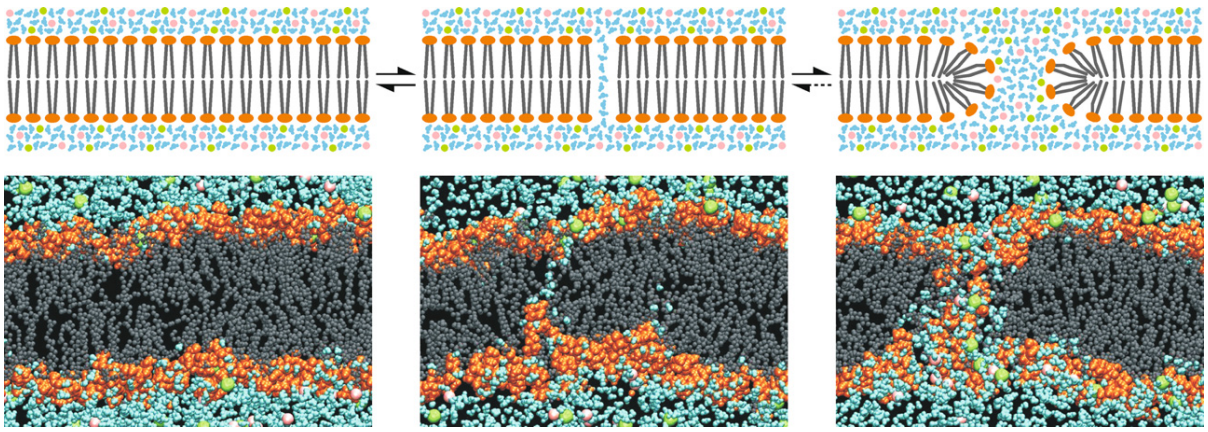
1.2 Elektroporacija in elektrofuzija

Elektroporacija in elektrofuzija sta znani že več desetletij. Prve objave o elektroporaciji segajo v leto 1958, kjer je bila dosežena prevodnost membrane pri vzdraženih celicah (Raniver node of myelinated axon) (Stämpfli, 1958) in leta 1967 pri nevzdržnih celicah (porirana bakterijska celična stena in membrana) (Hamilton in Sale, 1967). Membrane celičnih organel (chromafin granula) so bile elektroporirane leta 1972 (Neumann in Rosenheck, 1972), in ravninski lipidni dvosloji leta 1979 (Benz s sod., 1979). Elektrofuzija je bila prvič demonstrirana na živalskih celicah (z in brez celičnega jedra) leta 1980, in tudi na rastlinskih protoplastih, leta 1981 pa še na lipidnih veziklih (Zimmermann in Scheurich, 1981; Zimmermann s sod., 1981; Scheurich s sod., 1980; Neumann s sod., 1980).

Od odkritja naprej pa vse do danes sta elektroporacija in elektrofuzija predmet naraščajočega zanimanja in sta do danes našli mesto v številnih aplikacijah na področjih medicine in biotehnologije. Na področju medicine se reverzibilna elektroporacija uporablja kot metoda vnosa kemoterapevtikov v celice pri zdravljenju raka (Yarmush s sod., 2014; Miklavčič s sod., 2014; Mali s sod., 2013; Miklavčič s sod., 2012, 2010; Marty s sod., 2006; Miklavčič s sod., 2006, 2005, 2000, 1998) in kot metoda vnosa DNA pri genski terapiji (Zupanič s sod., 2012; Murakami in Sunada, 2011; Čemažar s sod., 2010; Miklavčič s sod., 2000). Ireverzibilna elektroporacija pa obeta velik potencial pri ablaciji tkiva (Zupanič s sod., 2012; Davalos s sod., 2005). Elektrofuzija postaja vse bolj zanimiva pri pridobivanju monoklonskih protiteles, tako za diagnostične kot za terapevtske namene (Rems s sod., 2013; Yu s sod., 2008). Na področju biotehnologije je ireverzibilna elektroporacija učinkovita metoda za ekstrakcijo biomolekul iz celic in tkiv (Mahnič-Kalamiza in Vorobiev, 2014; Sack s sod., 2010; Suga in Hatakeyama, 2009), hkrati pa je tudi zelo uporabna (v kombinaciji z morebitnimi termičnimi učinki ali brez njih) kot metoda za inaktivacijo ali uničenje mikroorganizmov (pasterizacija, sterilizacija) (Zupanič s sod., 2012; Gusbeth s sod., 2009).

Čeprav nekaj neznank še obstaja, je elektroporacija danes dobro poznana in preučena na ravni membrane in celice, v zadnjih letih pa napredek v simulacijah molekularne dinamike omogoča tudi vse boljši vpogled v ta pojav tudi na ravni posameznih molekul in atomov (Polak s sod., 2013; Marrink s sod., 2009; Böckmann s sod., 2008; Knecht in Marrink, 2007; Tarek, 2005; Leontiadou s sod., 2004). Kljub temu so mehanizmi prenosa s pomočjo elektroporacije (še posebej prenosa DNA molekul) še vedno predmet preučevanja. Če elektroporacijo povzroči obločna razelektritev, k rasti prepustnosti membrane poleg izpostavitve električnemu polju lahko prispevata še tlačni val (sonoporacija) in/ali segretje (termoporacija) (Jelenc s sod., 2012; Wolf s sod., 1989; Neumann s sod., 1982).

Elektroporacija je tako na podlagi teoretičnih izvajanj (Rems in Miklavčič, 2014; Weaver in Chizmadzhev, 1996; Sugar in Neumann, 1984) kot v skladu z rezultati simulacij molekularne dinamike (Polak s sod., 2013; Böckmann s sod., 2008; Tarek, 2005) posledica vodnih por v lipidnih dvoslojnih celičnih membran, ki nastanejo zaradi vsiljene napetosti, ki jo na membrani zgradi izpostavitev električnemu polju. Pri nadpragovni vrednosti te napetosti (nad nekaj sto milivolti) molekule vode prodro v lipidni dvosloj, njihova interakcija s sosednjimi lipidi pa povzroči reorientacijo slednjih s polarnimi glavami proti molekulam vode, kar ustvari polarno in s tem hidrofilno steno vodne pore (Slika 4). Zaradi hidrofilnosti je pora, s tem pa lokalno tudi membrana, prepustno za ione in v vodi raztopljene (prosto plavajoče) molekule. Elektroporacija je fizikalen pojav, ki nastopi tako v čistih lipidnih dvoslojih kot v naravnih membranah prokariotskih in evkariotskih celic, skozi katere lahko tako prehajajo različne molekule, tudi DNA, in to v obe smeri – v celice (Kotnik s sod., 2012; Neumann s sod., 1982) in iz njih (Kotnik s sod., 2012; Hamilton in Sale, 1967). Nastanek por je posledica zakonitosti elektrokemije in statistične termodinamike (Yarmush s sod., 2014; Weaver in Chizmadzhev, 1996; Sugar in Neumann, 1984). Zaradi statistične termodinamike nastanek pore ni deterministično pojav, ki bi vsakič nastopil pri enakih pogojih in bi bil povsem ponovljiv (Kramar s sod., 2012), sta pa tvorba por in transport skoznje močno korelirana z višino napetosti na membrani (transmembranske napetosti), ki jo ustvari električno polje – na področjih z višjo transmembransko napetostjo sta elektroporacija in na njej temelječi transport skozi membrano izrazitejša (Kotnik s sod., 2010). Transmembranska napetost pa je premo sorazmerna jakosti električnega polja, v katerem se nahaja celica (Kotnik s sod., 2010).

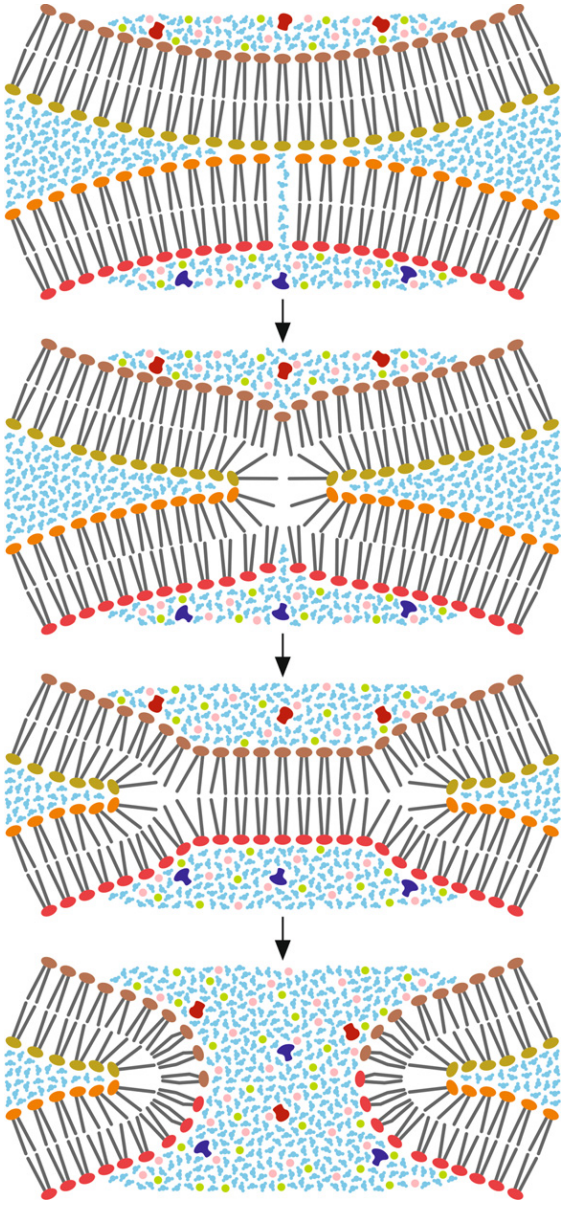


Slika 4: Idealizirana molekularna shema (zgoraj) in simulacija molekularne dinamike elektroporacije na atomskem nivoju (spodaj). Električno polje poteka pravokotno gledano na lipidni dvosloj, ki ga obdaja solna raztopina. Povzeto po Kotnik, 2013a.

Elektrofuzija dveh celic se lahko zgodi zgolj v primeru, če sta celici v neposrednem stiku ob izpostavitvi električnemu polju, ali pa če prideta v tak stik v ustrezno kratkem času (sekunde ali celo minute) po izpostavitvi (Trontelj s sod., 2010; Teissie in Rols, 1986; Sowers, 1986). Eksperimentalni rezultati so pokazali, da se ob elektrofuziji dveh lipidnih dvoslojev praviloma najprej zlijeta enosloja v neposrednem stiku, enosloja, ki nista v stiku, pa v tej fazi ostaneta nespremenjena (Stenger in Hui, 1986). Ti poskusi kažejo, da tudi elektrofuzija poteka skozi

podobne faze kot fiziološka fuzija dveh celic: (1) zunanja enosloja, od katerih je vsaj en lokalno destabiliziran, se zlijeta na območju lokalne nestabilnosti; (2) zlita enosloja se pričneta oddaljevati v radialni smeri, nezlita enosloja pa tako prideta v stik diskaste oblike in naraščajočega radija; (3) diskasta stična površina med enoslojema poči in se preoblikuje v kanal (poro), ki povezuje citoplazmi obeh celic; ta kanal se nato razširja, s čimer se oblika nastale hibridne celice spremeni iz »osmičaste« v stabilnejšo okroglo oziroma ovalno, fuzija pa se uspešno zaključi (Cohen in Melikyan, 2004) (Slika 5). Fiziološka celična fuzija se od elektrofuzije razlikuje predvsem po mehanizmu, ki lokalno destabilizira zunanji enosloj vsaj ene celice in s tem sproži fuzijo – pri fiziološki fuziji je to vgradnja fuzogenih proteinov v ta enosloj (Chernomordik in Kozlov, 2008), pri elektrofuziji pa nastanek por zaradi elektroporacije ob izpostavitvi celice električnem polju (Abidor in Sowers, 1992).

Na učinkovitost vnosa genskega materiala v bakterije z elektroporacijo (elektrotransformacijo) vpliva več dejavnikov: vrsta bakterije; značilnosti molekule DNA; parametri električnega polja, ki so mu bakterije v procesu elektroporacije izpostavljene; sestava medija, v katerem se bakterije nahajajo. Učinkovitost elektrotransformacije bakterij izražamo s število transformantov zmožnih tvorbe kolonij (Colony Forming Units – CFU) na µg dodane DNA (CFU/µg DNA).



Slika 5: Idealizirana molekularna shema fuzije dveh celic zaradi elektroporacije. Povzeto po Kotnik, 2013a.

1.2.1 Vloga vrste bakterij

Elektrotransformacija je izvedljiva tako pri Gram-negativnih kot pri Gram-pozitivnih bakterijah, a je pri slednjih zaradi dodatne peptidoglikanske celične stene za uspešno elektrotransformacijo potrebno večje električno polje, najvišja dosegljiva učinkovitost elektrotransformacije pa je pri Gram-pozitivnih bakterijah nekaj velikostnih redov nižja kot pri Gram-negativnih. Pri različnih Gram-pozitivnih bakterijah je tako mogoče doseči učinkovitosti med 10^8 in 10^{10} CFU/µg DNA, pri Gram-negativnih pa med 10^6 in 10^7 CFU/µg DNA (Chassy s sod., 1988; Miller, 1994). Še nekaj nižja je najvišja dosegljiva učinkovitost pri bakterijah, obdanih s polisaharidno kapsulo, a je tudi pri teh elektrotransformacija izvedljiva; najvišje učinkovitosti tu znašajo okoli 10^4 CFU/µg DNA in jih dosežemo, če so bakterije ob izpostavitvi električnim pulzom v eksponentni fazi rasti (Fournet-Fayard s sod., 1995), ko se proizvodnja polisaharida zmanjša

(Dzul s sod., 2011; Cunnion s sod., 2001). Vsi zgoraj navedeni podatki o učinkovitosti elektrotransformacije se nanašajo na plazmidno DNA, ki se največkrat uporablja v ta namen in je v splošnem tudi najbolj učinkovita.

1.2.2 Vloga značilnosti molekule DNA

Učinkovitost elektrotransformacije se močno razlikuje tudi glede na značilnosti molekule DNA, ki jo želimo vnesti v celice. V smeri od najvišje proti najnižji dosegljivi učinkovitosti si molekulske oblike DNA sledijo takole:

- *dodatno zvita krožna dvojna vijačnica* (supercoiled circular double-stranded DNA), ki je avtohtona oblika DNA pri mnogih prokariotih;
- *sproščena (nezvita) krožna dvojna vijačnica* (relaxed circular double stranded DNA);
- *krožna enojna vijačnica* (circular single-stranded DNA), ki je avtohtona pri večini ssDNA virusov,
- *linearna dvojna vijačnica z homolognima koncema* (linear double stranded DNA with homologous ends), avtohtona pri evkariotih,
- *linearna dvojna vijačnica z nehomolognima koncema* (»linear double stranded DNA with nonhomologous ends«).

Kimoto in Taketo sta leta 1996 opravila obsežno študijo, v kateri sta primerjala učinkovitost elektrotransformacije pri *E. coli* z več oblikami DNA (Kimoto in Taketo, 1996). S plazmidno DNA pACYC177, ki je dvojna vijačnica (dsDNA), sta dosegla naslednje učinkovitosti: 1.9 x 10^9 CFU/µg DNA z dodatno zvito krožno dsDNA, 9.6 x 10^8 CFU/µg DNA s sproščeno krožno dsDNA in 1.2 x 10^5 CFU/µg DNA z linearno dsDNA s homolognima koncema. Z bakteriofagno DNA iz virusa *α3*, ki je krožna enojna vijačnica (ssDNA), sta dosegla učinkovitost 8.0 x 10^6 CFU/µg DNA, z bakteriofagno DNA iz virusa *ΦKh-1*, ki je prav tako krožna enojna vijačnica, pa 3.2 x 10^5 CFU/µg DNA.

King in sodelavci pa so leta 1993 primerjali učinkovitost elektrotransformacije z dvema različnima linearnima oblikama plazmidne DNA pBR322, ki sta bili obe dsDNA z nehomolognima koncema, in z eno dosegli učinkovitost 1.6 x 10^3 CFU/µg DNA, z drugo pa 4.0 x 10^2 CFU/µg DNA (King s sod., 1993).

1.2.3 Vloga koncentracije DNA

Tipične koncentracije DNA se gibljejo od *pg/ml* do *µg/ml*, v več poskusih pa so pokazali, da je učinkovitost elektrotransformacije konstantna; to pomeni, da je pri nespremenjenih eksperimentalnih pogojih verjetnost, da se bo posamezna bakterija transformirala s prosto plavajočo DNA iz svoje okolice, premo sorazmerna koncentraciji te DNA (Dower s sod., 1988).

1.2.4 Vloga parametrov električnega polja

Parametri električnega polja, s katerimi dosežemo optimalno (najvišjo dosegljivo) učinkovitost elektrotransformacije, se razlikujejo glede na vrsto organizmov, ki jih želimo transformirati. Okvirno se za elektrotransformacijo bakterij optimalne jakosti električnega polja gibljejo med 2 in 30 kV/cm, trajanja izpostavitve polju pa od nekaj milisekund do nekaj deset milisekund (Aune in Aachmann, 2010; Miller, 1994; Chassy s sod., 1988). Pri evkariotskih celicah so te jakosti tipično nekajkrat nižje, učinkovitost vnosa in izražanja DNA pa se še dodatno izboljša, če kratkemu in močnemu električnem pulzu, s katerim povzročimo elektroporacijo, sledi daljši in šibkejši električni pulz (nekaj deset V/cm), ki ustvari elektroforetsko silo na DNA molekule in s tem povzroči njihovo gibanje (Šatkauskas s sod., 2012). Ta učinek je še posebej izrazit pri poskusih z nizko koncentracijo DNA (Kandušer s sod., 2009; Wolf s sod., 1994). Pri prokariotskih organizmih (bakterijah in arhejah) o izboljšanju učinkovitosti elektrotransformacije z dodatnimi, elektroforetskimi pulzi zaenkrat še niso poročali.

Danes se za elektrotransformacijo zaradi boljše opredeljenosti (determiniranosti) parametrov električnega polja in ponovljivosti eksperimentalnih pogojev vse pogosteje uporabljajo električni pulzi pravokotne oblike (Miklavčič in Towhidi, 2010); tako je za elektroforetski vlek potrebno prvemu, visokemu in kratkemu pravokotnem pulzu, pripeti še en pulz nižje jakosti in daljšega trajanja. Klasični generatorji, namenjeni elektrotransformaciji, pa temeljijo na preprostem principu razelektritve kondenzatorja, ki daje pulze eksponentno padajoče oblike; pri takšnih pulzih pa maksimumu jakosti sledi njeno postopno upadanje, zaradi katerega nastane tudi elektroforetski vlek, dodatni pulzi pa niso potrebni. Kot smo že opisali, imajo časovni potek, podoben razelektritvi kondenzatorja – hiter dvig električnega toka in z njim ustvarjenega električnega polja do maksimuma, nato pa postopno eksponentno upadanje – tudi naravne nevihtne strele.

1.2.5 Vloga sestave medija

Na učinkovitost elektrotransformacije vpliva tudi medij, v katerem pride do izpostavitve električnemu polju, predvsem njegova osmolarnost. Več avtorjev je poročalo, da se vnos DNA poveča, če je medij hiperosmolaren, kar lahko v laboratorijskih poskusih dosežemo z dodano 0.5-1.5 M koncentracijo sorbitola ali manitola (Ito in Nagane, 2001; Xue s sod., 1999). V naravi je hiperosmolarnost najpogosteje posledica raztopljene soli, a morebitnega vpliva takšne hiperosmolarnosti medija na učinkovitost elektrotransformacije še niso preučevali, verjetno tudi zato, ker v nasprotju z manitolom ali sorbitolom dodana sol močno poveča električno prevodnost medija, kar bi močno povečalo potrebni tok za dosego enake jakosti polja v mediju, s tem pa bi se, tudi če bi zagotovili ustrezno sposoben generator, znatno povečalo tudi segrevanje medija in elektrolitski procesi v njem.

1.3 Evolucija in prenos genov

1.3.1 Evolucija

Več vej znanosti si še vedno prizadeva ugotoviti, kako je življenje nastalo. Čeprav so si teorije nastanka življenja na Zemlji v podrobnostih lahko močno različne, so si v grobem enotne, da se živa bitja skozi naravno selekcijo prilagajajo okolju, v katerem se nahajajo, temu procesu prilagajanja skozi čas pa pravimo evolucija. Vemo, da so bila enocelična bitja prisotna pred večceličnimi, celice brez jedra (prokarioti) pa pred celicami z jedrom (evkarioti). Vemo tudi, da se življenje na Zemlji ni moglo pričeti prej kot pred približno štirimi milijardami let, ko se je zemeljska površina ohladila pod vrelišče vode, pa tudi ne kasneje kot pred tremi milijardami let, saj iz teh časov že izvirajo zanesljivi dokazi o razširjenosti živih organizmov; nekateri posredni dokazi kažejo, da je bilo življenje prisotno že pred 3.5 ali celo 3.8 milijardami let (Taylor s sod., 2009; Mojzsis s sod., 1996; Oro s sod., 1990).

Do devetdesetih let prejšnjega stoletja je veljalo tudi prepričanje, da so bile najpomembnejši vir inovacij v evoluciji mutacije, ki so se z delitvijo celice (vertikalnim prenosom genov) prenašale na njene potomke. Ta teorija se je porušila, ko so znanstveniki pričeli sorodnost organizmov vrednotiti glede na sorodnost njihovih genomov (temu postopku pravimo filogenetska analiza) in pri tem ugotovili, da pri sledenju sorodnosti različnih genov pridejo do različnih sorodnostnih struktur (filogenetskih dreves). Študije genomov so pokazale tudi, da nekateri organizmi vsebujejo gen, ki ga njihovi bližji sorodniki nimajo, najdemo pa ga (enakega ali zelo podobnega) pri nekaterih evolucijsko zelo oddaljenih organizmih. Iz teh ugotovitev izhaja, da so organizmi v splošnem prevzemajo gene ne le od celice, iz katere izvirajo, temveč tudi iz okolice oziroma od drugih organizmov (Pennisi, 1999; Doolittle, 1998; Deckert s sod., 1998; Pennisi, 1998; Fleischmann s sod., 1995). Ta proces imenujemo horizontalni prenos genov (HGT). HGT pri prokariotih je bil opisan že mnogo prej (Woese, 1987; Akiba s sod., 1960), vendar mu niso pripisovali pomembne vloge v razvoju življenja. Rezultati filogenetskih študij kažejo, da je bil HGT skozi evolucijo in je še danes pomemben vir inovacij, ki so omogočile hiter razvoj zgodnjega življenja (Gogarten in Townsend, 2005; Doolittle, 2000).

V literaturi zasledimo tri mehanizme naravnega HGT: naravno kompetenco, konjugacijo in virusno transdukcijo (Mercier s sod., 2006; Trevors, 1999).

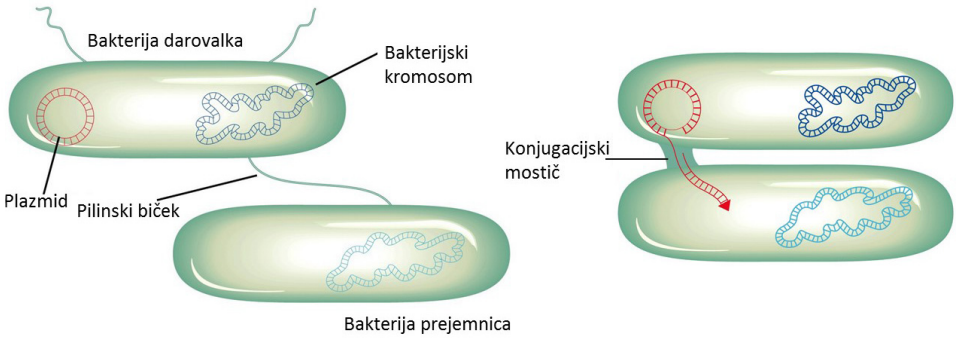
1.3.2 Konjugacija

Konjugacija je proces, pri katerem en prokariotski mikroorganizem (bakterija ali arheja), ki pride v neposreden fizični stik z drugim, s pomočjo posebnih proteinov vzpostavi kanal, ki poveže citosola obeh mikroorganizmov, skozi ta kanal pa eden od mikroorganizmov iz drugega potegne vase del genskega materiala. Ta proces ni recipročen – eden izmed mikroorganizmov je »darovalec«, drugi pa »prejemnik« DNA.

Konjugacijo sta leta 1946 odkrila in dokazala Joshua Lederberg in Edward Tatum (Tatum in Lederberg, 1947), ko sta iskala odgovor na vprašanje, ali obstaja pri bakterijah možnost prenosa genov, podobna spolnemu razmnoževanju pri večceličnih organizmih. Uporabila sta

dva heterotrofna seva bakterij *E. coli*, od katerih je bil vsak nezmožen sinteze nekaterih nujno potrebnih snovi, zato mu jih je bilo potrebno zagotoviti v gojišču (enemu sevu metionin in biotin, drugemu pa treonin, levcin in tiamin), ju zmešala in pokazala, da je bila po določenem času na vsakih 10^7 bakterij ena avtotrofna – sposobna sama sintetizirati vse naštete spojine in tako preživeti tudi, če niso bile dodane v gojišče.

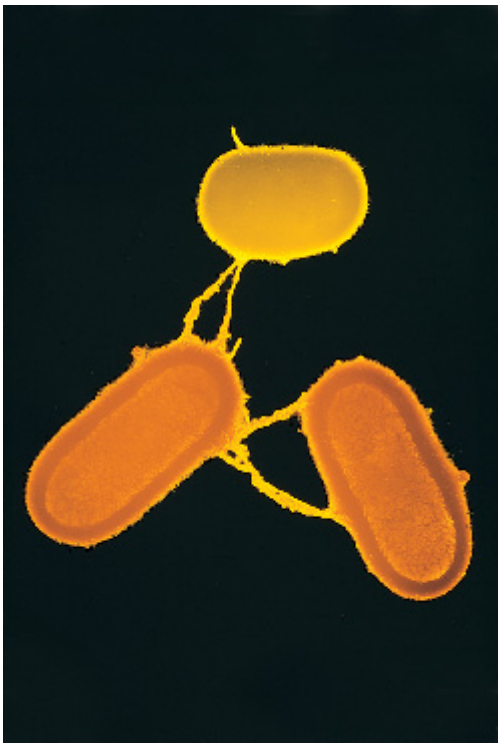
Bernard Davis je kasneje izključil možnost, da bi bila to posledica navzkrižnega hranjenja (ang. *cross fedding*), v katerem bi vsak sev v okolje izločal sestavine, ki bi jih drugi sev potreboval za rast (Griffiths s sod., 2000a). Leta 1953 pa je William Hayes ugotovil, da izmenjava delov genoma med bakterijami ni recipročna, in predlagal hipotezo, da vsebuje bakterija darovalka za takšno izmenjavo posebne molekule, ki jih bakterije prejemnice nimajo; te hipotetične molekule je poimenoval »plodnostni faktorji« oziroma faktorji F, bakterije darovalke je označil za F-pozitivne oziroma F^+, bakterija prejemnice pa za F-negativne oziroma F^- (Hayes, 1953).



Slika 6: Shematski prikaz konjugacije. Pilinski biček bakterije darovalke potegne bakterijo prejemnico k sebi, se z njo poveže s kanalom in skozenj prenese enojno vijačnico plazmidne DNA v bakterijo prejemnico. Povzeto po Griffiths s sod., 2000a, fig. 7–5.

Danes vemo, da se v konjugaciji prenašajo molekule plazmidne DNA, ki se v citosolu bakterije darovalke (F^+) razmnožujejo neodvisno od kromosomske DNA, ob stiku z bakterijo prejemnico (F^-) pa se lahko prenesejo vanjo, kot prikazuje Slika 6. Če je zmožnost konjugacije kodirana z genom, ki se nahaja na prenešeni plazmidni DNA, se zmožnost darovanja prenese na bakterijo prejemnico (ta se iz F^- pretvori v F^+). Takšna plazmidna DNA vsebuje gen za sintezo pilinskega bička, ki deluje kot nekakšna lovka, ki ujame bakterijo prejemnico, jo potegne v svojo bližino, vzpostavi direkten kanal med citosoloma, razcepi dvojno vijačnico svoje plazmidne DNA in eno od obeh enojnih vijačnic potisne skozi kanal v citosol bakterije prejemnice (Slika 7). Tam se ob njej zgradi še komplementarna veriga, s čimer postane plazmidna DNA dvojna vijačnica. Podobno pa se zgodi tudi v bakteriji darovalki, kjer tudi preostala enojna vijačnica tako spet postane dvojna vijačnica. V nekaterih primerih se gen za pilin integrira v kromosomsko DNA; tedaj govorimo o sevu Hfr (High-frequency recombination), pri katerem je učinkovitost konjugacije veliko večja, a se zmožnost konjugacije ne prenaša na bakterije F^-.

Podrobnejši pregled molekularnih procesov, ki skupaj tvorijo konjugacijo, presega okvir te disertacije, a jasno kaže, da je konjugacija zapleten proces, pravilni potek katerega zahteva krajevno in časovno natančno usklajeno delovanje več kompleksnih proteinov, ki so bržkone rezultat več sto milijonov let trajajoče evolucije.



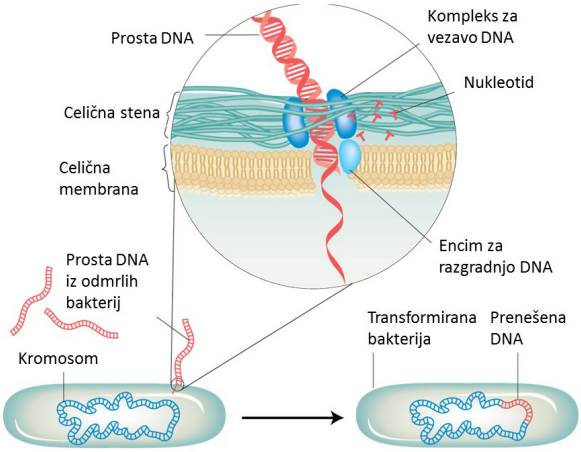
Slika 7: Konjugacija pod elektronskim mikroskopom. Vidni so pilinski bički, s katerimi bakterija darovalka vleče dve bakteriji prejemnici k sebi. Povzeto po http://www.nature.com/news/2001/011122/full/news011122-4.html.

1.3.3 Kompetenca

Določene vrste bakterij so sposobne prenesti vase prosto plavajočo DNA iz svoje okolice; za to uporabljajo vrsto specializiranih proteinov, s katerimi vežejo DNA in jo prenesejo torej preko celične stene in membrane v svoj citosol; temu procesu pravimo (naravna) kompetenca (Chen in Dubnau, 2004; Lorenz in Wackernagel, 1994).

Odkritje kompetence sega v leto 1928, ko je raziskovalec Frederick Griffith preučeval bakterijo *Streptococcus pneumoniae* (takrat imenovano *Pneumococcus*). To je patogena bakterija, a je nevarna le, če je obdana z polisaharidno kapsulo. V raztopino, v kateri so se nahajale mrtve bakterije *S. pneumoniae* iz seva, ki je imel polisaharidno kapsulo (njihovo smrt je povzročil s kuhanjem), je dodal žive bakterije *S. pneumoniae* iz seva, ki ni bil zmožen tvorbe kapsule, in po nekaj časa dobil aktivno populacijo *S. pneumoniae* s polisaharidno kapsulo (Griffith, 1928). Ker takrat še niso vedeli, da je dedni zapis shranjen v DNA, je prvotno obveljalo prepričanje, da so se bakterije brez kapsule transformirale v takšne s kapsulo z vnosom proteinov, ki so iztekli v iz mrtvih bakterij, zmožnih tvorbe kapsule. Leta 1944 pa je Oswald Avery s sodelavci pokazal, da do transformacije bakterij brez kapsule v takšne s kapsulo sicer res pride, ko te vase vnesejo snov, ki je iztekla iz mrtvih bakterij s kapsulo, vendar ta snov niso proteini, temveč DNA (Avery s sod., 1944).

Kompetence so zmožni le nekateri prokariotski mikroorganizmi, pa tudi med njimi večina le prehodno – pojavi se v pogojih, ki otežujejo rast in delitev teh organizmov, predvsem v gosto koncentriranih bakterijskih populacijah in ob pomanjkanju hrane (Lorenz in Wackernagel, 1994). Evolucijsko gledano je to v takšnih razmerah koristno, saj kompetenca omogoči dodaten vir genskih inovacij; ker je v danem okolju največ prosto plavajoče DNA iz tistih organizmov, ki jih je v tem okolju največ, lahko prevzem takšne DNA izboljša prilagoditev kompetentnega organizma temu okolju. Kompetenca je biokemično zelo kompleksen mehanizem; pri bakteriji *Bacillus subtillis* je tako potrebno pravilno delovanje kar 40 genov, ki omogočajo, da bakterija pritrdi nase molekulo DNA iz okolice, jo prenese skozi celično steno in membrano v citosol in jo vgradi v svoj genom (Solomon in Grossman, 1996). V splošnem je verjetnost uspešnega vnosa in izražanja pri kompetenci večja, če DNA iz okolice prihaja od sorodnih vrst organizmov, z naraščanjem genetske oddaljenosti pa ta verjetnost hitro upada. Določitev števila kompetentnih vrst prokariotskih organizmov je težavna, saj je, kot smo že omenili, kompetenca prehodne narave; kompetentnost posamezne bakterije se lahko izkazuje le v točno določenih pogojih, ob njihovi spremembi pa se preneha izkazovati, zato je ne zaznamo. Kompetenco so dokazali pri vsaj 67 različnih vrstah bakterij, dejansko pa je takšnih vrst zagotovo mnogo več, a fiziološki pogoji za njeno izkazovanje še niso bili določeni oziroma ugotovljeni (Johnsborg s sod., 2007; Lorenz in Wackernagel, 1994).

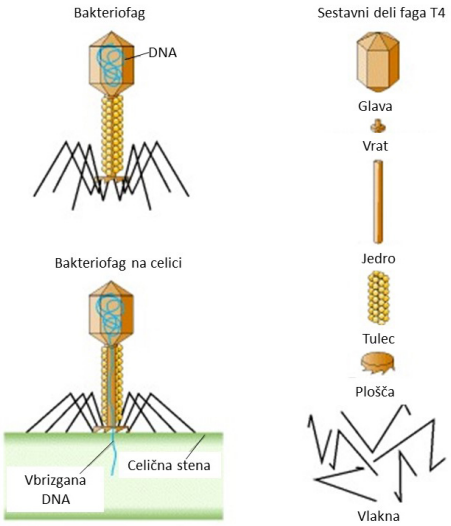


Slika 8: Shematski prikaz kompetence. Povzeto po Griffiths s sod., 2000b, Fig. 7–16.

Podobno kot pri konjugaciji, ali pa še bolj, tudi pri kompetenci velja, da temelji na vrsti zapletenih proteinov, ki morajo za pravilen potek procesa delovati krajevno in časovno usklajeno, evolucijski razvoj takšnega procesa pa je verjetno trajal več sto milijonov let.

1.3.4 Transdukcija

Transdukcija je prenos genskega material med organizmi preko virusov. Virusi so zelo majhni, tvori pa jih molekula RNA ali DNA (kromosom), ki ga obdaja ovoj iz proteinskih molekul (kapsida). Viruse, ki imajo sposobnost prenašati genski material med bakterijami, imenujemo *bakteriofagi* (Slika 9).

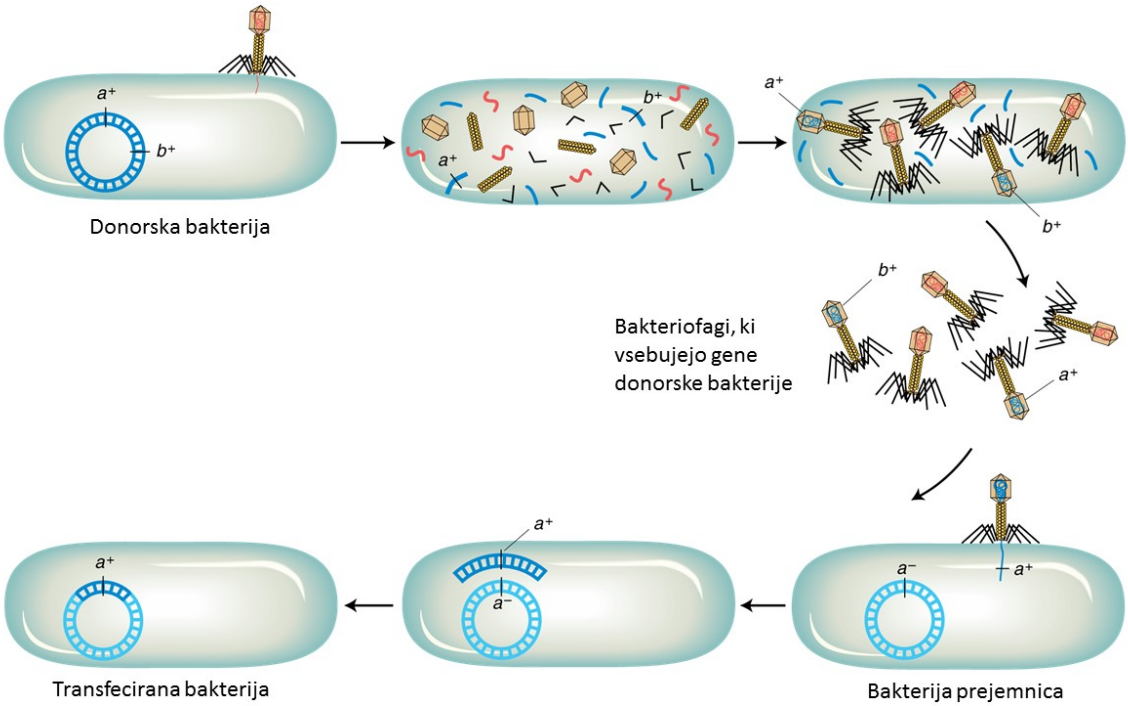


Slika 9: Sestavni deli bakteriofaga. Povzeto po Griffiths s sod., 2000b, fig. 7–17.

Transdukcijo sta leta 1952 odkrila Joshua Lederberg in Norton Zinder, ko sta izvajala poskuse rekombinacije dveh heterotrofnih sevov *Salmonelle typhimurium* in *E. coli* (Zinder in Lederberg, 1952) ter dobila nov avtotrofni sev. Na začetku sta to pripisovala konjugaciji, s kasnejšimi poskusi pa sta ugotovila, da je za rekombinacijo odgovoren bakteriofag *P22*.

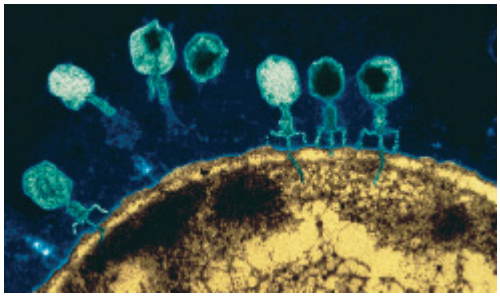
Bakteriofag okuži bakterijo tako, da se pritrdi na njeno površje (celično steno ali zunanjo ovojnico), nato pa s pomočjo kompleksnih proteinov prebode steno in membrano, tako vzpostavi kanal do citosola, nato pa vanj izbrizga svoj genski material (Slika 9). Izražanje tega materiala ustvari snovi, ki v bakteriji zavro sintezo njenih lastnih molekul in sprožijo sintezo molekul bakteriofaga – podvajanje njegove DNA in sintezo proteinov njegove kapside. Tako bakterija postane »tovarna« bakteriofagov, ki deluje toliko časa, dokler nakopičeni bakteriofagi ne povzročijo njenega razpočenja (celične lize), bakteriofagi pa se sprostijo v okolico, od koder lahko okužijo novo bakterijo.

Pri nastajanju bakteriofagov v bakteriji se lahko zgodi, da katera od novonastalih kapsid ne obda le bakteriofagne DNA, temveč tudi del kromosomske DNA bakterije. Ko nato tak bakteriofag okuži drugo bakterijo, skupaj z svojim genskim materialom izbrizga vanju tudi genski material prejšnje bakterije gostiteljice (Slika 10).



Slika 10: Shematski prikaz transdukcije. Povzeto po Griffiths s sod., 2000b, fig. 7–26.

20 Kljub svoji majhnosti so tudi bakteriofagi zapleteni organizmi, ki za svoje delovanje potrebujejo vrsto kompleksnih proteinov, kar pomeni, da so tudi bakteriofagi rezultat dolgotrajnega evolucijskega razvoja.



Slika 11: Več virusov na površini bakterijske celice. Povzeto po Griffiths s sod., 2000c.

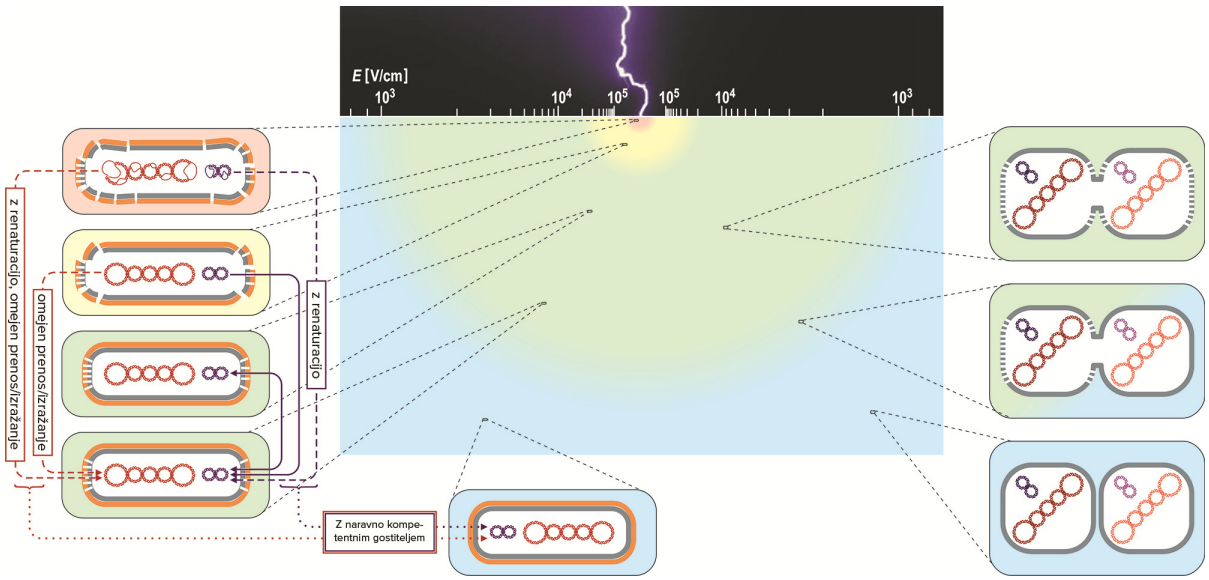
Ker vsi trije biološki mehanizmi horizontalnega prenosa genov temeljijo na proteinih in so kot taki morali nastati šele v določeni fazi evolucije, je smiselno vprašanje, ali obstaja tudi kak preprostejši, denimo povsem fizikalen mehanizem, ki je lahko deloval že vse od nastanka življenja. Eden najobetavnejših tovrstnih mehanizmov je elektroporacija.

1.3.5 Elektroporacija: četrti naravni mehanizem prenosa genov?

Ko strela udari v tla, se njen tok iz točke vstopa razširja radialno, v smereh navzdol in navzven od te točke; če je zgradba tal homogena, gostota toka in jakost električnega polja, ki ga ta tok ustvarja, upadata približno obratno sorazmerno s kvadratom razdalje od te točke.

Pri trdnih tleh je predpostavka homogene zgradbe tal vprašljiva, v vodnih okoljih, ki prekrivajo tri četrtine površja Zemlje in so bila večji del evolucije celo edini habitat živih organizmov, pa je upravičena. Zaradi boljše definiranosti in s tem možnosti kvantitativne obravnave se bomo v nadaljevanju omejili na obravnavo strel, ki vstopajo v vodna okolja, tam pa je tudi difuzija prosto plavajoče DNA najmanj omejena.

Jakost električnega polja se tako z oddaljevanjem od točke vstopa strele hitro in zvezno zmanjšuje; tako so v določenem pasu, ki je zelo blizu te točke, prisotni pogoji, v katerih pride do ireverzibilne elektroporacije in s tem iztekanja DNA, hkrati pa znatnega dviga temperature, ki lahko to DNA denaturira; nekoliko dlje od te točke so prisotni pogoji za ireverzibilno elektroporacijo in s tem iztekanje DNA brez denaturacije, še nekaj dlje pogoji za reverzibilno elektroporacijo in s tem vnos DNA, nato pa sledi območje, kjer je jakost polja premajhna za elektroporacijo. Pri dveh prokariotih brez celične stena bi lahko ob elektroporaciji vsaj enega od njiju prišlo tudi do njunega zlitja v hibridni organizem, ki bi izražal gene obeh zlitih organizmov.



Slika 12: Elektroporacija in elektrofuzija prokariotskih organizmov v vodnem habitatu ob udaru strele. Povzeto po Kotnik, 2013a.

1.3.6 Jakost električnega polja in dvig temperature pri elektrostatični razelektritvi

Kot smo že omenili, je 90% vseh strel, ki potekajo med oblakom in tlemi, tipa navzdol potekajočih negativnih, skoraj vse preostale pa so navzdol potekajoče pozitivne. Negativne strele pogosteje tvori več udarov, dodatni pa deloma ali v celoti potujejo po isti poti kot prvotni, ki je najmočnejši in traja najdlje, medtem ko pozitivne strele praviloma tvori le en udar.

Pri prvi negativni streli (natančneje njenem povratnem udaru) je medianska vrednost amplitude električnega toka okoli 30 kA, pri pozitivni razelektritvi pa 35 kA. Medianski čas vzpona električnega tok je pri negativni streli okoli 5 µs in pri pozitivni okoli 20 µs, medtem

čas upada do polovične vrednosti pa okoli 75 µs pri negativni streli in 230 µs pri pozitivni (Chowdhuri s sod., 2005; Rakov, 2003; Goto in Narita, 1995; Anderson in Eriksson, 1979; Berger, 1975). Standard IEC 60060-1 (Commission, 1989) definira tudi funkcijo, ki modelira časovni potek električnega toka pri streli:

$$I(t) = I_a\left(e^{-\frac{t}{\tau_2}} - e^{-\frac{t}{\tau_1}}\right). \qquad (1_a)$$

Da dobimo medianske vrednosti, ki smo jih opisali v zgornjem odstavku, moramo v enačbi (1_a) uporabiti naslednje vrednosti parametrov:

$$I_a = 31{,}76\ kA, \tau_1 = 1{,}077\ \mu s, \tau_2 = 106{,}6\ \mu s. \qquad (1_b)$$

Približek jakosti električnega polja v vodnem okolju lahko izračunamo, če predpostavimo, da je vodno okolje homogeno in neomejeno, kar pomeni, da električni tok teče enakomerno radialno od točke vstopa navzdol in navzen proti ozemljitvi, ki se nahaja v neskončnosti. Na razdalji *r* od točke vstopa je *I(t)* enakomerno porazdeljen po polkrogelni površini $2\pi r^2$, zato tam gostota toka znaša:

$$J(\mathrm{r},\mathrm{t}) = \frac{I(t)}{2\pi r^2}, \qquad (2)$$

takšna gostota toka pa v mediju z električno prevodnostjo σ povzroči jakost električnega polja:

$$E(r,t) = \frac{J(r,t)}{\sigma} = \frac{I(t)}{2\pi\sigma r^2}. \qquad (3)$$

Največjo vrednost jakosti električnega polja, kot funkcijo *r*, dobimo tako, da poiščemo največjo vrednost *I(t):*

$$E_{max}(r) = \frac{max(I(t))}{2\pi\sigma r^2}. \qquad (4)$$

Na časovni skali mikrosekund lahko odtekanje toplote zanemarimo, zato lahko spremembo temperature medija zaradi električnega toka *I(t)* zapišemo z izrazom:

$$\Delta T(r,t) \approx \frac{1}{\rho C_p}\int_0^t E(r,\theta)J(r,\theta)d\theta = \frac{1}{\rho C_p}\int_0^t \frac{I^2(\theta)}{4\pi^2\sigma r^4}d\theta = \frac{1}{4\pi^2\rho C_p\sigma r^4}\int_0^t I^2(\theta)d\theta, \qquad (5)$$

kjer je ρ masna gostota medija, C_p pa specifična toplota medija. V takšnem približku (z zanemarjenjem odtekanja toplote) bi maksimum spremembe temperature dobili z integriranjem na intervalu [0, +∞):

$$\Delta T_{max}(r) \approx \frac{1}{4\pi^2\rho C_p\sigma r^4}\int_0^\infty I^2(\theta)d\theta. \qquad (6)$$

Če uporabimo izraz *I(t)* iz enačbe (1_a), dobimo izraz:

$$\Delta T_{max}(r) \approx \frac{1}{4\pi^2\rho C_p\sigma r^4}\int_0^\infty {I_a}^2\left(e^{-\frac{\theta}{\tau_2}} - e^{-\frac{\theta}{\tau_1}}\right)^2 d\theta = \frac{{I_a}^2}{8\pi^2\rho C_p\sigma r^4}\frac{(\tau_2-\tau_1)^2}{\tau_2+\tau_1}. \qquad (7)$$

Če upoštevamo še dejstvo, da je $\tau_1 << \tau_2$ in torej: $(\tau_2 - \tau_1) \approx (\tau_2 - \tau_1) \approx \tau_2$, dobimo še dodatno poenostavljen približek:

$$\Delta T_{max}(r) \approx \frac{{I_a}^2 {\tau_2}^2}{8\pi^2 \rho C_p \sigma r^4}. \qquad (8)$$

Za vodne medije lahko izberemo ρ = 1 g/cm^3, C_p = 4.2 J/(gK). Za oceno *E(r,t)* potrebujemo samo še električno prevodnost medija σ.

Električna prevodnost (σ) različnih naravnih vodnih okolij se lahko zelo razlikuje. Pri deževnici so vrednosti σ med 0.02 mS/cm (približno dvojna vrednost tiste pri povsem čisti vodi) in 0.15 mS/cm (Jonsson in Vonnegut, 1991; Sequeira in Lung, 1995), pri rekah med 0.1 mS/cm in 0.5 mS/cm (Hoffman s sod., 1983; Snorrason s sod., 2002), pri jezerih segajo od 1 mS/cm vse do 160 mS/cm v najbolj slanih jezerih kot je npr. Mrtvo morje (Talling in Talling, 1965), v morjih in oceanih pa od 20 mS/cm do 60 mS/cm (Riley s sod., 1975; Thomas s sod., 1934). Tako lokalna jakost električnega polja (*E*) kot sprememba temperature (ΔT) pri udaru strele upadata z naraščanjem *r* in σ, kar pomeni, da njuna vrednost upada stran od točke vstopa strele v vodno okolje, pa tudi z naraščanjem slanosti vodnega okolja. Večja kot sta *r* in σ, manjša sta torej *E* in ΔT.

Tabela 1 povzema izračunane radialne razdalje od točke vstopa strele v vodno okolje, pri katerih pride do različnih značilnih vrednosti dviga temperature in jakosti električnega polja, za okolja z različno električno prevodnostjo, pridobljeno iz enačb (4) in (6) ter vrednosti po (1_b). Vidimo, kako s povečevanjem *r* in σ upada tako *E* kot ΔT.

Tabela 1: Radialne razdalje od točke vstopa strele v vodno okolje, na katerih pride do značilnih vrednosti dviga temperature in jakosti električnega polja, za okolja z različno električno prevodnostjo. TP - termične poškodbe DNA; IEP - ireverzibilna elektroporacija; REP - reverzibilna elektroporacija; EF - elektrofuzija (pri organizmih brez stene)

Učinek	Komentar	Razdalja od točke vstopa (*r*)			
		σ = 0.1 mS/cm	**σ = 1 mS/cm**	**σ = 10 mS/cm**	**σ = 100 mS/cm**
ΔT = 70° C	TP	14.6 cm	8.19 cm	4.60 cm	2.59 cm
ΔT = 30° C	mogoče TP	18.0 cm	10.1 cm	5.69 cm	3.20 cm
E = 30 kV/cm	IEP	39.9 cm	12.6 cm	3.99 cm	1.26 cm
E = 10 kV/cm	REP in EF, mogoče IEP	69.1 cm	21.9 cm	6.91 cm	2.19 cm
E = 3 kV/cm	REP in EF	126 cm	39.9 cm	12.6 cm	3.99 cm
E = 1 kV/cm	mogoče REP in EF	219 cm	69.1 cm	21.9 cm	6.91 cm

1.4 HGT kot posledica elektrostatičnih razelektritev

Ob primerni prevodnosti vodnega habitata in dovolj visoki koncentraciji prokariotskih organizmov v njem se ob vstopu strele v tak habitat vzpostavijo pogoji, ki povzročijo iztekanje DNA, nekaj dlje od točke vstopa strele pa pogoji, ki omogočajo elektrotransformacijo s prosto plavajočo DNA iz okolice tamkajšnjih organizmov; če DNA, ki

je iztekla iz ireverzibilno elektroporiranega prokariota, vstopi v reverzibilno elektroporiranega in ga transformira, to predstavlja HGT med tema organizmoma.

Seveda je mogoč pomislek, ali so koncentracije prokariotov v naravnih okoljih, dosegljivih udaru električnih strel, zadostne, da bi udar strele lahko privedel do iztekanja DNA iz enega prokariota in njenega vnosa v drugega. Vendar mnoge študije kažejo, da v različnih naravnih vodnih okoljih prihaja do HGT zaradi naravne kompetence bakterij, kar pomeni, da so v takšnih okoljih koncentracije prokariotov dovolj velike, da prosto plavajoča DNA, izločena iz enega prokariota, lahko doseže drugega in ga transformira, še preden bi prišlo bodisi do njene prevelike degradacije (zaradi v okolju prisotnih encimov, predvsem endonukleaz), bodisi do njenega metaboliziranja (vnosa v kakega od organizmov, ki bi jo razgradil kot hranilno snov). Vsaj v takšnih okoljih lahko tudi DNA, katere izlitje povzroči strela, doseže kakšnega od drugih prokariotov, še preden je razgrajena.

Ireverzibilna elektroporacija kot posledica elektrostatične razelektritve je eden izmed mnogih mehanizmov, ki povzročijo iztekanje oziroma sproščanje DNA iz celic, upoštevajoč pogostost strel pa tudi ni nujno zanemarljiva v primerjavi z ostalimi mehanizmi, ki delujejo v naravi. V kontekstu zgodnje evolucije se je v prejšnjih desetletjih uveljavila hipoteza, da je življenje nastalo in se še dolgo zatem razvijalo le v bližini globokomorskih termalnih vrelcev (Martin s sod., 2008), takšni habitati pa strelam seveda niso dostopni, a pred tremi leti objavljena študija je ponudila prepričljive argumente, da je takšna hipoteza zmotna in da se je življenje dejansko razvilo v plitvih celinskih jezercih (Mulkidjanian s sod., 2012), ta pa so seveda zlahka dostopna udarom strele.

Kot je razvidno iz enačb (4) in (7) in prikazano v Tabeli 1, se s povečano električno prevodnostjo medija zmanjša tako prostornina območja ireverzibilne kot reverzibilne elektroporacije. Torej se s povečano električno prevodnostjo medija zmanjša tudi število prokariotskih organizmov, iz katerih pride do izlitja DNA, kot tudi tistih, ki so dovzetni za elektrotransformacijo s prosto plavajočo DNA iz svoje okolice. Prav tako lahko iz tabele razberemo, da pri udaru strele v morsko vodo ireverzibilno elektroporacijo praviloma spremlja tudi nekaj denaturacije DNA. Iz tega lahko zaključimo, da je verjetnost za HGT zaradi udara strele v sladkovodnih habitatih večji kot v morskih. Še največjo verjetnost takšnega prenosa genov bi šlo pričakovati v sladkovodnih okoljih, ki so najbolj bogata z raznolikimi mikroorganizmi; primer takega habitata so odpadne vode.

V preteklosti je bilo opravljenih kar nekaj raziskav, ki nam lahko pomagajo pri tehtanju verjetnosti, da bi strele lahko prispevale k HGT med prokariotskimi organizmi. V začetku 1990-ih let, ko se je elektroporacija uveljavljala kot dominantna laboratorijska metoda umetne transformacije bakterij, je več raziskovalnih skupin začelo razmišljati, kako bi lahko čim bolj poenostavili sistem za prenos genov med bakterijami. Pri standardnem pristopu, ki je v veljavi še danes, bakterije darovalke DNA razgradimo z močnim detergentom, izločeno DNA očistimo s centrifugiranjem v gosti gradientni raztopini cezijevega klorida, jo dodamo celicam prejemnicam in jih elektroporiramo. Da bi se izognili uporabi sorazmerno zapletene in drage standardne opreme za kemično ekstrakcijo DNA, so nekateri od že omenjenih raziskovalcev poskusili tudi ekstrakcijo povzročiti z električnimi pulzi – torej doseči

ireverzibilno elektroporacijo bakterij darovalk DNA. V najpreprostejši izvedbi so izpostavili mešanico dveh vrst bakterij enemu samemu močnemu električnemu pulzu in dobili ponovljiv prenos iz *E. coli* v *Salmonello typhimurium* in v nasprotni smeri (Summers in Withers, 1990), oziroma iz *E. coli* v *Pseudomonas aeuriginosa* (Kilbane in Bielaga, 1991), a je bila učinkovitost takšnega pristopa za vsaj tisočkrat manjša kot pri kemični ekstrakciji in očiščenju DNA (Kilbane in Bielaga, 1991). Če so celice darovalke izpostavili močnejšemu pulzu (da so pri večini dosegli ireverzibilno elektroporacijo), nato pa dodali celice prejemnice in dovedli šibkejši pulz (da so pri večini teh celic dosegli reverzibilno elektroporacijo), je bila učinkovitost znatno višja, a še vedno približno desetkrat nižja kot pri standardnem pristopu s kemično ekstrahirano in očiščeno DNA (Kilbane in Bielaga, 1991). Zato je obveljalo, da takšna poenostavitev eksperimentalnega sistema ne odtehta močno zmanjšane učinkovitosti elektrotransformacije, (Kilbane in Bielaga, 1991; Ward in Jarvis, 1991).

Da ponovljiva elektrotransformacija, dosežena z enim samim pulzom, dovedenim v mešanico bakterij darovalk in prejemnic DNA, načeloma namiguje, da bi lahko do elektrotransformacije prišlo tudi ob udaru strele, sta že leta 1990 opozorila James Pfau in Philip Youderian, a sta tej hipotezi posvetila le en stavek in jo poimenovala za »špekulacijo« (Pfau in Youderian, 1990).

Morebitno vlogo strel in elektroporacije v evoluciji bakterij je leta 1995 omenil tudi Jack Trevors, ki je to zamisel povzel kot »zanimiv koncept«, a dodal, da »verjetno ne bo nikoli znano, kakšen je dejanski vpliv strel na HGT med bakterijami, vendar to zamisel omenja kot spodbudo drugim, da razmišljajo o tem« (Trevors, 1995).

Šest let kasneje, ko so filogenetske analize že kazale, da vloga HGT v evoluciji prokariotov ni bila zanemarljiva, se je raziskovanja strel kot morebitnega mehanizma prenosa genov lotila skupina Pascala Simoneta in eksperimentalno preučevala vnos DNA v elektroporirane bakterije *E. coli* (Demaneche s sod., 2001) in dve vrsti bakterij roda *Pseudomonas* (Cérémonie s sod., 2006). Metodološko sta bili ti študiji podobni že prej omenjenim, oziroma celo šibkejši, saj so preučevali le elektroporacijski vnos umetno dodane DNA v bakterije, ne pa tudi elektroporacijsko izločanje DNA iz bakterij, a so bili prvi, ki so poskušali vsaj delno emulirati naravnim podobne pogoje. Tako so za medij uporabili zemljo (prst), ki sicer ni naravni habitat *E. coli*, je pa naravni habitat ene od uporabljenih vrst *Pseudomonas*, poleg tega pa so poudarili pomembnost nastavitve parametrov napetostnega generatorja tako, da bi ti čim bolje odražali strelo. Kljub temu zapisanemu poudarku pa so pulze dovajali preko ploščatih elektrod v neposrednem stiku s prstjo (brez razelektritvenega obloka ter spremljajočih toplotnih, optičnih in tlačnih pojavov), zaradi omejene zmogljivosti generatorja, ki je v vzorcu ustvarjal jakosti električnega polja do največ 6.3 kV/cm, za dosego elektroporacije uporabili pulze s časovno konstanto kar 6 ms, torej kar okoli 100-krat daljšo kot pri tipičnih naravnih strelah.

1.5 Izdelava sistema za preučevanje naravnim podobnih pogojev

Za preučevanje elektroporacije kot naravnega mehanizma HGT je potrebno opraviti biološke poskuse, kjer se v kontroliranih laboratorijskih pogojih čim bolj približamo naravnim razmeram ob udaru strele. V ta namen je bilo potrebno razviti napravo, ki nam take poskuse omogoča.

V tej disertaciji opisujemo načrtovanje, izdelavo in testiranje modularnega sistema (sistem »Znanstveni Emulator eVolucijskih Strel« – ZEVS) in pripadajočega visokonapetostnega generatorja za izpostavitev bioloških vzorcev emulacijam električne strele (generator ZEVS). Sistem ZEVS nam omogoča, da biološke vzorce (celice ali tkiva) v kontroliranem okolju (točno določena dolžina obloka razelektritve, merjenje časovnega poteka električnega toka, ki teče skozi vzorec, snemanje poteka poskusov s hitro kamero) izpostavimo elektrostatični razelektritvi z nastavljivo amplitudo električnega toka (do nekaj sto amperov). To predstavlja ponovljivo emulacijo elektrostatične razelektritve, kakršna poteka pri naravni streli. Sistem ZEVS z generatorjem ZEVS raziskovalcem omogoča ponovljivo generiranje elektrostatičnih razelektritev z ustrezno ozemljitveno elektrodo in prilagodljivo dolžino obloka. Na sistem ZEVS lahko priklopimo tudi poljubni visokonapetostni generator. Modularna zasnova sistema omogoča hitro montažo in demontažo, kot tudi preprosto in temeljito čiščenje.

2 MATERIALI IN METODE

2.1 Razvoj sistema ZEVS

2.1.1 Računalniško modeliranje

Komponente sistema, kot tudi zgradbo celotnega sistema smo najprej načrtovali na osebnem računalniku z modeliranjem v okolju SolidWorks 2013 (Dassault Systems, Solidworks Corporations, ZDA). Računalniški model nam je omogočal, da smo mehanske dele še pred samo fizično izdelavo ustrezno preizkusili in po potrebi prilagodili. Računalniški model nam je bil v pomoč pri izbiri materialov, saj smo lahko numerično ocenili ustreznost njihovih mehanskih lastnosti. Za numerično analizo razelektritev in njihovih fizikalnih učinkov pa smo uporabljali okolje COMSOL MultiPhysiscs 4.2 (Comsol, Stockholm, Švedska).

2.1.2 Materiali

Osnovni material, ki smo ga izbrali pri sestavi našega sistema, je bila polietilenska plastika (PE 500 Natur, Simona AG, Nemčija), ki ima dobre električno izolativne lastnosti, je cenovno dostopna, hkrati pa jo je preprosto čistiti. Sistem smo načrtovali tako, da omogoča opazovanje vzorca med samim poskusom, ki pa je moral biti ob tem zaščiten pred kontaminacijo s prašnimi delci in mikroorganizmi iz okolišnjega zraka, zato smo posamezne komponente sistema naredili iz prozornega materiala, za kar smo uporabili pleksi steklo (PLEXIGLASS XT Tube Clear 0A070GT, Evonik Industries, Nemčija). Tudi pleksi steklo je zelo dober električni izolator in se preprosto očisti.

Elektrode smo prvotno izdelali iz bakra, a smo ugotovili, da razelektritve povzročajo njihovo korozijo, zato smo baker v nadaljevanju razvoja nadomestili z nerjavečim jeklom, ki se je izkazalo za ustrezno odporno proti razelektritveni koroziji. Pri ozemljitveni elektrodi, ki je v neposrednim stikom z biološkim vzorcem, je nerjaveče jeklo ustreznejše tudi zato, ker se v primerjavi z bakrom precej manj elektrolitsko raztaplja in posledično manj kontaminira vzorec. Podrobnejša utemeljitev izdelave elektrod je opisana v poglavju 3.2.

2.1.3 Izdelava

Vse komponente sistema ZEVS smo izdelali po meri z uporabo stroja CNC (High-Z 720, CNC-STEP, Nemčija), rezkarjev (CMT Utensili, Italija) in stružnice (F-900 A/G, Voest Alpine, Avstrija). Razpolaganje s to opremo in izdelava po meri sta nam omogočila hitre iteracije in izboljšave sistema.

Sistem smo zasnovali tako, da je zmožen čim bolj realistično emulirati nevihtne strele, ob tem pa se lahko hitro sestavi in razstavi, kar omogoča enostavno transportiranje in s tem izvajanje poskusov v različnih laboratorijih. Pozorni smo bili tudi na to, da je mogoče sistem enostavno in temeljito očistiti, kar bistveno zmanjša tveganje kontaminacije, hkrati pa omogoča ponovljivost poskusov.

2.1.4 Generator razelektritev

Za sistem ZEVS smo razvili še pripadajoči generator, ki je opisan kasneje. Sistem smo najprej testirali s komercialno dostopnim paralizatorjem (Great Power 750000, Great Power, South Korea), ki smo ga predelali, tako da smo ga lahko priklopili na sistem in proizvajali in ga prožili preko računalnika. Paralizator uporablja ojačevalno vezje, da ojača napajalno napetost, s pomočjo oscilatorja pa napolni kondenzator, ki je priklopljen na zunanje elektrode. Kondenzator se sprazni skozi zunanje (izhodne) elektrode, ko je presežena prebojna napetost zraka med njimi, kar povzroči električni oblik (ionizirani prevodni kanal), ki kratko sklene zunanje elektrode in se tako kondenzator sprazni.

2.2 Testiranje sistema ZEVS na bakterijskih celicah

2.2.1 Gojenje bakterij *Escherichia coli*

Kot model za testiranje sistema na bakterijskih celicah smo uporabili bakterije *Escherichia coli* seva K12 ER1821 (New Englin BioLabs, Frankfurt, Nemčija). Gojili smo jih v gojišču Luria Broth (Sigma-Aldrich, Munchen, Nemčija), s katerim smo jih zmešali v posodi (erlenmajerici), to pa smo nato za 24 ur postavili v inkubator z regulirano temperaturo 37 °C in stalnim tresenjem (I-50, Kambič laboratorijska oprema, Slovenija). Po inkubaciji smo posodo vzeli iz inkubatorja in jo centrifugirali 15 minut na 4 °C pri 4200 obratih na minuto, s čimer smo bakterije zbrali na dnu posode. Nato smo supernatant odstranili, v posodo nalili sterilno vodo (Aqua B. Braun, Braun Melsungen, Nemčija) in vsebino posode premešali s pipeto, da so se bakterije resuspendirale. Suspenzijo smo nato z dodajanjem sterilne vode razredčili do končne koncentracije *E. coli* v višini 5.5 x 10^6 CFU/ml.

Petrijevke z agarjem smo pripravili tako, da smo destilirani vodi dodali agar Luria (Sigma-Aldrich, Munchen, Nemčija) v koncentraciji 40 g/l in mešanico sterilizirali s segretjem na 121 °C za 15 minut v avtoklavu (A-11, Kambič laboratorijska oprema, Slovenija). Ko se je mešanica ohladila na 55 °C, smo jo s pipeto prenesli v petrijevke z notranjim premerom 86 mm (90 mm Sterilin plates, Thermo Scientific, VB) in sicer po 10 ml na petrijevko in enakomerno po vsem dnu vsake petrijevke. Ko se je agar zgostil in ohladil na 21 °C, smo nanj nanesli 1 ml suspenzije *E. coli*, tudi to tako, da je suspenzija enakomerno pokrivala celotno površino agarja. Po 1 min smo del suspenzije, ki se ni absorbiral v agar, odstranili s pipeto. Plošče smo nato pustili odkrite v laminarju še nadaljnjih 10 min, tako da je še preostala suspenzija, ki jo nismo uspeli odstraniti s pipeto, izhlapela. Tako smo dosegli, da na površini agarja ni bilo več nobene tekočine, s katero bi se bakterije *E. coli* lahko premikale zaradi njenega pretakanja. Z merilnikom LCR (Agilent 4284A Precision LCR meter, Agilent Technologies, ZDA) smo izmerili električno prevodnost agarja v petrijevkah pri frekvenci 1 MHz, ki je po pripravi na zgoraj opisan način znašala 22.9 mS/cm. Frekvenco 1 MHz smo izbrali zato, da smo smo lahko izmerili električno prevodnost agarja v znotraj podobnega časovnega razpona kot traja razelektritev pri paralizatorju.

2.2.2 Ireverzibilna elektroporacija

Petrijevke z konfluentno nasajenimi bakterijami *E. coli* na agarju (opis nasaditve je zgoraj) smo vstavili v naš sistem ZEVS in prek elektrod sistema v središče petrijevk dovedli 10 razelektritev. Električni tok vsake razelektritve je imel največjo vrednost ~100 A, dvižni čas od nič do največje vrednosti ~0.1 µs in čas upada do polovične vrednosti ~0.3 µs. Dolžina obloka pri vsaki razelektritvi je znašala ~15 mm. Kontrolna petrijevka je bila vstavljena v sistem na enak način kot vse ostale petrijevke, vendar brez razelektritev. Vse petrijevke smo po poskusu inkubirali za 18 ur pri 37 °C.

2.2.3 Ocena območja ireverzibilne elektroporacije

Po končani 18-urni inkubaciji po izpostavitvi elektrostatičnim razelektritvam smo petrijevke vzeli iz inkubatorja in vsako slikali s fotoaparatom z ločljivostjo 8 milijonov točk (iPhone 5, Apple, ZDA) od zgoraj pravokotno na središče plošče. Območje ireverzibilne elektroporacije je bilo jasno razvidno tudi s prostim očesom, saj na tem območju praktično ni bilo bakterijskih kolonij, temveč le prosojni agar, preostala površina petrijevke oziroma agarja v njej pa je bila prekrita z bakterijami in motna.

2.3 Testiranje sistema ZEVS na celicah sesalca

2.3.1 Gojenje celic CHO

Kot vzorčne sesalčje celice smo uporabili ovarijske celice kitajskega hrčka (Chinese Hamster Ovary cells – CHO-K1, Evropska zbirka celičnih kultur, VB). Nasadili smo jih v petrijevke z notranjim premerom 53 mm (60 mm TPP tissue culture dishes, Trasdingen, Švica) pri koncentraciji 1.5 x 10^5 celic/ml. Gojišče smo sestavili iz medija F-12 HAM (Dulbecco's modification of Eagle's Minimum Essential Medium; Sigma-Aldrich Chemie, Deisenhofen, Nemčija) z dodanim 10% zarodnega telečjega seruma in 0,15 mg/ml L-glutamina (oba Sigma-Aldrich Chemie, Deisenhofen, Nemčija). Električno prevodnost medija smo izmerili z merilnikom LCR (Agilent 4282A Precision LCR Meter, Agilent Technologies, ZDA) in je pri frekvenci 1 MHz znašala 14.9 mS/cm.

Celice CHO smo po nasaditvi v petrijevke 24 ur inkubirali pri 37 °C in 5% CO_2 (inkubator I-CO2-235, Kambič Laboratorijska oprema, Slovenija), ob koncu te inkubacije pa so dosegle gostoto približno 4 x 10^5 celic na petrijevko. Nato smo petrijevke s celicami CHO vstavili v naš sistem in jih izpostavili elektrostatičnim razelektritvam.

2.3.2 Genska elektrotransfekcija

Neposredno pred izpostavitvijo elektrostatičnim razelektritvam smo iz petrijevk odstranili gojišče in nato dodali 1.5 ml svežega gojišča z 4 µg/ml plazmidne DNA pEGFP-N1 (Clonotech, ZDA, 4649 baznih parov), ki izraža zeleno fluorescentni protein (GFP, vzbujanje pri valovni dolžini svetlobe 488 nm, emisija pri 520 nm), in počakali 3 minute, da se je plazmidna DNA posedla na dno petrijevke in s tem na celice, ki so bile tam pritrjene. Nato smo vsako

petrijevko posebej vstavili v sistem in dovedli 10 razelektritev. Električni tok vsake razelektritve je imel največjo vrednost ~14 A, dvižni čas od nič do največje vrednosti ~0.5 µs in čas upada do polovične vrednosti ~1.5 µs. Dolžina obloka pri vsaki razelektritvi je znašala ~7 mm. Kontrolna petrijevka je bila vstavljena v sistem na enak način kot vse ostale, vendar brez razelektritev. Vse plošče smo po poskusu 5 min inkubirali pri 37 °C in 5% CO_2, nato pa dodali v vsako petrijevko še 3.5 ml svežega gojišča in nadaljevali z inkubacijo še 24 ur.

2.3.3 Ocena deleža celic z vnosom plazmidne DNA in njenim izražanjem

Po končani 24-urni inkubaciji po izpostavitvi plošč elektrostatičnim razelektritvam smo vsako petrijevko posebej pregledali z fluorescenčnim mikroskopom (Axioverz 200, Zeiss, Nemčija) pri 100-kratni povečavi. Slike smo zajemali s koračnimi premiki vzdolž notranjega premera petrijevke, s čimer smo zajeli 76 slik, ki so skupaj tvorile trak dolžine 46 mm in širine 0.7 mm, segajoč od enega roba petrijevke prek njenega središča do nasprotnega roba. Celice, pri katerih je prišlo do vnosa in izražanja plazmidne DNA (torej tiste celice, ki so proizvajale GFP), smo zaznali po emisiji fluorescence pri valovni dolžini 520 nm.

2.4 Razvoj generatorja ZEVS

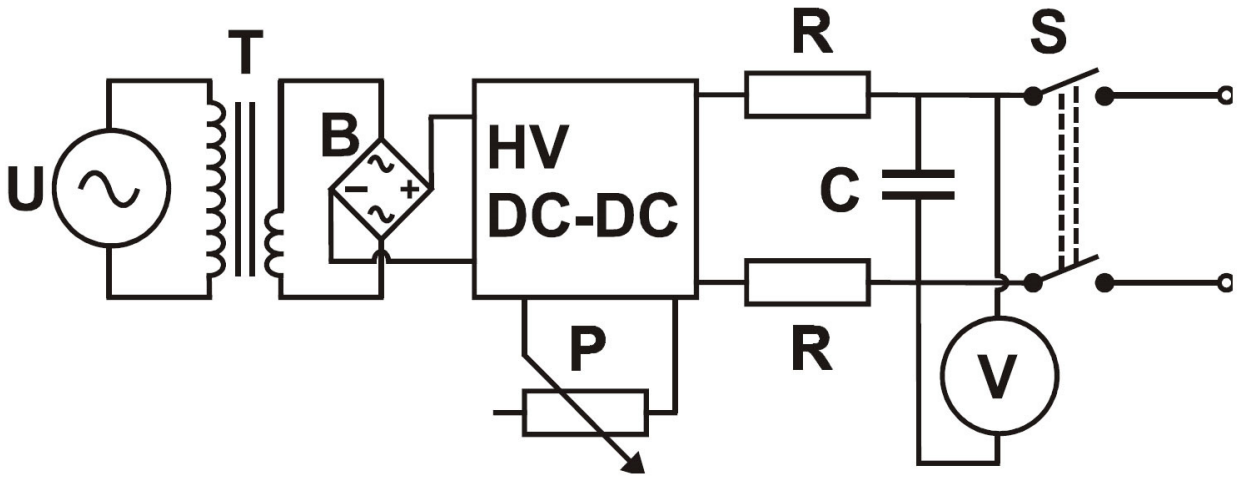
Na začetku smo sistem ZEVS preizkušali z generatorjem, ki smo ga dobili s predelavo komercialnega električnega paralizatorja – taserja (Great Power 750000, Great Power Co., Južna Koreja). Sočasno pa smo se lotili še razvoja lastnega visokonapetostnega generatorja, temelječega na krmiljeni 5 kV razelektritvi kondenzatorja s kapacitivnostjo 1 µF in je po časovnem poteku generiranega razelektritvenega toka precej bolj podoben dejanskim nevihtnim strelam (dvig toka z ničle do maksimuma v približno 5 µs, nato pa eksponentno upadanje s časovno konstanto približno 75 µs). To napravo smo poimenovali generator ZEVS.

2.4.1 Razvoj in uporaba

Električna shema napetostnega generatorja ZEVS, ki smo ga razvili, je prikazana na Sliki 13. Za vir visoke napetosti in razelektritve v breme smo izbrali kondenzator (Reberšek in Miklavčič, 2011), saj je pri hitri izpraznitvi kondenzatorja časovni potek toka zelo podoben tistemu pri naravni streli (Kotnik, 2013b). Zaradi dodatne varnosti in čim manjšega neželenega praznjenja kondenzatorja preko parazitnega toka smo napetostni izhod generatorja izolirali od omrežja z ločilnim transformatorjem (18 V - 60 VA, ELMA, Slovenija). Za stabilno napajanje kondenzatorja smo uporabili visokonapetostni pretvornik toka (RB60-6P, Matsusada, Japonska). Izhodno napetost pretvornika smo nastavljali s potenciometrom, v območju 0 do 5 kV in jo merili z voltmetrom. Pretvornik smo zaščitili pred visoko napetostjo na izhodu kondenzatorja z visokoimpedančnimi upori. Naboj za razelektritev se ustvarja s polnjenjem kondenzatorja s kapacitivnostjo 1 µF (MOP105-5MN, Condenser Products, ZDA) preko pretvornika toka. Iz kondenzatorja, s sklenitvijo para

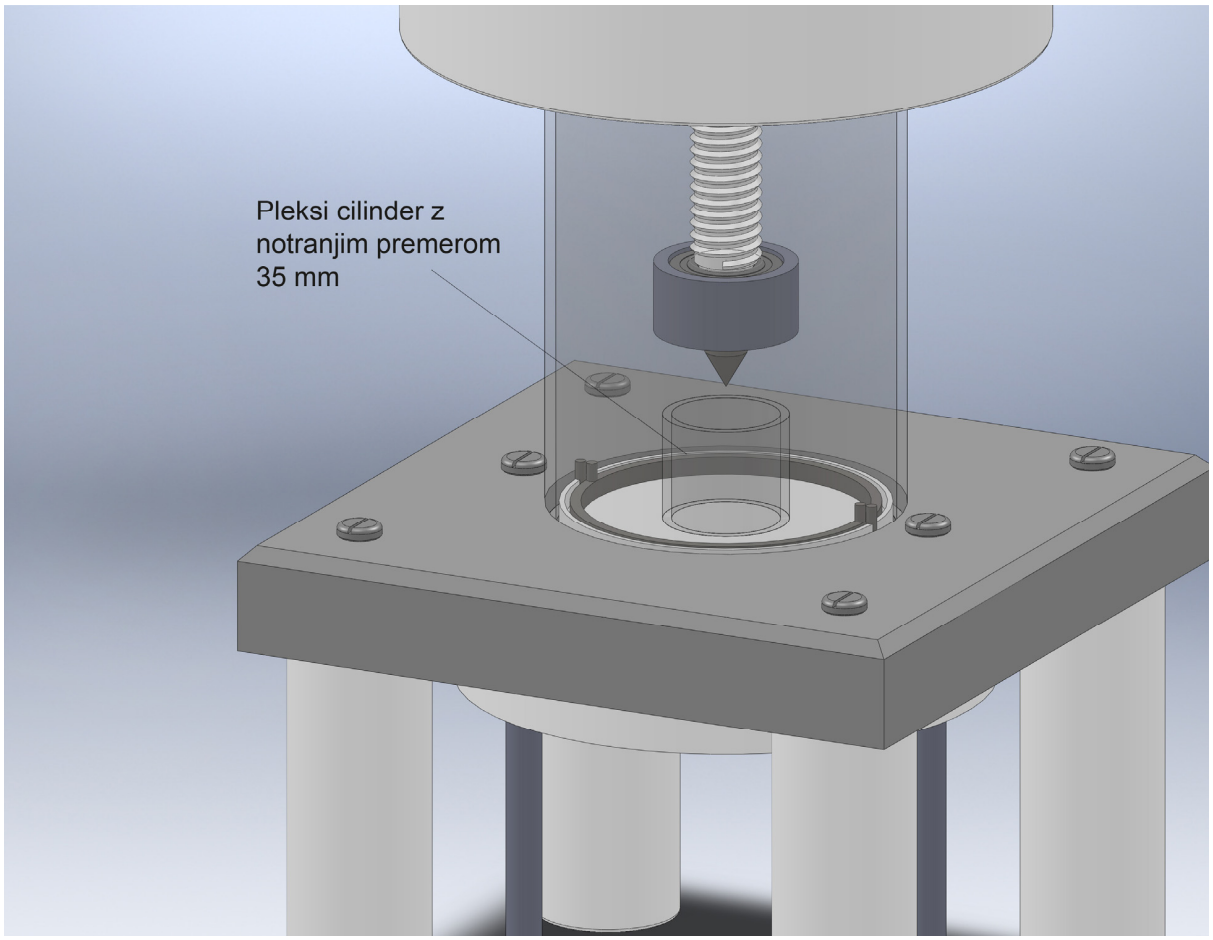
visokonapetostnih relejev (RL 42, SPS electronic, Nemčija) dovedemo razelektritev do elektrod in prek njiju v breme.

Z izbiro kapacitivnosti kondenzatorja v višini 1 µF smo dosegli, da je bil pri izmerjeni skupni impedanci sistema ZEVS in priključenega bremena (biološkega vzorca v petrijevki), ki je znašala ~100 Ω, časovni potek toka razelektritve podoben tistemu pri povratnem udaru strele – torej dvižni čas nekaj µs in razpolovni čas reda ~100 µs (Chowdhuri s sod., 2005; Kotnik, 2013). Da bi z generatorjem ZEVS generirali in dovedli elektrostatično razelektritev, smo kondenzator v njem naelektrili na napetost 5 kV ter ga izpraznili v vzorec preko releja in elektrod sistema ZEVS.



Slika 13: Električna shema generatorja. T: ločilni transformator; HV DC-DC: visokonapetostni pretvornik toka; P: potenciometer; V: voltmeter; R: visokoimpedančni upori; C: kondenzator; S: dva visokonapetostna releja.

V prvem sklopu preizkusov generatorja ZEVS smo ga priklopili na sistem ZEVS na enak način, kot smo prej pri poskusih z ireverzibilno elektroporacijo *E. coli* in vnosom DNA v celice CHO priklapljali prirejeni električni paralizator: konična emisijska elektroda se je nahajala na določeni razdalji nad središčem, obročasta ozemljitvena elektroda pa ob notranjem robu petrijevke (Marjanovič in Kotnik, 2013). Pri uporabi smo s slikanjem z ultrahitro kamero ugotovili, da obloki razelektritve, krajši od 3 µs, skozi svoje celotno trajanje vstopajo navpično v osrednji del petrijevke, pri daljših razelektritvah pa temu sledi postopno odmikanje spodnje točke obloka proti obročasti elektrodi, s čimer oblok vstopa v medij vse bolj diagonalno, dokler pri pulzih, daljših od 8–12 µs, ne sklene kratkega stika med konično in obročasto elektrodo ter se tako povsem izogne vstopu v vzorec. Zato smo sistem nadgradili tako, da smo v prostor pod konično elektrodo vstavili dodaten, manjši votel cilinder iz pleksi stekla s premerom 35 mm (Slika 14). To je omejilo diagonalno potovanje obloka in onemogočilo tvorbo kratkega stika med elektrodama, v tako nadgrajenem sistemu pa so tudi obloki, daljši od 12 µs, vstopali v medij pod koničasto elektrodo v smeri navzdol, električni tok pa je odtod tekel po mediju radialno proti obročasti elektrodi.

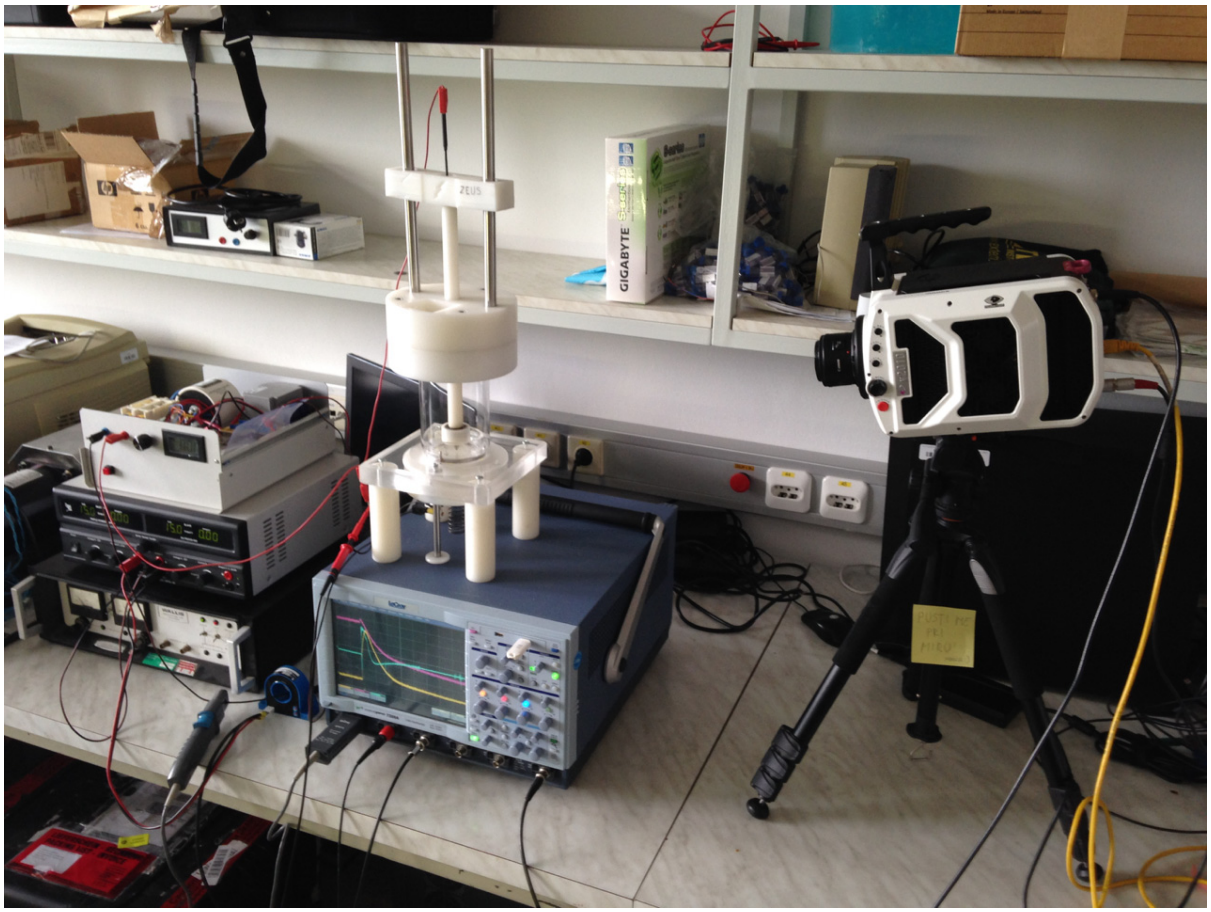


Slika 14: Sistem ZEVS z dodatnim 35 mm cilindrom iz pleksi stekla.

2.4.2 Meritve

Med samimi poskusi smo razelektritve beležili z zajemanjem slik z ultrahitro kamero (Phantom® v2010, Vision Research, ZDA) in objektivom z fiksno goriščno razdaljo (EF 50 mm f/1.8, Canon, Japonska). Slike smo zajemali z ločljivostjo 256 x 128 točk in 12 biti na točko. Hitrost zajemanja slik je bila 341000 slik na sekundo, izpostavitveni čas pa 2.55 µs. V primerih, ko je prišlo med elektrodama do kratkega stika, smo slike zajemali skozi zaščitno steklo za varjenje (90x110 mm Shade 11, Technolit, Nemčija), saj so bile sicer svetlobno zasičene, v ostalih primerih pa zaščitnega stekla nismo uporabljali.

Zajem slik med razelektritvijo smo dosegli s proženjem ultrahitre kamere preko osciloskopa, ki je generiral prožilni signal, ko je zaznal električni tok (Slika 15). Časovni potek toka in napetosti razelektritve smo merili s tokovno in napetostno sondo (AP015 in PPE6kV, obe LeCroy, ZDA), priklopljenima na osciloskop (WavePro 7300A, LeCroy, ZDA), katerega napajanje je bilo ločeno od napajalnega omrežja z ločilnim transformatorjem. Akustične komponente razelektritev smo zajemali z mikrofonom z merilnim dosegom zvočnega tlaka do 120 dB (Yeti, Blue Microphones, ZDA). Mikrofon se je nahajal 60 cm od razelektritve, ki jo je od mikrofona ločilo pleksi steklo sistema ZEVS in tako nekoliko zadušilo jakost zvočnega udara.



Slika 15: Snemanje razelektritve v sistemu ZEVS s hitro kamero, ki jo proži osciloskop.

2.5 Inaktivacija spor

2.5.1 Priprava bakterijskih spor

Bakterijske spore *Bacillus pumilus* (ATCC 27142) smo inkubirali 5 dni pri 37°C v sporulacijskem mediju Difco (mediju DSM) (Schaeffer s sod., 1965). Nezaželene bakterije, ki so se lahko iz spor razvile med inkubacijo, smo uničili s toplotnim šokom (80°C za 20 min) in encimskim šokom (izpostavitvijo 50 µg/ml lizocima v 50 mM Tris-HCl pri pH 6.2 in 37°C za 60 min). Spore smo nato centrifugirali (5 min, 10000 g) in oprali najprej z deonizirano vodo, potem z 0.02% raztopino natrijevega dodecil sulfata (SDS), nato pa še trikrat z deonizirano vodo. Pri vsakem poskusu smo na petrijevko z agarjem enakomerno razlili 1 ml suspenzije s sporami, nato odstranili ~900 µl tekočine nad agarjem in petrijevko še 15 min sušili v inkubatorju, preden smo jo vstavili v sistem ZEVS.

2.5.2 Izpostavitev spor razelektritvam

Z sporami nasajeno petrijevko z agarjem smo vstavili v sistem ZEVS ter med obročasto in končno elektrodo postavili cilinder iz pleksi stekla, da smo preprečili kratek stik med elektrodama. Razelektritve smo dovajali z dvema generatorjema: predelanim električnim

paralizatorjem (glej poglavje 2.4), ki je generiral razelektritve trajanja ~0.5 µs (~12 kV, ~100 A), in generatorjem ZEVS, ki je generiral razelektritve trajanja ~20 µs (~5kV, ~50 A).

2.5.3 Določitev področja inaktivacije

Gostoto spor, nasajenih na agarju, smo ocenili s štetjem kolonij pri različnih razredčitvah suspenzije in inkubaciji čez noč pri 37°C. Območje inaktivacije smo določili z analizo fotografij petrijevk s programom ImageJ 1.46r (National Institutes of Health, ZDA). Stopnjo inaktivacije smo izračunali kot razmerje gostote spor pri kontrolnih pogojih in gostote spor, izpostavljenih razelektritvam. Področje in stopnja inaktivacije sta bila izračunana iz treh neodvisnih poskusov.

3 REZULTATI IN RAZPRAVA

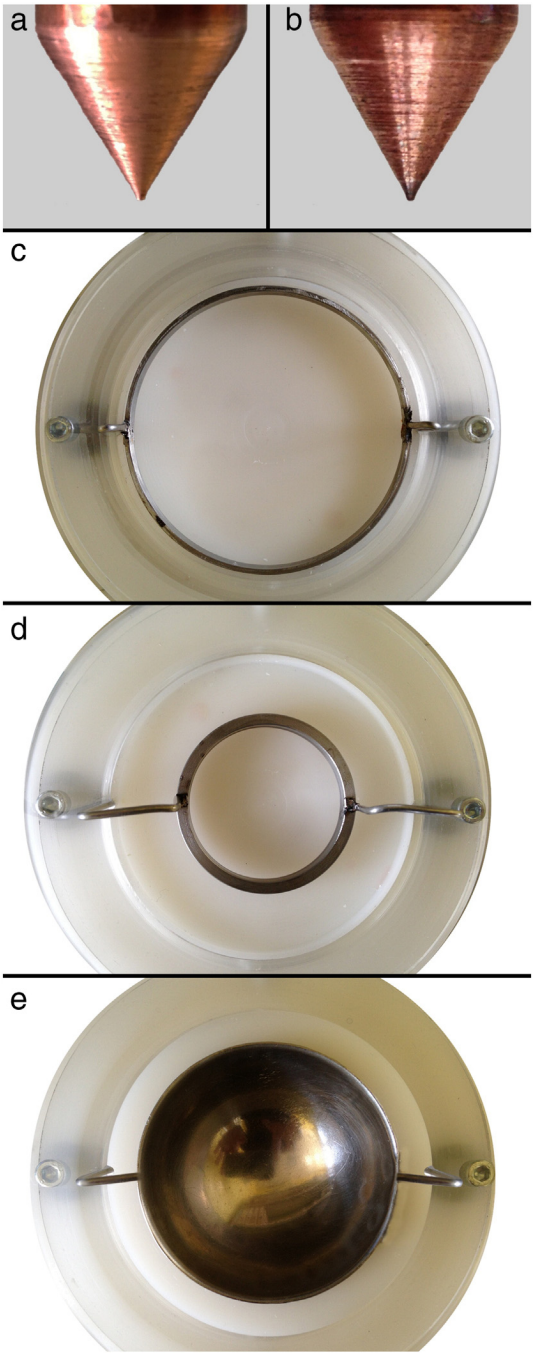
3.1 Razvoj sistema ZEVS

3.1.1 Izdelava emisijske elektrode

Emisijsko elektrodo smo oblikovali konično (v obliki konusa, t.j. stožca), kot prikazuje Slika 16a. Pri običajni uporabi v sistemu ZEVS je konični vrh elektrode obrnjen navzdol in se nahaja nad središčem vzorca, v katerega želimo dovesti razelektritev. Konična oblika omogoča nadzor nad točko izstopa razelektritvenega obloka iz emisijske elektrode, zgoraj opisana postavitev pa zagotavlja, da je zračna razdalja od elektrode najkrajša prav do središča vzorca. Tako lahko natančno predvidimo lokacijo obloka elektrostatične razelektritve in njegovo dolžino.

Prvi prototip konične elektrode smo izdelali iz bakra, a so preliminarni poskusi pokazali, da razelektritve privedejo do izrazito močne oksidacije; ta učinek se je z nadaljnjimi razelektritvami stopnjeval, najbolj pa je bil izražen povsem pri konici elektrode, torej v točki izstopa obloka in njeni neposredni okolici (Slika 16b). Zaradi oksidacije je na konici elektrode nastajal sloj bakrovega oksida, ki je izolator, posledično pa je prevodnost naše elektrode z razelektritvami počasi upadala, kar je vplivalo na ostale električne parametre (upad maksimalnega toka in spremenjen časovni potek razelektritve). Če smo z zelo finim brusnim papirjem bakrov oksid odstranili z elektrode, so se električni parametri povrnili v bližino začetnega stanja, a je tak poseg okrnil želeno sterilnost, otežil pa je tudi hitro menjavanje vzorcev pri poskusih. Prav tako nam je brušenje elektrode onemogočalo, da bi na istem vzorcu v kratkem času izvedli več razelektritev z enakim časovnim razmakom. Vse našteto je botrovalo temu, da smo baker kot izbrani material za konično elektrodo opustili.

Končno različico konične elektrode smo tako izdelali iz nerjavečega jekla. Čeprav ima baker nekaj višjo električno prevodnost (5.9 x 10^5 S/cm pri 20 °C) kot nerjaveče jeklo (1.4 x 10^4 S/cm pri 20 °C), je upornost teh dveh materialov veliko nižja od upornost kateregakoli vodnega medija (fiziološka raztopina ima npr. prevodnost 12 mS/cm pri 20 °C), seveda pa tudi od še precej manj prevodnega zraka. Zato je bil učinek razlik v prevodnosti bakra in nerjavečega jekla na potek razelektritev v našem primeru zanemarljiv; če smo razelektritev ustvarili z istim generatorjem in pod enakimi ostalimi pogoji, menjava materiala konične elektrode iz bakra z nerjavečim jeklom tako ni imela opaznega učinka na vrednost električnega toka in njegov časovni potek. Poleg tega pa se je elektroda iz nerjavečega jekla izkazala za veliko bolj odporno proti koroziji, saj se električni parametri razelektritev tudi po 1000 zaporednih električnih razelektritvah s testnim generatorjem še niso zaznavno spremenili. Obenem je elektroda iz nerjavečega jekla veliko bolj odporna na korozijo, ki jo povzroči voda in se zato tudi lažje čisti kot bakrena elektroda, če je potrebno, pa jo lahko umivamo tudi z detergenti.



Slika 16: (a-b) Oksidacija emisijske elektrode iz bakra. (c) obročasta ozemljitvena elektroda za petrijevke z 90 mm. (d) obročasta ozemljitvena elektroda za 60 mm petrijevke. (e) ozemljitvena elektroda v obliki polkrogelne lupine z notranjim premerom 70 mm.

3.1.2 Izdelava ozemljitvenih elektrod

Ozemljitvena elektroda je narejena tako, da pride v stik z biološkim vzorcem. Za material te elektrode smo že v samem začetku izbrali nerjaveče jeklo, ker je odporno na korozijo tudi v vodnih okoljih, ni elektrolitsko toksično za biološki vzorec in je enostavno za čiščenje. Da smo lahko zagotovili enakomerno razširjanje električnega toka na vse strani od točke vstopa v vzorec, smo oblikovali več ozemljitvenih elektrod: dve različni obročasti elektrodi (Slika 16c,d) in elektrodo v obliki polkrogelne lupine (Slika 16e).

Obročasti elektrodi smo dimenzionirali tako, da sta s svojim zunanjim premerom malo manjši od notranjega premera dveh standardnih tipov petrijevk (takih z zunanjim premerom 90 mm in 60 mm). To omogoča postavitev elektrode v petrijevko na način, da krožno obda vzorec v njej, s tem pa zagotavlja enakomerno dvodimenzionalno radialno razširjanje električnega toka od središčne točke vstopa v vzorec proti elektrodi.

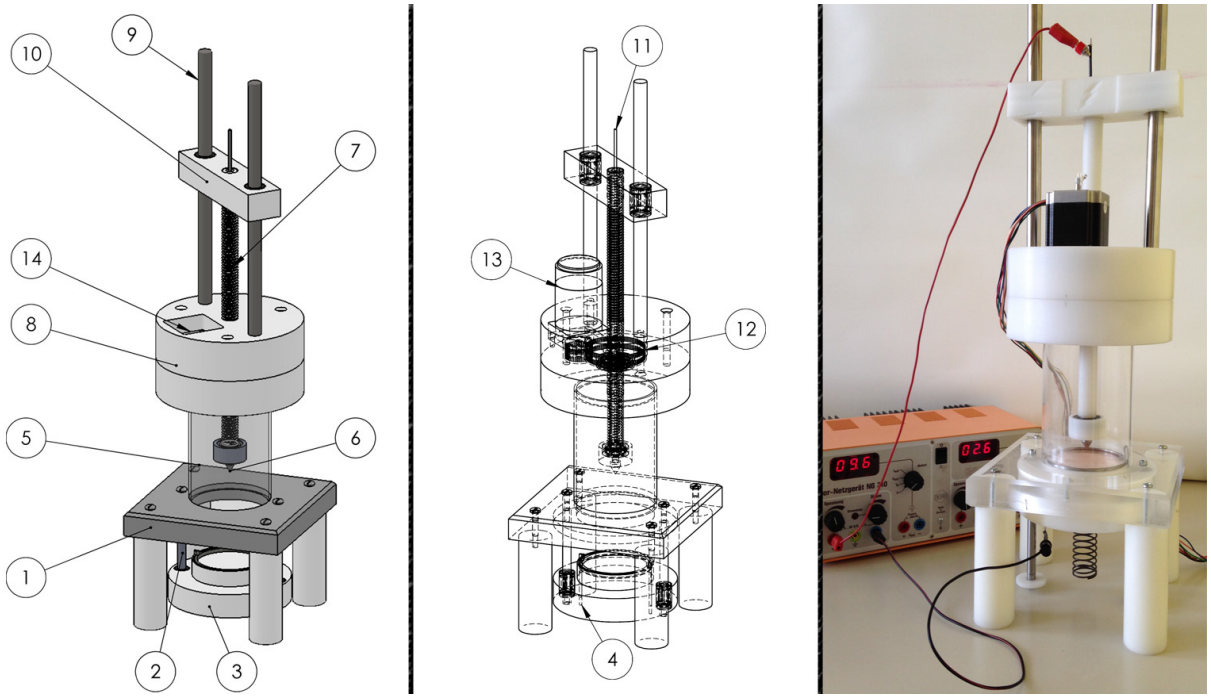
Elektroda v obliki polkrogelne lupine pa omogoča enakomerno radialno razširjanje električnega toka v treh dimenzijah, hkrati pa služi tudi kot posoda za biološki vzorec. Slabost takšne elektrode je v oteženem opazovanju dogajanja, saj je to mogoče le z vrha.

3.1.3 Izdelava sistema ZEVS

Pri izdelavi sistema ZEVS smo poskušali zadostiti naslednjim pogojem:

- preprosto vstavljanje in odstranjevanje biološkega vzorca iz sistema;
- zaščita biološkega vzorca pred delci v zraku (prah, mikroorganizmi, itd…);
- možnost spremljanja razelektritev med poskusom s prostim očesom ali kamero;
- preprost priklop poljubnega generatorja elektrostatičnih razelektritev;
- preprosta in natančna nastavitev vertikalne zračne razdalje med emisijsko (konično) elektrodo in vzorcem;
- zanesljiva električna izolacija povsod tam, kjer je to potrebno.

Sistem je sestavljen iz 14 različnih komponent, kot prikazuje Slika 17. Relativno široka in masivna osnova (Slika 17-1) sistema zagotavlja nizko težišče, kar zagotavlja fizično stabilnost. Na osnovo je preko dveh vodil iz nerjavečega jekla (Slika 17-2) pritrjena nalagalna postaja (Slika 17-3), ki vsebuje tudi ozemljitveno elektrodo (Slika 17-4). Nalagalna postaja se premika vertikalno vzdolž vodil in ima dva končna položaja: odprti položaj (postaja je spuščena do dna vodil) omogoča preprosto vstavitev vzorca, zaprti položaj (postajo dvignemo po vodilih do vrha, kjer se magnetno zaklene) pa je namenjen izvajanju poskusov na vzorcu. V zaprtem položaju se nalagalna postaja z vstavljenim vzorcem nahaja na dnu navpične prozorne cevi iz pleksi stekla (Slika 17-5). Nad vrhom vzorca je navzdol usmerjena konica emisijske elektrode (Slika 17-6), ki se navzgor nadaljuje kot kovinska žica, obdana s plastjo polivinilklorida in vstavljena v votel cilinder iz polietilena z zunanjim navojem (Slika 17-7), napeljan skozi jedro (središčni del) sistema (Slika 17-8). Žica, ki dovede tok razelektritve do konice emisijske elektrode, je tako obdana s kar tremi plastmi električne izolacije: plastjo polivinilklorida (debelina 0.3 mm), zračno režo (širina 4 mm) in steno iz polietilena (debelina 6 mm); ta izolacija vzdrži napetosti do vsaj 120 kV celo v primeru dolgotrajne enosmerne napetosti.



Slika 17: Sistem ZEVS. Levo: model v polnem prikazu. V sredini: model v prosojnem prikazu. Desno: dejanski prototip z naloženo petrijevko v zaprtem položaju in s koračnim motorjem, priklopljen na napetostni generator. Glavne komponente sistema: (1) osnova, (2) vodili za nalagalno postajo, (3) nalagalna postaja, (4) ozemljitvena elektroda, (5) prozorna cev, (6) konica emisijske elektrode, (7) neprevodno ohišje emisijske elektrode, (8) jedro sistema, (9) vodili za emisijsko elektrodo, (10) zgornji stabilizator, (11) konektor za emisijsko elektrodo, (12) zobnik z notranjim navojem, (13) koračni motor z zobnikom, (14) odprtina za koračni motor. (c).

Iz jedra naprave navzgor izstopa ohišje emisijske elektrode, ki poteka navpično vzporedno z dvema vodiloma iz nerjavečega jekla (Slika 17-9) do zgornjega stabilizatorja (Slika 17-10). Ta povezuje ohišje emisijske elektrode z vodiloma, na njem pa se nahaja tudi priklop za to elektrodo (Slika 17-11). Zgornji stabilizator se premika vertikalno po vodilih, medtem ko je ohišje zgornje elektrode togo pritrjeno na ta stabilizator. Na zunanji strani ohišja emisijske elektrode je navoj, ki se ujema z notranjim navojem zobnika (Slika 17-12) v jedru naprave, z vrtenjem katerega premikamo emisijsko elektrodo z njenim ohišjem v vertikalni smeri in tako prilagajamo vertikalno oddaljenost konice elektrode od vzorca pod njo. Za avtomatizirano nastavljanje te oddaljenosti lahko na zobnik priključimo koračni motor (Slika 17-13), ki ga vstavimo v prilagojeno odprtino (Slika 17-14) v jedru sistema.

3.2 Testiranje sistema

Sistem ZEVS smo najprej preizkušali s prilagojenim električnim paralizatorjem v vlogi generatorja, v dveh različnih poskusih; v prvem smo preučevali ireverzibilno elektroporacijo bakterij *E. coli*, v drugem pa vnos DNA v celice CHO.

3.2.1 Testiranje sistema ZEVS na bakterijskih celicah - ireverzibilna elektroporacija

Slika 18 prikazuje učinek 10 elektrostatičnih razelektritev na bakterije *E. coli*, merjen z njihovo zmožnostjo tvorbe kolonij na agarju v petrijevki premera 90 mm. Vsaka razelektritev je imela naslednje lastnosti: največjo vrednost električnega toka 97 ±8 A, dvižni čas od nič do največje vrednosti toka 0.11 ±0.01 µs, upadni čas od največje do polovične vrednosti toka 0.17 ±0.03 µs, dolžino obloka 15.0 mm in zamik med zaporednimi razelektritvami 29 ±2 ms. Obročasta ozemljitvena elektroda je imela zunanji premer 86 mm in notranji premer 82 mm.

Ko je razvidno iz slike, so razelektritve povzročile, da je bilo osrednje območje z radijem ~5 mm skoraj popolnoma brez kolonij *E. coli* (Slika 18c). Vrednost tokovne gostote skozi ravninski sloj agarja debeline *d* = 1.72 mm (10 ml agarja razlitega po celotni površini agar plošče z premerom 86 mm) pri električnem toku *I* = 100 A na razdalji *r* = 5 mm od središča lahko ocenimo kot:

$$J \approx \frac{I}{2\pi r d} \approx 185\ A/cm\ ;$$

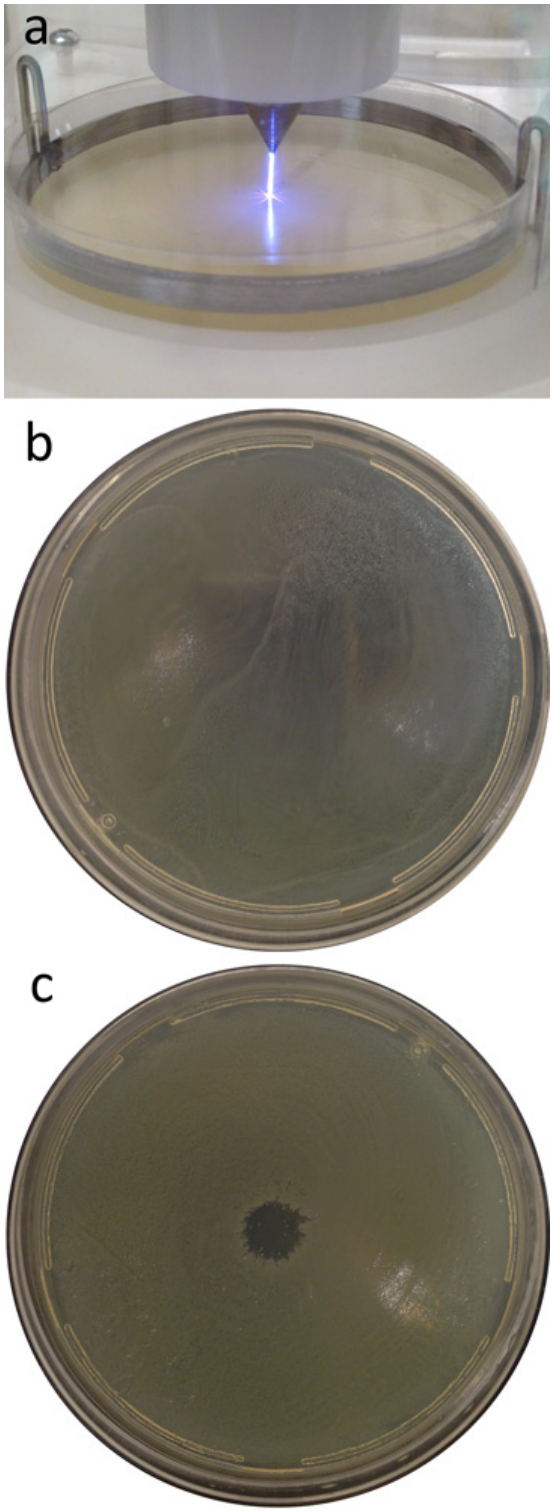
(9)

pripadajoča jakost električnega polja, ki ga ta tokovna gostota *J* ustvari v agarju z električno prevodnostjo σ = 22.9 mS/cm, pa znaša:

$$E = \frac{J}{\sigma} \approx 8.08\ kV/cm.$$

(10)

Ta ocenjena vrednost *E* se dokaj dobro sklada z vrednostmi iz dveh raziskav elektroporacije *E. coli*, kjer so poročali, da jakost električnega polja v območju 6-10 kV/cm povzroči 50% upad preživetja, oziroma v območju 9-12 kV/cm 75% upad preživetja (Calvin in Hanawalt, 1988; Dower s sod., 1988). Potrebno je omeniti, da je pri teh primerih šlo za električne pulze precej daljšega trajanja (nekaj ms), saj so bili ti poskusi usmerjeni v poskus optimizacije učinkovitosti genske elektrotransformacije.

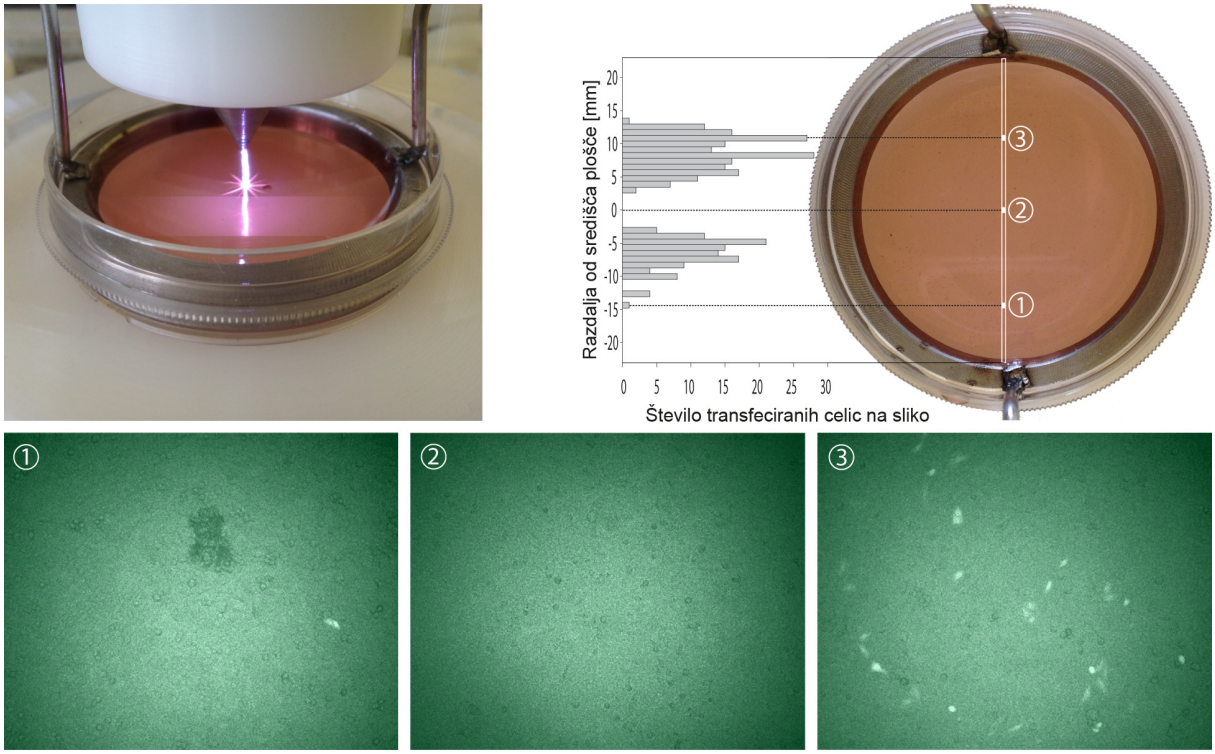


Slika 18: Ireverzibilna elektroporacija *E. coli*, z 10 razelektritvami. a) Izpostavitev, b) kontrolna petrijevka, c) petrijevka z dovedenimi razelektritvami. Prosojno področje je brez kolonij.

3.2.2 Testiranje sistema ZEVS na sesalčjih celicah – vnos in izražanje DNA

Slika 19 prikazuje učinek 10 elektrostatičnih razelektritev na pritrjene celice CHO v petrijevki premera 60 mm. Vsaka razelektritev je imela naslednje lastnosti: največjo vrednost električnega toka 14 ±1 A, dvižni čas od nič do največje vrednosti toka 0.48 ±0.05 µs, upadni

čas od največje do polovične vrednosti toka 0.97 ±0.06 µs, dolžino obloka 7.0 mm in zamik med zaporednimi razelektritvami 10 ±1 ms. Obročasta ozemljitvena elektroda je imela zunanji premer 52 mm in notranji premer 46 mm.



Slika 19: Vnos in izražanje DNA pri celicah CHO.

Kot je prikazano na sliki, smo dobili vnos in izražanje DNA na območju 3 do 15 mm od središča plošče. Na ostalih razdaljah izražanja ni bilo zaznati, a zgolj iz teh rezultatov ne moremo zanesljivo sklepati, ali je to posledica slabega prenosa genov ali pa padca preživetja CHO celic. Vsekakor pa rezultati, ki smo jih dobili pri poskusu ireverzibilne elektroporacije v prejšnjem poglavju, nakazujejo znatno verjetnost, da je v osrednjem območju pomanjkanja izražanja posledica ireverzibilne elektroporacije. To tezo podpre tudi izračun jakosti električnega polja; če je električni tok 14 A po vstopu v gojišče potoval skozi 0.9 mm debelo plast z električno prevodnostjo 14.9 mS/cm proti elektrodi (1.5 ml gojišča, razlitega po 46 mm premera velikem območju v obliki diska), in če iz teh podatkov izračunamo jakost električnega polja na enak način kot v prejšnjem poglavju, sledi, da znaša na razdalji 15 mm od središča petrijevke jakost električnega polja 1.11 kV/cm, na razdalji 3 mm od središča pa 5.54 kV/cm. Ti jakosti električnega polja sta približno trikrat večji od najnižjih jakosti, pri katerih so poročali o vnosu DNA oziroma o ireverzibilni elektroporaciji, a so bili slednji rezultati doseženi z električnimi pulzi, kakršni se običajno uporabljajo pri vnosu DNA (Marjanovič s sod., 2010; Rols in Teissié, 1998) in imajo več kot stokrat daljše trajanje, kar z elektroforetsko silo na DNA prispeva k dodatni učinkovitosti njenega vnosa v celico (Kandušer s sod., 2009; Wolf s sod., 1994).

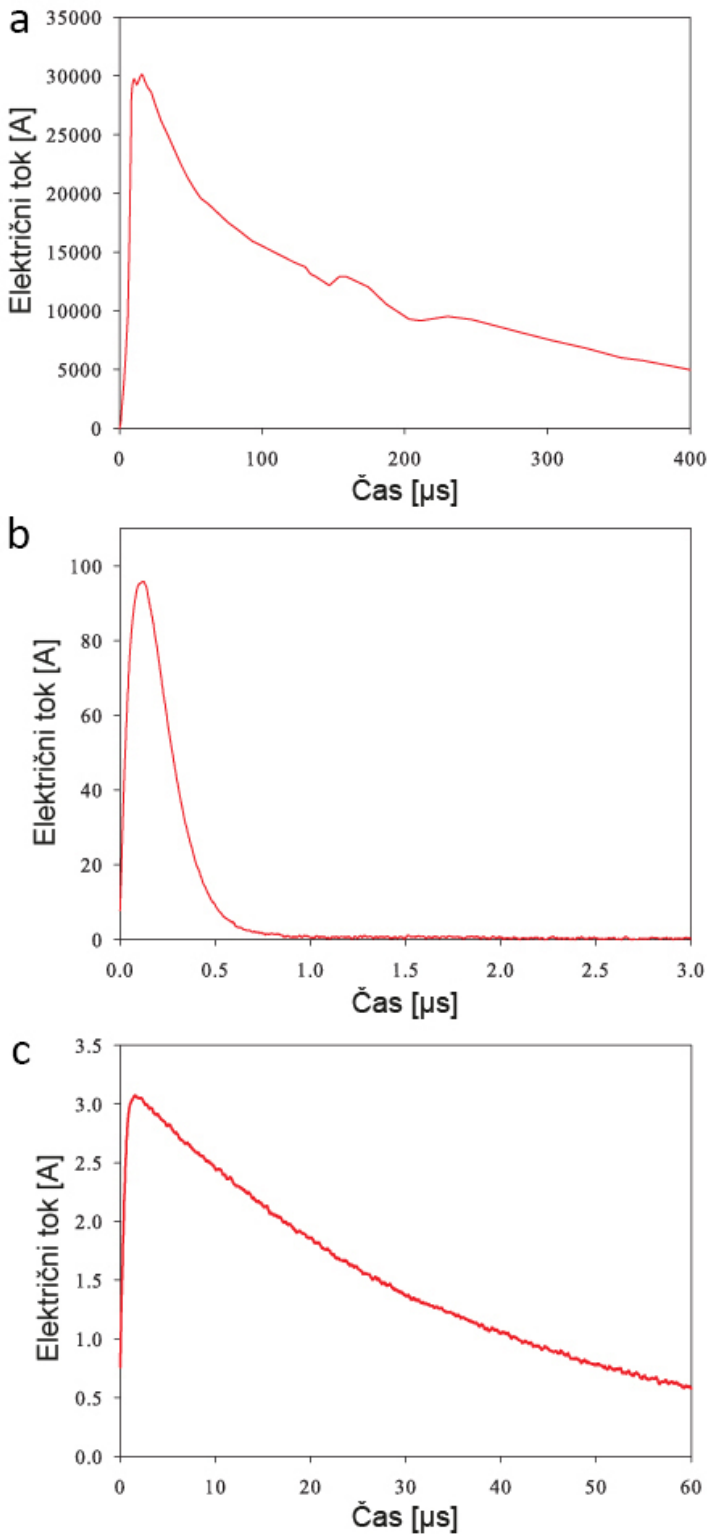
3.3 Napetostni generator

Danes je na izbiro kar nekaj razelektritvenih generatorjev, ki so sposobni ustvariti oblok skozi nekaj centimetrov zraka. Ti generatorji omogočajo, da se z eno elektrodo izognemo neposrednemu stiku z vzorcem in dovedemo električni tok preko obloka, ki se generira skozi zrak med elektrodo in vzorcem. Razelektritve skozi oblok spremlja tudi akustična komponenta v obliki tlačnega vala in je, podobno kot električna, sicer po jakosti manjša, a po drugih značilnostih podobna kot pri naravnih strelah. Če akustična komponenta, ki spremlja obločno razelektritev, pripomore k dodatni prepustnosti celičnih membran, govorimo o kombinaciji elektroporacije in sonoporacije.

Če sistem ZEVS uporabimo za analizo učinkov naravnih strel na živo snov, se ne moremo ogniti zmanjšanju toka, saj znaša maksimalna medianska vrednost električnega toka pri strelah kar 30 kA pri navzdol potekajočih negativnih in 35 kA pri navzdol potekajočih pozitivnih strelah tipa CG (Chowdhuri s sod., 2005). Ker se tok obločne razelektritve po vstopu v tekočo ali trdno snov razširja radialno od točke vstopa, sta vrednosti tokovne gostote (J) in električnega polja, ki ga ta ustvarja (E), obratno sorazmerni z kvadratom razdalje od točke vstopa. Zato so električni pogoji, ki jih ustvari tok naravne strele s časovnim potekom $I(t)$ na neki razdalji od točke vstopa, doseženi tudi v našem sistemu, če ima tok razelektritve časovni potek $I(t)/X$, kjer je $X >> 1$, le na ustrezno manjši razdalji od točke vstopa.

Seveda povsem ob točki vstopa naravne strele v medij nastanejo pogoji, ki jih s pomočjo našega sistema ne moremo ustvariti. Vendar so ti pogoji takšni, da uničijo vso živo snov, to pa se zgodi skozi dobro poznane fizikalne in kemične mehanizme segrevanja, vretja, elektrolize, oksidacije ipd.

Če bi želeli preučevati tudi nepravilnosti časovnega poteka električnega toka tipične strele, kakršne vidimo denimo na Sliki 20a, bi lahko ta časovni potek generirali s programirljivim funkcijskim generatorjem in njegov izhod preko vezja za ojačevanje električnega toka (Flisar s sod., 2003) priklopili na naš sistem. Ta princip bi lahko uporabili za generiranje poljubnih časovnih funkcij električnega toka pri poskusih na sistemu.



Slika 20: Časovni potek električnega toka od različnih virov. a) navzdol potekajoča negativna strela, b) razelektritev paralizatorja preko sistema ZEVS (skozi oblok), c) spraznitev 400 V, 0.25 µF preko Sistema ZEVS v agar ploščo, ki je v neposrednem stiku s konično elektrodo.

3.3.1 Testni generator (paralizator)

Pri razelektritvi, ki je posledica dielektrične porušitve zraka, je časovni potek določen z zakonitostmi fizike. Kot primer navedimo najbolj pogosto prvo negativno strelo (Slika 20a), ki
jo lahko primerjamo z razelektritvijo našega sistema, generirano z električnim paralizatorjem (Slika 20b). Vidimo, da se razelektritev paralizatorja ne razlikuje od naravne strele le v času trajanja in amplitudi električnega toka, temveč tudi v časovnem poteku same razelektritve – električni tok naravne strele ima veliko daljši upadni čas glede na dvižni čas, hkrati pa je tudi celotna oblika toka razelektritve drugačna. Nepravilnosti v toku strele lahko pojasnimo ob upoštevanju dejstva, da strela potuje skozi zrak po korakih skozi različne sloje zraka, ko išče najbolj prevodno pot proti Zemlji. Daljši upadni čas glede na dvižnega pa je razumljiv zato, ker nevihtni oblak in tla v približku predstavljata naelektreni vzporedni plošči ploščatega kondenzatorja, zrak med njima pa izolacijo med ploščama. Pri ploščatih kondenzatorjih so naboji porazdeljeni po celotni površini plošče in v primeru, ko se ustvari prevodni oblok med ploščama, naboji najprej stečejo po plošči proti točki, kjer se je ustvaril oblok med ploščama predno lahko vstopijo vanj. V primeru našega sistema pa je celotni naboj skoncentriran v konici zgornje elektrode, kar nam omogoča, da imamo točno določeno točko, kjer se bo generiral oblok, kar je nujen pogoj, če hočemo delati kontrolirane in ponovljive poskuse. Koničasta elektroda tako močno skrajša čas razelektritve.

Da bi dosegli čimbolj streli podoben časovni potek razelektritve, bi načeloma lahko konično emisijsko elektrodo nadomestili s ploščato, ki bi tako skupaj z površino petrijevke tvorila ploščat kondenzator, ampak v tem primeru bi bila točka izstopa obloka iz emisijske elektrode nepredvidljiva, kar bi močno otežilo opravljati kontrolirane in ponovljive poskuse.

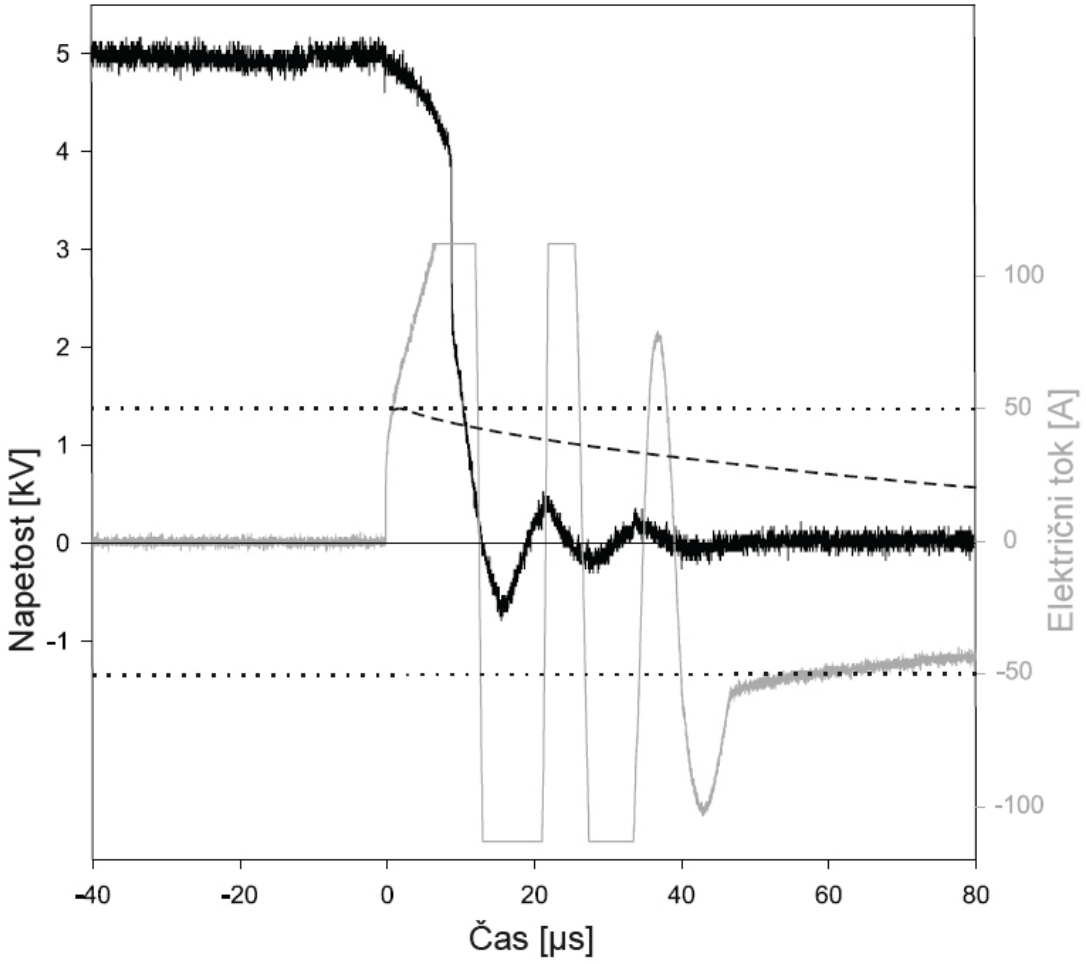
Na sliki 20c pa je prikazano, kako smo preko našega sistema izpraznili 0.25 µF kondenzator, z napetostjo 400 V, preko polprevodniškega hitrega stikala (čas preklopa 0.1 µs), ko je bila zgornja elektroda v neposrednem stiku z vzorcem. V tem primeru je časovni potek toka razelektritve veliko bolj podoben tistemu pri streli – ima kratek dvižni čas in veliko daljši upadni čas. Največji tok pri tem načinu razelektritve je odvisen od kapacitete in maksimalne napetosti kondenzatorja ter je v tem primeru občutno manjši kot v prejšnjem primeru, ko smo za generator razelektritev uporabljali paralizator, a znatno daljšega trajanja, ki je že podobnega velikostnega reda kot pri streli. Ta ugotovitev nam je ponudila iztočnico, da smo se odločili razviti lasten generator, katerega delovanje temelji na obločni razelektritvi kondenzatorja.

3.3.2 Generator ZEVS

Največjo dilemo pri izdelavi generatorja je predstavljala izbira ustreznega visokonapetostnega stikala, ki poveže kondenzator z bremenom. Tehtali smo med bipolarnim tranzistorjem (»insulated-gate bipolar transistor« (IGBT), stikalom preko iskre (»spark-gap switch«) in relejem (Reberšek s sod., 2014, p. 3; Reberšek in Miklavčič, 2011). Vsa ta stikala so bila dobavljiva za maksimalno izhodno napetost 5 kV, a se je za tranzistorje IGBT izkazalo, da imajo omejitev pri amplitudi kot tudi dvižnem času električnega toka, poleg

tega pa so manj vzdržljivi in dražji od drugih dveh stikal. Odločili smo se za izbiro releja, ker stikalo preko iskre predstavlja že sam sistem, saj je razelektritev v vzorec dovedena skozi zrak.

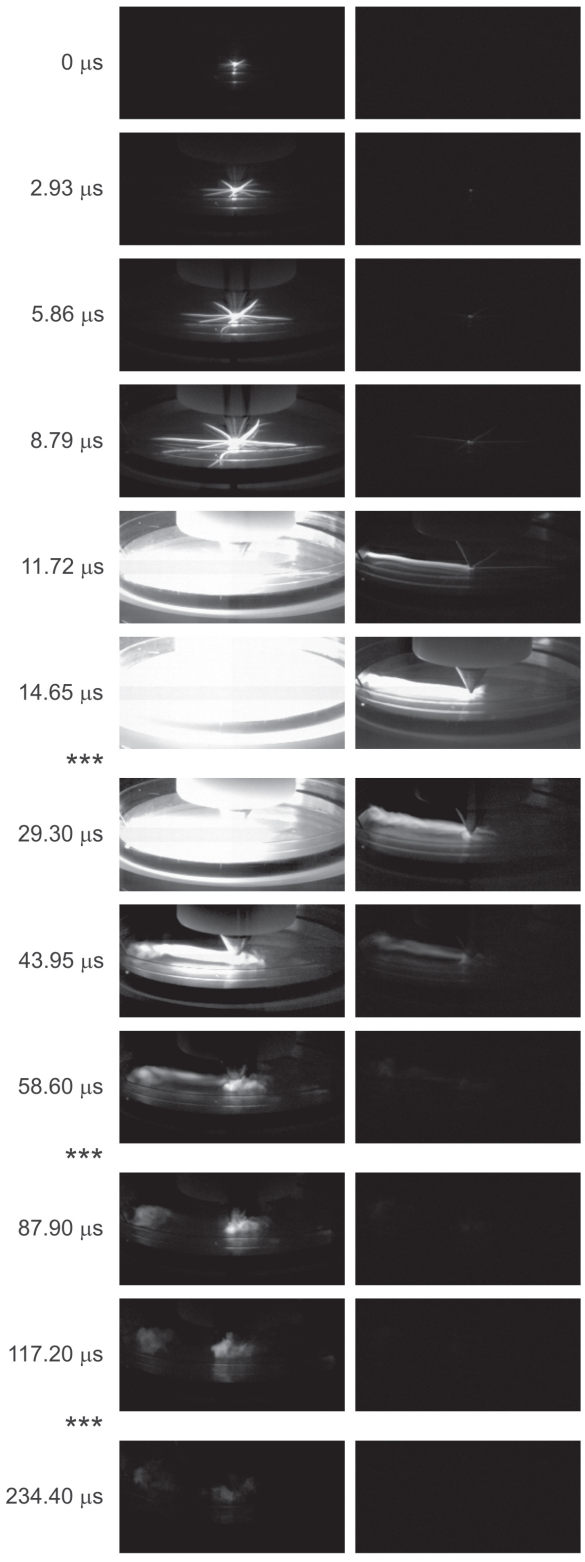
Ko smo testirali sistem ZEVS z električnim paralizatorjem (čas trajanja razelektritve ≤ 2 µs), je sama razelektritev potekala po obloku skozi zrak med konično elektrodo in vzorcem, po približno navpični poti in vstopila v vzorec tik pod elektrodo. Nato je električni tok tekel od točke vstopa v vzorec približno enakomerno na vse strani proti obročasti elektrodi, ki je ga je obdajala (Slika 18a, 19a). Generator ZEVS pa smo zasnovali tako, da z njim dovajamo v vzorec razelektritve trajanja ~100 µs. Ko smo izvedli prve poskuse s tem generatorjem, smo opazili, da je razelektritev vstopila v vzorec preko obloka, podobno kot pri poskusih s paralizatorjem – tok je v nekaj µs narasel na ~50 A, napetost pa je z začetne vrednosti 5 kV postopoma upadala. Po ~10 µs pa je električni tok nenadno narasel in močno presegel vrednost 50 A, ki jo je bila sposobna izmeriti tokovna sonda, napetost pa se je skoraj hipoma sesedla. Posledica tega je bila, da je razelektritev trajala le ~10 µs, nato so še nadaljnjih ~35 µs sledile oscilacije napetosti in toka, ki so bile posledica induktivnosti žic, preko katerih je bil sistem ZEVS priklopljen na generator (Slika 21).



Slika 21: Časovni potek električnega toka (siva) in napetosti (črna) pri razelektritvah z generatorjem ZEVS, kjer pride do kratkega stika med elektrodama preko obloka. Točkasti vodoravnici predstavljata ±50 A

delovno območje tokovne sonde, črtkasta krivulja pa prikazuje pričakovan potek toka v primeru, če ne bi prišlo do kratkega stika.

Pri tej razelektritvi smo opazili zelo svetel oblok, ki je potekal od konične elektrode proti obročasti elektrodi v vodoravni smeri in posledično kratko sklenil elektrodi. Sama razelektritev se je tako izognila vstopu v vzorec. Zaradi zelo močne svetlobe obloka s prostim očesom ni bilo mogoče opazovati prehodnega pojava, zato smo se odločili, da ga preučimo z hitro kamero. Uporabili smo Vision Research Phantom® v2010, trenutno najhitrejšo komercialno dosegljivo kamero, ki nam jo je proizvajalec Vision Research Europe prijazno posodil. Kamera ima zmožnost zajema slik s hitrostjo milijona slik na sekundo, vendar je pri tej hitrosti ločljivost posnetka prenizka, da bi omogočala ustrezno opazovanje pojava. Posnetki pri hitrosti 341000 slik na sekundo (slika na vsake 2.39 µs) so se izkazali za optimalno kombinacijo med ločljivostjo posnetka in hitrostjo zajemanja slik, prikazani pa so na Sliki 22.



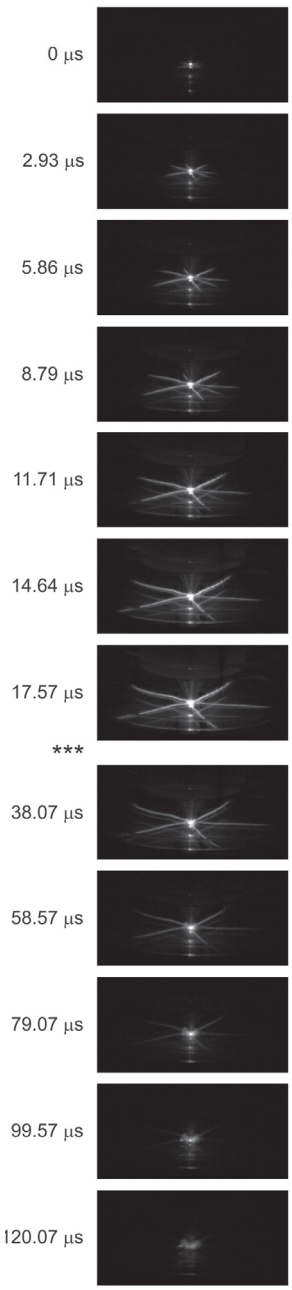
Slika 22: Razvoj obloka pri razelektritvi brez notranjega cilindra. Po 8-12 µs pride preko obloka med elektrodama do kratkega stika. Razelektritev posneta s hitro kamero brez filtra (levo), oziroma skozi zaščitno steklo za varjenje (desno).

Kot prikazuje levi stolpec Slike 22, razelektritev preko obloka (zračnega kanala) potuje najprej navpično navzdol proti vzorcu. Po približno ~3 µs se oblok razveji in električni tok teče delno skozi vzorec, odtod pa tik nad njegovo površino, proti obročasti elektrodi. Po ~9

µs (v konkretnem primeru na sliki, v splošnem pa med 8 in 12 µs) je nad površino že velika večina dolžine posameznih oblokov, a še vedno vstopajo v vzorec, preden dosežejo obročasto elektrodo. Ko jo prvi oblok doseže, pa se preko zraka sklene prevodna pot (vodilni kanal), ki povzroči kratek stik med elektrodama, po tej poti pa nato steče ves električni tok, kar še poveča njegovo prevodnost in ustvari tudi močan svetlobni blisk. Ta je tako močan, da povsem zasiči (presvetli) zajeto sliko, zato smo bili primorani te slike zajeti skozi filter, za katerega smo uporabili zaščitno steklo varilne čelade (Slika 22, desni stolpec). Na Sliki 22 vidimo tudi, da zrak ostane ioniziran in oddaja svetlobo še do ~200 µs potem, ko tok preneha teči.

Pri nevihtnih strelah obročasto elektrodo našega sistema zamenja Zemlja, zato do takih pojavov v naravi ne more priti. Ta pojav je artefakt, ki je nastal kot stranska posledica zasnove sistema ZEVS. Če električni tok teče skozi vzorec približno radialno proti obročasti elektrodi, potem sistem ZEVS dobro emulira razširjanje električnega toka strele ki po Zemlji teče proti oddaljeni masi. Če pa pride do prej opisanega artefakta, kjer električni tok namesto skozi vzorec steče po vodilnem kanalu in posledično kratko sklene elektrodi, emulacija razelektritve izgubi podobnost z nevihtno strelo. Sistem ZEVS smo zato predelali tako, da smo med elektrodi postavili dodatno oviro v obliki cilindra iz pleksi stekla, postavljenega tako, da se konična elektroda nahaja na njegovi sredini (nad njim, a poravnana z osjo cilindra), spodnji del cilindra pa se dotika površine vzorca (Glej poglavje 2.4.1). Ta cilinder je ovira, ki prisili električni tok, da namesto po zraku teče skozi vzorec.

Najprej smo uporabili cilinder, ki se je s svojim zunanjim obodom prilegal notranjemu obodu obročaste elektrode. Notranji premer cilindra je bil 70 mm, zunanji pa 80 mm, medtem ko je bil notranji premer obročaste elektrode 82 mm. Razelektritev s tem obročem je prikazana na Sliki 23. V ~5 µs je električni tok narastel na ~50 A in potem eksponencialno upadal in dosegel polovično vrednost po 65 µs. Vendar pa je pri tej razelektritvi vstopna točka oblok v vzorec kljub temu postopoma potovala vstran, po ioniziranem zraku nad vzorcem, dokler oblok ni naletel na cilinder in se ob njem potopil v vzorec. Pri potovanju skozi vodilni kanal do cilindra je bil blisk najbolj viden med 15 in 25 µs, potem pa je postopoma upadal. Ioniziran zrak tik pod koničasto elektrodo pa je žarel še nadaljnjih ~100 µs. Pri kasnejših poskusih smo uporabili cilinder z manjšim premerom (Metode, Slika 14), s čimer smo dosegli, da je oblok vseskozi vstopal v vzorec skoraj navpično in je njegov električni tok skoraj v celoti tekel skozi vzorec.

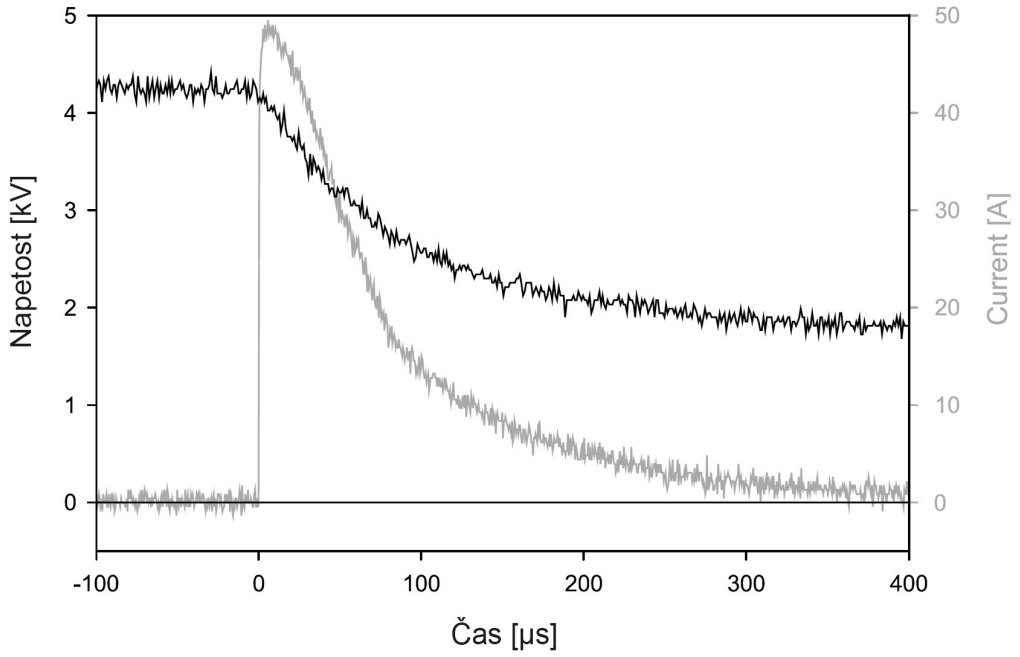


Slika 23: Razvoj obloka pri razelektritvi z dodanim notranjim cilindrom, ki prepričuje nastanek kratkega stika med elektrodama preko obloka.

Pri izdelavi sistema ZEVS smo se trudili, da razelektritve čimbolj približamo nevihtnim strelam in omogočimo poskuse v kontroliranem laboratorijskem okolju, na podlagi katerih lahko preučujemo, kaj se dogaja z organizmi, ki živijo v habitatu izpostavljenemu nevihtnim strelam. Največji električni tok, ki je bil doveden v vzorec s pomočjo sistema, je za faktor ~600 manjši kot pri streli (~50 A proti ~30 kA), vendar pa je časovni potek toka pri razelektritvi na sistemu ZEVS podoben tistemu pri streli. Ko enkrat električni tok vstopi v vzorec, se širi radialno in zvezno od točke vstopa proti obročasti elektrodi, ki obdaja vzorec. Gostota električnega toka in jakost električnega polja tako z oddaljevanjem od točke vstopa upadata zvezno.

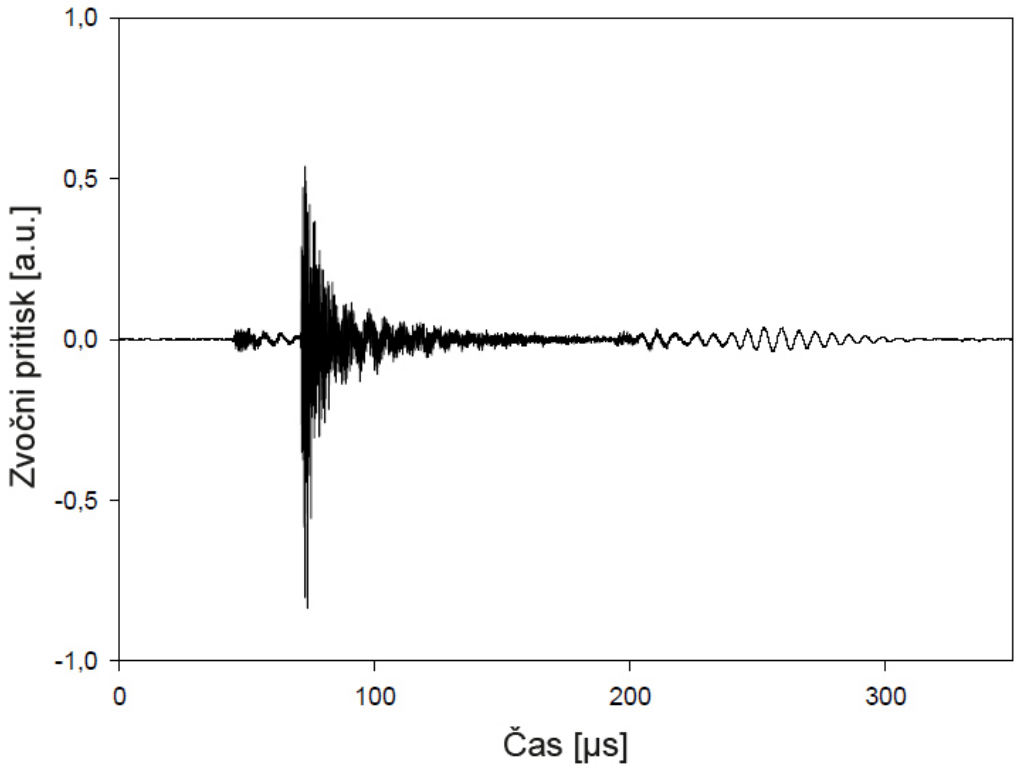
Pri udaru strele zato dobimo enake vrednosti *J* in *E*, le pri večji oddaljenosti od točke vstopa. Za primer vzemimo strelo z maksimalno vrednostjo električnega toka 30 kA (medianska vrednost pri navzdol potekajoči negativni streli), kjer se tokovna gostota v mediju znižuje zvezno navzdol in navzven od točke vstopa (tridimenzionalno); tokovno gostoto $J = 50$ A/cm^2 dobimo na razdalji $r = 9.77$ cm. Če pa s sistemom ZEVS dovedemo razelektritev na ploščati vzorec debeline 5 mm, ki ga obdaja obročasta elektroda, tok teče v dveh dimenzijah, torej od točke vstopa proti obročasti elektrodi, in dobimo isti *J* na razdalji $r = 3.18$ mm (če je medij enak kot pri streli, pa je pri tej razdalji tudi *E* enak kot pri streli na razdalji $r = 9.77$ cm).

S sistemom ZEVS ne moremo doseči največjih vrednosti *J* in *E*, ki ustvari strela, a tako visoke vrednosti imajo smrtonosen učinek na vse žive organizme zaradi dobro poznanih fizikalnih mehanizmov.



Slika 24: Časovni potek električnega toka (črna) in napetosti (siva) pri razelektritvah generatorja ZEVS z dodanim vmesnim cilindrom, ki prepričuje kratek stik med elektrodama preko obloka.

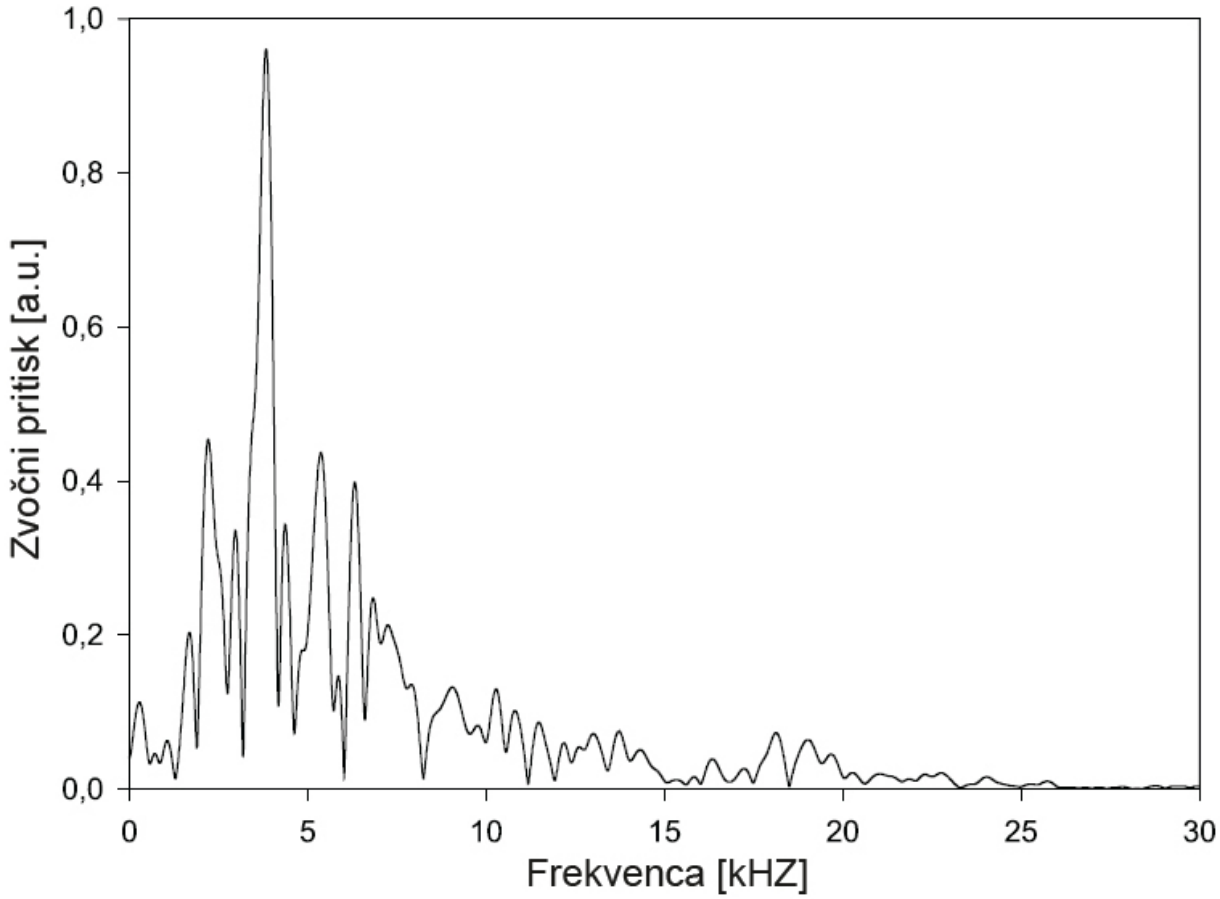
50



Slika 25: Časovni potek tlačnega vala, kot stranski produkt razelektritve na našem sistemu.

Če pri poskusih na sistemu ZEVS, kot je opisano zgoraj, uporabimo cilinder, ki prepreči kratek stik med elektrodama, lahko ta sistem emulira električne pogoje nevihtne strele dokaj natančno. Pri razelektritvi pride tudi do tlačnega vala, ki se sliši kot glasen pok (pri streli grmenje). Tlačni val ob razelektritvi smo posneli z mikrofonom (glej Poglavje 2.4.2), vendar nam ni uspelo izmeriti maksimalne amplitude tlačnega vala, saj je ta presegla 110 dB tudi na razdalji 2 m od sistema. Na Sliki 25 je prikazan izmerjeni časovni potek zvočnega tlaka. Čeprav nam ni uspelo natančno izmeriti maksimalne amplitude, saj je bil razpon mikrofona 120 dB presežen že na razdalji 60 cm od razelektritve, pa iz tega dejstva sledi, da je bila dejanska maksimalna amplituda zvočnega pritiska večja od 120 dB.

Nato smo preučili še frekvenčno sestavo zvoka razelektritve in sicer tako, da smo zvočni posnetek analizirali s hitro Fourierjevo transformacijo (FFT). Ko smo odstranili resonančno frekvenco, ki je bila posledica odboja zvoka v prostoru, kjer smo razelektritev posneli, in dodatno resonanco, ki je bila posledica odboja zvoka od pleksi cilindra, smo dobili zvočni spekter (Slika 26), ki je podoben spektru, ki ga proizvede strela (Coleman s sod., 2009).



Slika 26: Frekvenčni spekter zvočne komponente razelektritve, ustvarjene s sistemom in generatorjem ZEVS.

3.4 Inaktivacija spor

3.4.1 Bakterijske spore

Bakterijske spore so ena izmed najbolj trdoživih oblik živih organizmov, z zelo visoko odpornostjo na toplotne, kemične in mehanske obremenitve (Setlow, 2006). Spore lahko v hibernaciji preživijo ekstremne pogoje, celo v vesolju (Vaishampayan s sod., 2012), zato lahko povzročijo kontaminacijo v primeru medplanetarnih misij (Moeller s sod., 2012). Spore v hibernaciji lahko preživijo zelo dolgo časa. Spore *Bacillus subtilis* so bile najdene v 25 do 40 milijonov let starih fosilnih ostankih že izumrlih čebel (Cano in Borucki, 1995). Tolikšno odpornost in vzdržljivost bakterijskih spor pripisujemo večplastni ovojnici, ki obdaja bakterijo in je sestavljena iz notranje zelo selektivno prepustne membrane, temperaturno odpornega peptidoglikanskega področja, zunanje membrane in kemično odpornega proteinskega ovoja (McKenney s sod., 2013). Poleg tega pa vitalni deli same spore (genom in ribosomi) za svoje delovanje potrebujejo manj tekočine kot ostali organizmi. Patogene spore so vzrok številnim boleznim in njihova odpornost predstavlja velik problem pri inaktivaciji. Med drugim lahko povzročijo okužbo dihal, kontaminacijo hrane in smrtonosno paralizo (Enticknap s sod., 1968; Graham s sod., 1922; Oie s sod., 2011). Zaradi tega so učinkovite

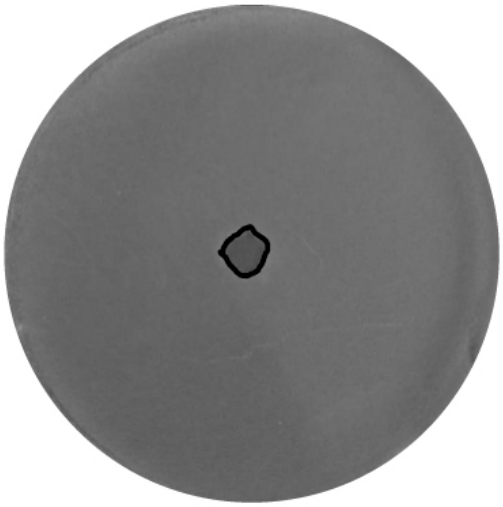
metode inaktivacije patogenih spor zelo pomembne. Klasična učinkovita metoda pri pasterizaciji hrane je segrevanje nad 100 °C (Norcross s sod., 1957), ki pa je ne moremo uporabljati pri dekontaminaciji temperaturno občutljivih snovi. Poznani sta tudi metodi dekontaminacije z gama žarki (Horne s sod., 1959) in izpostavitev etilen oksidu (Whitney s sod., 2003), ki sta sicer učinkoviti metodi dekontaminacije, a dragi in hkrati tudi velikokrat škodljivi za snov, ki jo želimo dekontaminirati.

Ena izmed možnih metod inaktivacije je tudi ireverzibilna elektroporacija, ki je učinkovita metoda pri inaktivaciji mnogih vrst bakterij, ko preko elektrod v neposrednem stiku z vzorcem dovajamo električne pulze. Ta metoda pri bakterijskih sporah ni učinkovita (Yonemoto s sod., 1993). Pri razelektritvi preko sistema ZEVS je poleg električnega polja vzorec izpostavljen še ultravijolični svetlobi (Edebo, 1968) in zvočnemu pritisku (Edebo in Selin, 1968), ki lahko povzroči kavitacijo (Boussetta s sod., 2013). Takšna metoda inaktivacije je bila sicer opisana že leta 1962 za bakterije v vodi (Brandt s sod., 1962), vendar do naših poizkusov, ki bodo opisani v naslednjih odstavkih, še ni bila preizkušena za inaktivacijo bakterijskih spor.

Poskuse inakativacije smo opravili na bakterijskih sporah *Bacillus pumilus* z razelektritvami, generiranimi s električnim paralizatorjem in generatorjem ZEVS.

3.4.2 Inaktivacija spor z 0.5 µs razelektritvami

Slika 27 prikazuje območje inaktivacije spor na petrijevki z agarjem po dovedenih ~20 razelektritvah, vsaka z dolžino ~0.5 µs, v intervalih ~300 ms. Te razelektritve so bile dovedene s paralizatorjema ter niso bile nastavljive in povsem ponovljive, vendar smo jih spremljali z osciloskopom in tako zagotovili, da pri nobenem izmed poskusov dejanska vrednost ni odstopala od zgoraj navedenih za več kot 10%.



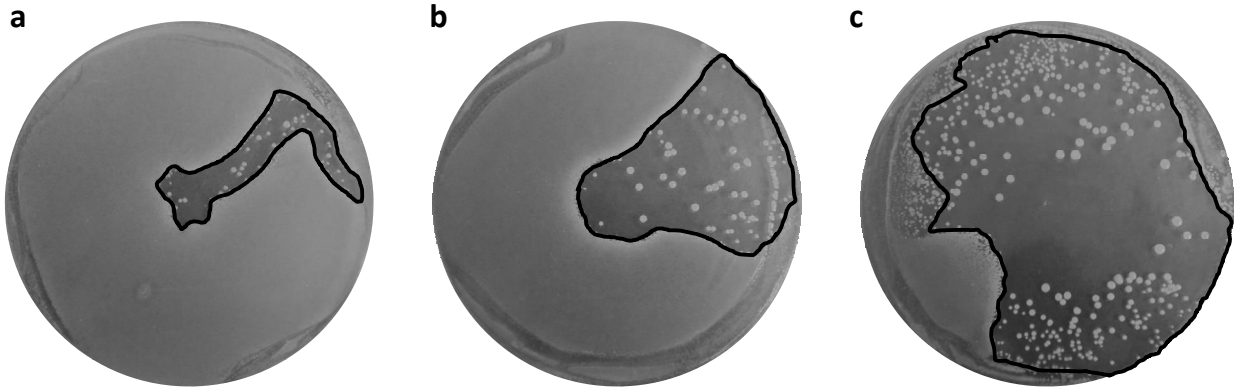
Slika 27: Inaktivacija spor z ~20 razelektritvami dolžine ~0.5 µs. Območje inaktivacije je obrobljeno s črno.

Pri teh razelektritvah je oblok iz konične elektrode potoval navpično proti središču petrijevke z agarjem, ko je vstopil v agar, pa je električni tok tekel radialno skoznjo proti

obročasti elektrodi (kot na Slikah 18a in 19a). Pri teh poskusih sta gostota električnega toka in jakost električnega polja upadala radialno, zvezno in obratno sorazmerno z razdaljo od točke vstopa razelektritve. Kot prikazuje Slika 27, je bilo območje inaktivacije prisotno le v okolici točke vstopa obloka v petrijevko in je zavzemalo ~0.65% celotne površine agarja v njej.

3.4.3 Inaktivacija spor z 20 µs razelektritvami

Slika 28 prikazuje območje inaktivacije spor na agar plošči, ko smo dovedli različno število razelektritev (od 1 do 50) pri dolžini razelektritve ~20 µs, z intervalom ~3s.



Slika 28: Inaktivacija spor z eno (a), desetimi (b) in petdesetimi (c) razelektritvami dolžine 20 µs. Območje inaktivacije je obrobljeno s črno.

Pri teh izpostavitvah je oblok na začetku prav tako potoval navpično od konične elektrode proti središču petrijevke z agarjem, a se je nato začel uklanjati in tako v 8 – 10 µs ustvaril kratkostični kanal med konično in obročasto elektrodo, tik nad površino vzorca (Slika 22). Iz Slike 27a lahko glede na območje inaktivacije, dosežemo s pravokotno vstopajočim oblokom, razberemo, po kateri poti se je gibala točka vstopa obloka v vzorec. Zaporedne razelektritve so potovale po ločenih oblokih, ki so se postopoma raztrosili, tako da je večje število razelektritev pokrilo večino področja petrijevke in sicer približno 7% površine pri eni razelektritvi (Slika 28a), 27% pri desetih (Slika 28b) in 55% pri petdesetih razelektritvah (Slika 28c).

3.5 Varnostne opombe

Z vidika varnosti uporabe sistema ZEVS je najpomembnejše, da preprečimo nezaželene elektrostatične razelektritve, bodisi v upravljalca sistema ali ostalega osebja, bodisi v ostalo opremo, ki se nahaja v bližini sistema. Da se to ne zgodi, je pomembno, da je razelektritveni generator v času, ko ga priklapljamo ali odklapljamo iz sistema, izklopljen. Prav tako mora biti izklopljen, ko vstavljamo ali odstranjujemo vzorec iz sistema. Preden se generator vklopi, se mora upravljavec sistema prepričati, da je ta zanesljivo priključen na sistem.

Ko se odločimo, kateri razelektritveni generator bomo priključili na naš sistem, moramo izbrati tudi primerne žice, katerih izolacija vzdrži prebojno trdnost (z kar nekaj dodatne

rezerve), ki jo zahteva maksimalna napetost generatorja. Od vseh delov je v sistemu ZEVS najmanj izoliran priklopni del emisijske elektrode, a tudi ta vzdrži električne napetosti vsaj do 120 kV. Če bi uporabili generator, ki bi bil sposoben dovajati večje napetosti, pa bi morali zgornjo elektrodo dodatno izolirati.

Članek 1

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An experimental system for controlled exposure of biological samples to electrostatic discharges

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ABSTRACT

Electrostatic discharges occur naturally as lightning strokes, and artificially in light sources and in materials processing. When an electrostatic discharge interacts with living matter, the basic physical effects can be accompanied by biophysical and biochemical phenomena, including cell excitation, electroporation, and electrofusion. To study these phenomena, we developed an experimental system that provides easy sample insertion and removal, protection from airborne particles, observability during the experiment, accurate discharge origin positioning, discharge delivery into the sample either through an electric arc with adjustable air gap width or through direct contact, and reliable electrical insulation where required. We tested the system by assessing irreversible electroporation of *Escherichia coli* bacteria (15 mm discharge arc, 100 A peak current, 0.1 μs zero-to-peak time, 0.2 μs peak-to-halving time), and gene electrotransfer into CHO cells (7 mm discharge arc, 14 A peak current, 0.5 μs zero-to-peak time, 1.0 μs peak-to-halving time). Exposures to natural lightning stroke can also be studied with this system, as due to radial current dissipation, the conditions achieved by a stroke at a particular distance from its entry are also achieved by an artificial discharge with electric current downscaled in magnitude, but similar in time course, correspondingly closer to its entry.

1. Introduction

Electrostatic discharges have long been known to humans; in nature we encounter them in the form of atmospheric lightning strokes, while artificial electric arcs – the first one generated by Humphry Davy in 1809 [1] – have many fields of application, ranging from light sources (arc lamps, including fluorescent tubes) to a wide span of tools for processing of materials, particularly for welding, heating (arc furnaces) and plasma cutting. Recently, it was reported that nanosecond electric arcs (sparks) applied to living skin lead to a more efficient DNA uptake and expression than the standard approach in which electric pulses are delivered through electrodes in direct contact with the skin [2], and it was also suggested that lightning strokes could have contributed to DNA transfer during evolution [3–5].

The physics of the effects caused by electrostatic discharges interacting with simple materials is well understood. Most technological processes thus exploit the heat dissipated by the electric current of the arc (flowing either through the material or through the air nearby), while emission of light from arc lamps also involves ionization and quantum excitation of the gas through which this current flows. When flowing through a material, the electric current of the arc also induces an electric field in the material, which is the strongest at the current's point of entry, then gradually decreases as the current flow is dispersed over larger cross-sections inside the material, and – provided that its exit is also point-like – increases again to reach another peak at the point of exit.

When an electrostatic discharge interacts with living matter, the basic physical effects – the induced electric field, the temperature increase, and possibly ionization caused by the electric current – are the same as in simpler materials, but they can be accompanied by a range of biophysical and biochemical phenomena. Electric fields as weak as 60 mV/cm (for durations over 100 μs) excite nerve and muscle fibers, while much stronger fields (hundreds of V/cm or more) cause – in all cells, both excitable and non-excitable – a considerable increase of their membrane permeability (electroporation) and/or their merger (electrofusion). If the field is neither too strong nor too long-lasting, electroporation is reversible, while otherwise it becomes irreversible, resulting in cell death. With sufficient power dissipation, exposures to strong electric fields also cause thermal damage to the cell and its molecules (protein denaturation, DNA melting).

The phenomena of electroporation and electrofusion have been known for several decades. Electroporation was thus first reported for an excitable cell plasma membrane (Ranvier node of a myelinated axon) in 1958 [6], for a non-excitable cell plasma membrane (bacterial outer and cytoplasmic membrane) in 1967 [7], for organelle membrane (of a chromaffin granule) in 1972 [8], and for a planar lipid bilayer (oxidized cholesterol/n-decane) in 1979 [9]. Electrofusion was first demonstrated for animal cells (both anucleate and nucleated) in 1980, and for plant protoplasts and lipid vesicles in 1981 [10–13].

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Since their discovery, electroporation and electrofusion have been extensively investigated and have to date found multiple applications in medicine and biotechnology. In medicine, reversible electroporation is used for delivery of chemotherapeutics in cancer treatment [14] and of DNA in gene therapy [15], irreversible electroporation is a promising technique of tissue ablation [16], while electrofusion holds some promise in preparation of monoclonal antibodies for both diagnostics and therapeutics [17]. In biotechnology, irreversible electroporation is an efficient technique for extraction of biomolecules from cells and tissues [18,19], and is also useful – either accompanied by thermal effects or not – for inactivation or/and destruction of microorganisms [20].

Although some unknowns still remain, the physical properties of electroporation and electrofusion are by now generally well understood on the cellular and membrane level, while the recent molecular dynamics simulations are also improving our understanding of both phenomena on the molecular and atomic level [21–25]. Nevertheless, the mechanisms of electroporation-mediated transport, particularly of macromolecules such as DNA, are still a subject of vigorous investigation, as they likely involve several concurrent phenomena generated by the electric field pulses either directly (electroporation) or indirectly — by the resulting pressure waves (sonoporation) and/or thermal effects (thermoporation) [26,27].

Electroporation is, according to both theoretical considerations [28,29] and molecular dynamics simulations [22,23], an electric field-induced formation of aqueous pores in lipid parts of biological membranes. In this process, the water molecules penetrate into the lipid bilayer and interact there with adjacent membrane lipids that consequently reorient with their polar heads towards these water molecules, thus forming a polar pore wall. These pores render the membrane locally permeable to both ions and molecules. Electroporation occurs in the lipid bilayer of the membranes of all prokaryotic and eukaryotic cells, with the pores in the plasma membrane providing a pathway for transport of a wide range of molecules, including DNA, into [26] and out of the cell [7]. Pore formation is governed by electrochemistry and statistical thermodynamics [28,29] and due to the latter it is not strictly a threshold event, in the sense that the pores would only form in electric fields exceeding a certain level, but transport across the electroporated membrane is strongly correlated with transmembrane voltage induced by the electric field [30], which is in turn proportional to the strength of this field [30].

Electrofusion of two cells can occur both if they are in direct contact during the exposure to the electric pulses, or if they are brought into such contact within a sufficiently short time (seconds or even minutes) after the exposure [31,32]. Experiments also show that in electrofusion of two lipid bilayers, the monolayers in direct contact often fuse first, while the other two monolayers still appear intact [33]. This suggests that electrofusion proceeds in the same three stages broadly recognized in the physiological fusion of two cells in direct contact: first, their membranes' external monolayers, at least one of which is locally destabilized, fuse within the area containing the instability, forming a stalk; second, the fused monolayers move apart radially, forming a disk-shaped diaphragm and bringing the internal monolayers into contact; and finally, the rupture of the diaphragm creates a pore connecting the cytoplasms of the two cells, thus completing the fusion [34]. Physiological fusion and electrofusion then differ mainly in the trigger of local destabilization of the exterior monolayer that initiates the fusion — various fusogenic membrane proteins in the former case [35], and electric pulses in the latter [36].

In medical and biotechnological applications of electroporation and electrofusion, as well as in basic research of these phenomena and the accompanying thermal effects, the required electric fields are generated by a suitable voltage source and delivered through electrodes in direct contact with the sample. Furthermore, while the early voltage sources used for electroporation and electrofusion were based on a capacitor discharge and as such delivered exponentially decaying pulses, the modern sources largely deliver rectangular pulses, with the voltage turned on stepwise, sustained at a constant level for a preset duration of the pulse, and then turned off stepwise. Such a description is a slight idealization, but the rise- and falltimes of the commercially available rectangular pulse generators for electroporation are now well below a microsecond, and the variability of the pulse amplitude is in general within a few percent of the preset value. In this manner, the electrical parameters used for electroporation and/or electrofusion are well controlled, making the basic studies reproducible and the resulting applications reliable.

Electric fields with amplitudes and durations adequate for electroporation and electrofusion can also occur in natural environments when these are hit by a lightning stroke [4,5]. But unlike with the laboratory studies and applications of electroporation and electrofusion described above, a stroke proceeds through a highly conductive channel (electric arc) created by electrical breakdown of the air separating the cloud and the ground, and the time course of the electric current and the electric field induced by it as it flows through the ground are neither rectangular nor purely exponentially decaying. Furthermore, in the ground the current does not flow towards a well-defined electrode, but dissipates downward and outward from its point of entry, and consequently the amplitude of the electric field it induces decreases rapidly with increasing distance from this point.

To adequately study lightning-induced electroporation, electrofusion, and the accompanying physical, biophysical and biochemical effects, the standard equipment used in the studies and applications of electroporation and electrofusion would thus have to be adapted to reflect the abovementioned distinctions — the delivery of the electric current to the sample through an electric arc formed in the air, the current's roughly radial dissipation in the sample, and preferably also its specific time course.

In this article, we describe the design, construction and testing of a scalable modular exposure system built along the above-mentioned guidelines, allowing to expose samples of biological cells and tissues to an electrostatic discharge with an adjustable peak current (up to several hundred amperes) in a controlled environment (with a precisely defined arc length, and with monitored time course of the current flowing through the sample). This provides a reproducible emulation of a downscaled lightning stroke. The exposure system allows the researchers to incorporate the electrostatic discharge generator and the ground-simulating electrode (s) of their choice, to quickly adjust the arc length, and due to its modular nature the system also allows for quick assembly, disassembly, and thorough cleaning.

2. Materials and methods

2.1. System development

2.1.1. Computer modeling

The components of the exposure system and the construction of the system as a whole were first modeled computationally in SolidWorks 2013 (Dassault Systèmes SolidWorks Corporation, USA), which was used both for mechanical design and for material selection, with the latter also comprising a basic numerical assessment of the mechanical strength of the materials. For more advanced numerical calculations of lightning stroke simulations and electric field distribution within the exposed sample, we used COMSOL MultiPhysics 4.2 (Comsol, Stockholm, Sweden).

2.1.2. Materials

The main construction material was polyethylene plastic (PE 500 Natur, Simona AG, Germany), as it is a good electrical insulator, widely available, affordable, and easy to clean. To allow for visual monitoring of the sample during the experiment, transparent parts of the system were constructed from plexi-glass (PLEXIGLAS XT Tube Clear 0A070GT, Evonik Industries, Germany), which is also a good insulator and easy to clean. For the electrodes, we tested both copper and stainless steel,

opting for the latter in the final design, with the reasons for this choice described in Section 3.

2.1.3. Fabrication

All the components of the exposure system except for the electrostatic discharge generator were custom fabricated using CNC machinery (High-Z 720, CNC-STEP, Germany), routing bits (CMT Utensili, Italy) and universal router (F-900 A/G, Voestalpine, Austria). The reasons for custom fabrication were to construct an exposure system emulating as closely as reasonably achievable an actual lightning stroke, and to allow for modular assembly and simple disassembly of the components for cleaning and transportation.

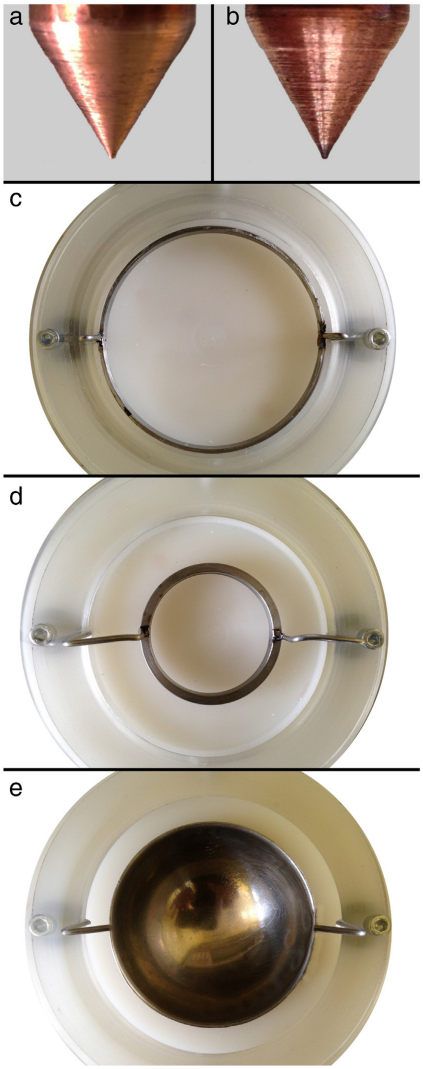

Fig. 1. (a–b) Corrosion of an emitting electrode made of copper. The electrode is shown before (a) and after (b) delivery of 500 discharges with approx. 100 A peak current, 0.1 μs zero-to-peak time, and 0.3 μs halving time into an agar plate. For an arc to form, the distance from the cone tip to the plate surface had to be gradually reduced from 15 mm for the first discharge to 11 mm for the 500th discharge. To avoid corrosion, we chose stainless steel for the final version of the emitting electrode. (c–e) Shapes of receiving electrodes made of stainless steel. (c) A ring electrode fitting into a 90 mm dish used for agar plating of bacteria. (d) A ring electrode fitting into a 60 mm dish for culturing of eukaryotic cells. (e) A hemispherical bucket electrode with an inner diameter of 70 mm suitable for three-dimensional cell populations growing in suspensions, gels, or soil, which are filled directly into the bucket. With a ring electrode the radial current dissipation is planar, while with the bucket electrode it is three-dimensional.

2.1.4. Discharge generator

Electrostatic discharges applied in our system testing (see Sections 2.2 and 2.3) were generated by commercially available taser gun (Great Power 750000, Great Power, South Korea). This device uses an amplifier circuit to amplify the source voltage, and an oscillator to generate the current that charges the capacitor connected to external electrodes; the capacitor discharges through these electrodes when the air breakdown voltage is reached between them, causing an electric arc across the air gap between the electrodes.

2.2. System testing on bacteria

2.2.1. Cultivation of Escherichia coli bacteria

E. coli K12 ER1821 strain (New England BioLabs, Frankfurt, Germany) was used as a model of bacterial cells. The cells were mixed with Luria broth (Sigma-Aldrich, Munich, Germany) in an Erlenmeyer flask and placed into an incubator with continuous shaking (I-50, Kambič Laboratory Equipment, Slovenia) for 24 h at 37 °C. After incubation, the flask was removed from the incubator and centrifuged for 15 min at 4 °C at 4200 RPM. Supernatant was then carefully removed, and pure water (Aqua B. Braun, Braun Melsungen, Germany) was added and stirred gently to suspend the cell pellet, and diluted further with additional pure water to a concentration of 5.5×10^8 CFU/ml to yield the final suspension of *E. coli*.

Agar plates were obtained by dissolving Luria agar (Sigma-Aldrich, Munich, Germany) in distilled water at 40 g/l, boiling for 15 min at 121 °C in an autoclave (A-11, Kambič Laboratory Equipment, Slovenia), cooling to 55 °C and spreading the solution uniformly in round Petri dishes with an inner diameter of 86 mm (90 mm Sterilin dishes, Thermo Scientific, UK) at 10 ml per dish. After the agar solidified and agar plates cooled to 21 °C, the final suspension of *E. coli* was poured uniformly over the surface of the plates at 1 ml per plate. After 1 min the suspension that did not get absorbed into agar was removed with a pipette. The agar plates were then left uncovered for additional 10 min in the laminar, allowing the remaining suspension to evaporate. At this stage there was no fluid present on the agar that would let bacterial cells to float and migrate. The electrical conductivity of the agar prepared in this manner was measured with LCR meter (Agilent 4284A Precision LCR Meter, Agilent Technologies, USA) at 1 MHz frequency and amounted to 22.9 mS/cm.

2.2.2. Irreversible electroporation

For electric discharge application, an agar plate with bacterial cells prepared as described above was loaded into the exposure system and 10 electric discharges were applied. The current of each discharge had a peak value of ~100 A, zero-to-peak time of ~0.1 μs, and halving time of ~0.3 μs, with the arc length of ~15 mm (see Fig. 5b). The control agar plate was loaded into the exposure system in the same manner as the exposed plates, but no discharge was applied. All plates were then incubated for 18 h at 37 °C.

2.2.3. Assessing the range of irreversible electroporation

After 18 h of post-exposure incubation, the top view of the agar plates was photographed using an 8-Mpixel digital video camera (iPhone 5, Apple, USA). The region of irreversible electroporation was clearly detectable as the central area with almost no colonies formed (see Fig. 3).

2.3. System testing on mammalian cells

2.3.1. Cultivation of CHO cells

Chinese Hamster Ovary cells (CHO-K1; European Collection of Cell Cultures, Great Britain) were used as a model of mammalian cells, plated in round Petri dishes with an inner diameter of 53 mm (60 mm TPP tissue culture dishes, Trasadingen, Switzerland) at 1.5×10^5 cells/ml. The culture medium consisted of F-12 HAM (Dulbecco's modification of Eagle's Minimum Essential Medium; Sigma-Aldrich Chemie, Deisenhofen, Germany) supplemented with 10% fetal bovine serum and 0.15 mg/ml L-glutamine (both Sigma-Aldrich Chemie, Deisenhofen, Germany). The electrical conductivity of the culture medium was measured with LCR meter (Agilent 4284A Precision LCR Meter, Agilent Technologies, USA) at 1 MHz frequency and amounted to 14.9 mS/cm.

Before exposure to electric discharges, the Petri dishes were kept in an incubator (I-CO2-235, Kambič Laboratory Equipment, Slovenia) at 37 °C and 5% CO_2 for 24 h, yielding approximately 4×10^5 cells per dish in exponential growth phase at the time of exposure.

2.3.2. Gene electrotransfer

Before electric discharge application, the culture medium was removed from the Petri dishes and replaced in each dish by 1.5 ml of fresh culture medium containing 4 μg/ml of pEGFP-N1 plasmid DNA (Clontech, USA; 4649 base pairs), which expresses green fluorescent protein (GFP, excitation 488 nm, emission 520 nm). After 2–3 min of incubation at 21 °C, a Petri dish was loaded into our exposure system and 10 electric discharges were applied. The current of each discharge had a peak value of ~14 A, zero-to-peak time of ~0.5 μs, and halving time of ~1.5 μs, with the arc length of ~7 mm. The control dish was loaded into the exposure system in the same manner as the exposed dishes, but no discharge was applied. After the exposure, the Petri dishes were incubated at 37 °C and 5% CO_2 for 5 min, then 3.5 ml of culture medium was added per dish, and all dishes were incubated for 24 h at 37 °C and 5% CO_2.

2.3.3. Assessing the fraction of electrotransfected cells

After 24 h of post-exposure incubation, the expression of GFP was assessed by observing the cells under an inverted fluorescence microscope (Axiovert 200, Zeiss, Germany) at 100× magnification. The fluorescence images were taken along a band 46 mm long and 0.7 mm wide passing through the center of the Petri dish, yielding 75 contiguous microphotograph frames. The number of transfected cells (those expressing GFP) per frame was estimated by counting the cells emitting clearly detectable fluorescence at 520 nm (see Fig. 4).

3. Results and discussion

3.1. System development

3.1.1. Emitting electrode design

The emitting electrode was shaped conically, with the cone tip pointing vertically downward. This provides a precisely defined position of the origin of electric discharge with respect to the sample positioned below, and a controlled distance from the cone tip to the sample surface, with the electric arc formed in the air separating them.

Our first prototype emitting electrode was made of copper, but the experiments showed that discharges delivered from this electrode caused its oxidation. This effect was progressive and most pronounced at the very tip of the cone (Fig. 1a–b), with the resulting layer of copper oxide gradually reducing the electrical conductivity of the cone, thereby affecting the peak current and the typical shape of discharges generated in an otherwise fixed setup. The initial properties of the emitting electrode were restorable by removing the oxide layer with a fine sandpaper, but due to the unpredictability and poor reproducibility of arcs generated using this electrode we abandoned it.

The final emitting electrode was therefore made of stainless steel. While copper has a higher electrical conductivity (5.9×10^5 S/cm at 20 °C) than stainless steel (1.4×10^4 S/cm at 20 °C), the resistivity of an electrode made from either material is very low compared to that of aqueous solutions (physiological saline has an electrical conductivity

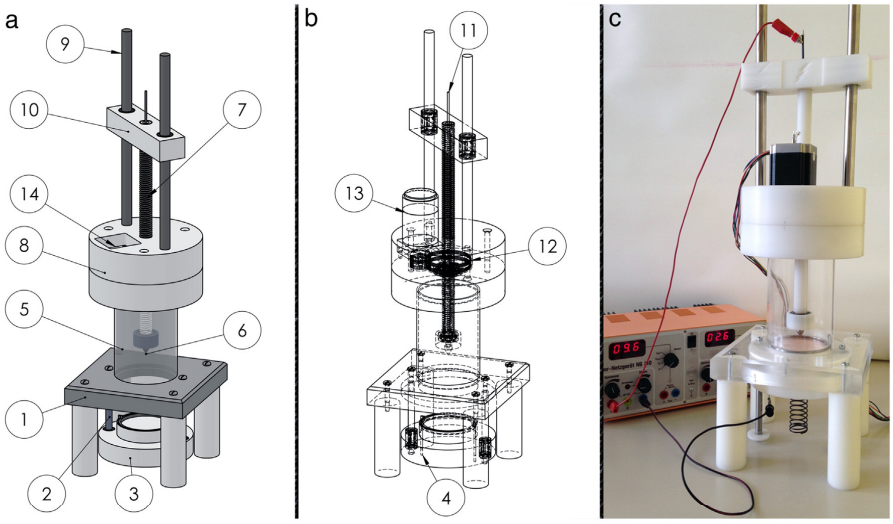

Fig. 2. The exposure system. (a–b) Major components in solid (a) and wireframe (b) representation: (1) base, (2) dock guide, (3) sample loading dock, (4) receiving electrode connector, (5) transparent tube container, (6) emitting electrode tip, (7) emitting electrode encasement, (8) core, (9) emitting electrode guide, (10) upper stabilizer, (11) emitting electrode connector, (12) central cogwheel, (13) stepper motor with its cogwheel, (14) stepper motor slot. (c) The actual prototype with a Petri dish in the loading dock locked in its upper position, a stepper motor in the core, and connected to a voltage generator.

of 12 mS/cm at 20 °C), and negligible compared to the resistivity of air. Thus, a change of emitting electrode material from copper to stainless steel had no detectable effect on the peak and time course of the electric current generated by the same arc-forming circuit. On the other hand, the stainless-steel emitting electrode proved much more resistant to discharge-induced corrosion, with thousands of reproducible consecutive discharges. In addition, stainless steel electrodes are also much more resistant to water-induced corrosion than copper electrodes, so they can also be washed with detergents if required.

3.1.2. Receiving electrode design

The receiving electrode was designed as to be in direct contact with a sample. Therefore, stainless steel was chosen as the construction material, as it is resistant to corrosion and dissolution in aqueous environments, nontoxic for biological samples, and easy to clean. To ensure a radial dissipation of the electric current from its point of entry into the sample, the receiving electrode was shaped either as a ring fitting inside the inner radius of a dish, ensuring two-dimensional current dissipation (Fig. 1c–d), or as a bucket shaped as a hemispherical shell, ensuring three-dimensional dissipation (Fig. 1e). In the former case, the dish is filled with the biological sample, while in the latter, the bucket electrode itself serves as a container.

3.1.3. Exposure system design

With the overall design of the exposure system, we tried to meet the following requirements:

- easy insertion and removal of a sample,
- protection of the sample from airborne particles (dust, microorganisms, etc.),
- observability of the sample during the experiment,
- easy connection of the discharge generator,
- easy and accurate adjustment of the emitting electrode vertical position,
- reliable electrical insulation where required.

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The system consists of fourteen major components as shown in Fig. 2. The relatively broad and heavy base provides a low center of system's mass, ensuring its stability. Attached to the base via two guides is the sample loading dock with the receiving electrode. The loading dock is moved vertically along the guides, with its lower position (Fig. 2a–b) allowing for easy sample loading as well as unloading, and its upper position (Fig. 2c) magnetically locked, so that the dock with the loaded sample forms the bottom of the hollow and transparent tube container. The conical tip of the emitting electrode approaches the sample from above within the tube container, with the cylindrical body of the electrode and its electrically insulating encasement protruding vertically upwards through the core. The electrode body has a 1.2-mm radius, while its encasement consists, concentrically outwards, of a 0.3-mm layer of polyvinyl chloride and a 6-mm layer of polyethylene, which prevents dielectric breakdown for delivered voltages up to at least 120 kV (even if such a voltage was sustained indefinitely).

Above the core, the electrode body and its encasement run in parallel with two guides, proceeding to and through the upper stabilizer, above which the emitting electrode has its connector. The upper stabilizer slides along the guides, while the electrode encasement is fixed to the stabilizer. The encasement is threaded, and the base contains a central cogwheel with a matching inner threading, so that the cogwheel's rotation causes vertical movement of the encasement and the electrode inside it. For automated adjustment of the vertical position of the emitting electrode tip, the central cogwheel can also be rotated by a stepper motor with a suitable cogwheel transmission, and the core provides a slot for such a motor.

In our system, the non-transparent electrically insulating parts (base legs, loading dock, core, emitting electrode encasement, upper stabilizer) were made of polyethylene plastic, and the transparent parts (base platform, tube container) of plexi-glass. The electrodes and the guides were made of stainless steel.

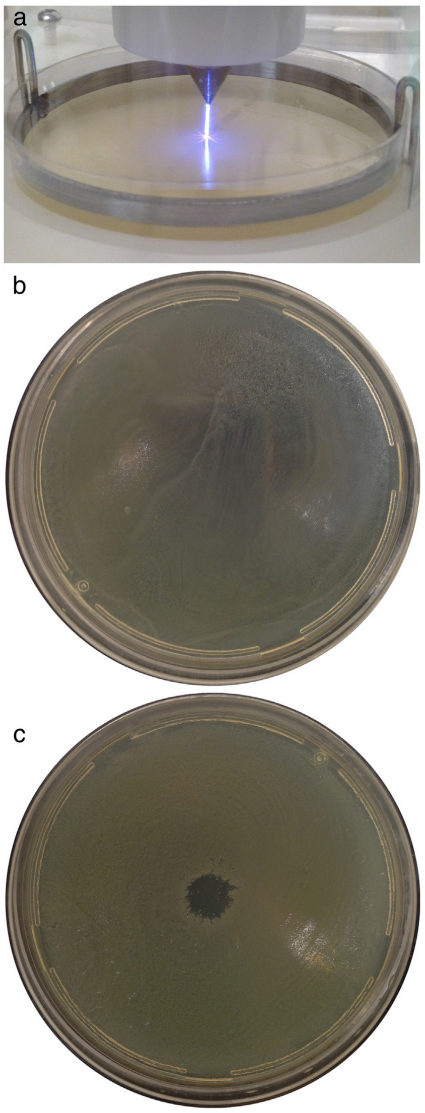

Fig. 3. Irreversible electroporation of *E. coli* with ten discharges of approx. 100 A peak current (for other parameter values, see main text). (a) The exposure. (b) The control plate (loaded into the system, but no discharge applied). (c) The exposed plate. The transparent areas correspond to regions with absence of *E. coli* colonies.

3.2. System testing

To test our exposure system, we performed two experiments. In the first experiment, we assessed irreversible electroporation of *E. coli* bacteria, while in the second experiment, we evaluated the fraction of electrotransfected CHO cells.

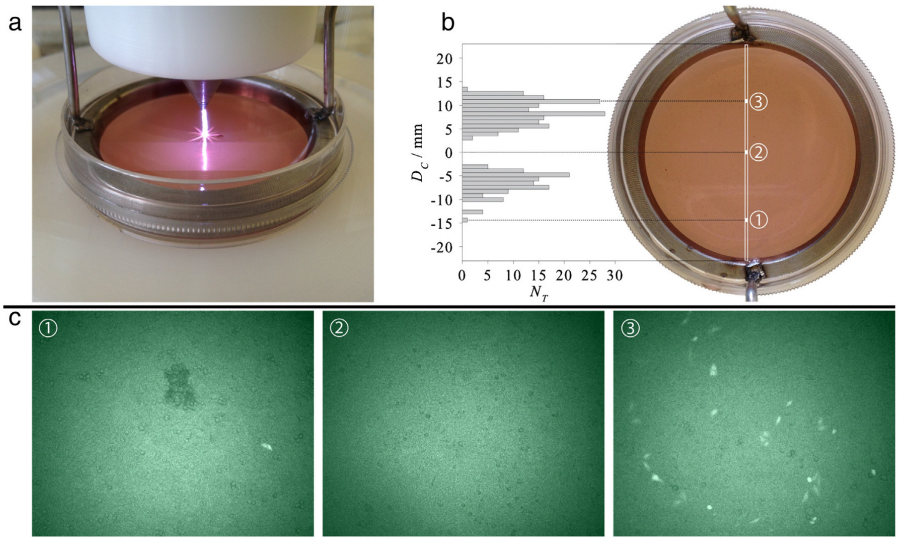

Fig. 4. Gene electrotransfer into CHO cells with ten discharges of approx. 14 A peak current (for other parameter values, see main text). (a) The exposure. (b) The exposed dish and the number of transfected cells per frame (N_T) as a function of the distance from the center of the Petri dish (D_C), counted along a band 46 mm long and 0.7 mm wide passing through the center of the dish. (c) Three microphotograph frames of the fluorescence emitted at 520 nm, indicating the transfected cells (location of these frames within the band is marked in the top right view of the dish).

3.2.1. Irreversible electroporation of bacteria

Fig. 3 shows the effect of ten discharges with a peak current value of 97(±8) A, zero to peak time of 0.11(±0.01) μs, peak to halving time of 0.17(±0.03) μs, arc length of 15.0 mm, and 29(±2) ms delay between consecutive discharges, on the colony forming ability of *E. coli* bacteria in a 90 mm agar plate. The receiving ring electrode had an outer diameter of 86 mm (fitting closely to the inner side of the plate wall) and an inner diameter of 82 mm.

As the figure shows, this exposure resulted in a central area of approximately 5 mm radius almost devoid of *E. coli* colonies. For a radial dissipation of electric current $I = 100$ A through a planar agar layer with thickness $d = 1.72$ mm (10 ml of agar spread across a disk with an 86 mm diameter), the electric current density at a distance $r = 5$ mm from the center can be estimated as

$$J \approx I/\langle 2\pi r d\rangle \approx 185\,\mathrm{A/cm^2}, \quad (1)$$

and the electric field it induces in a medium with electrical conductivity $\sigma = 22.9$ mS/cm (see Section 2.2.1) is

$$E = J/\sigma \approx 8.08\,\mathrm{kV/cm}. \quad (2)$$

This is in relatively good agreement with reports in the literature specifying field strengths in the ranges 6–10 kV/cm and 9–12 kV/cm as yielding 50% and 75% loss of viability, respectively, in electroporated *E. coli* [37,38]. It should be noted, however, that these literature data were reported for pulses with considerably longer durations (several ms) than the discharge in our setup, as they were all optimized for gene electrotransfer (see Section 3.2.2).

3.2.2. Gene electrotransfer into mammalian cells

Fig. 4 shows the effect of ten discharges with a peak current value of 14(±1) A, zero-to-peak time of 0.48(±0.05) μs, peak-to-halving time of 0.97(±0.06) μs, arc length of 7.0 mm, and 10(±1) ms delay between consecutive discharges, on gene transfer into CHO cells in a 60 mm culture dish. The receiving ring electrode had an outer diameter of 52 mm (fitting closely to the inner side of the dish) and an inner diameter of 46 mm.

As the figure shows, gene transfer with subsequent expression occurred at distances between approximately 3 and 15 mm from the center of the dish, while closer to the center and farther from it, no expression was detectable. From this experiment alone it is not possible to determine whether the lack of expression reflects the loss of cell viability or absence of gene transfer, but the results discussed in the preceding subsection and shown in Fig. 3 strongly suggest that in the central area the cells are irreversibly electroporated. This is also corroborated by the estimated electric field induced by radial dissipation of 14 A current through a culture medium layer with 0.90 mm thickness (1.5 ml of medium spread across a disk with a 46 mm diameter) and 14.9 mS/cm electrical conductivity, which – using the same estimation method as in the preceding section – amounts to 1.11 kV/cm and 5.54 kV/cm at 15 mm and 3 mm from the dish center, respectively. These are both roughly by a factor of 3 higher than the lowest field strengths yielding detectable gene electrotransfer and irreversible electroporation of CHO cells as obtained with pulse durations of several hundred μs to several ms – i.e., by two to three orders of magnitude longer than the discharge in our setup – that are routinely used for gene electrotransfer [39,40], for which the long "tail" of the pulse increases the efficiency due to its electrophoretic action on the DNA [41,42].

3.3. Operating considerations

3.3.1. Safety

From the safety point of view, the most important aspect is to avoid the possibility of uncontrolled discharge — either into the system operator, or other personnel or equipment nearby. For this, the discharge generator has to be disabled during its connection and disconnection to the system, as well as during the loading and unloading of the sample. Before the discharge generator is enabled, the operator has to ensure that it is connected to the system stably and reliably. In the prototype shown in Fig. 2c, the electrode connectors

are exposed, but for additional safety, connectors fully insulated from the outside can be used, yielding complete external insulation at the contact between a wire and an electrode connector.

After deciding on a generator with a given peak voltage output, wires for connection of the generator to the system have to be chosen so that their insulation is able to withstand this peak voltage with a substantial safety margin. Of the system components, the most susceptible to dielectric breakdown is the exposed part of the emitting electrode body; as explained in Section 3.1.3., it is able to withstand peak voltages at least up to 120 kV, and if the generator is capable of delivering higher voltages, the thickness of the electrode's insulation must be increased accordingly.

3.3.2. Choice of discharge generator

There are a number of power-electronic circuits able to generate an electric arc across at least several centimeters of air, the most widely commercially available being those of the self-defense electroshock weapons (tasers). Such generators allow to avoid direct contact of one electrode with the sample and deliver the current through an actual electric arc across the air separating this electrode from the sample. Such discharges also generate a distinct acoustic component, reflecting a pressure wave that accompanies the discharge, downscaled but of the same physical nature as with lightning strokes, potentially supplementing electroporation with sonoporation, and distinguishing such discharges from pulses delivered through direct contact between the electrodes and the exposed sample.

If the purpose of the setup is to study the effects of natural lightning strokes, some extent of downscaling is unavoidable, as the peak current of the most common negative strokes is ~30 kA [43]. Nonetheless, as the current of a stroke dissipates roughly radially downward and outward from its point of entry into the solid or aqueous environment, the current density and the electric field it induces are roughly inversely proportional to the square of the distance from this point. As a consequence, the conditions achieved by a stroke current with time course $I(t)$ in a given medium at 5 cm from its point of entry would also be achieved in the setup described in this article by a generator current with a time course $I(t)/100$ at 0.5 cm from its point of entry if the receiving electrode is a hemispherical bucket filled with the same medium, or at 2.5 cm if it is a ring encircling the medium 0.1 mm thick.

While the very highest current densities of lightning strokes and induced electric fields attained by lightning strokes but not attainable with downscaled exposure systems do not occur in downscaled exposure systems, those extreme conditions damage all living matter irreversibly and lethally. Moreover, they do this through rather elementary and well-understood physical and physico-chemical mechanims — heating, electrical breakdown, boiling, oxidation, etc.

Still, with electrostatic discharges triggered by dielectric breakdown, the time course of the electric current is predetermined by physics. As an example, a typical time course of the current in the most common, first negative lightning stroke (Fig. 5a), can be compared to the time course of the current in our experiment described in Section 3.2.1 (Fig. 5b). With respect to a lightning stroke (an electrostatic discharge between a cloud and the ground), the discharge generated by our setup is not only downscaled in its dimensions, current, and time, but also differs in shape — the current of a lightning stroke has a much longer falltime with respect to its risetime, and its overall time course is somewhat less regular.

The less regular time course of the current in a lightning stroke is understandable, as it propagates in stages and through different layers of air, while the longer falltime of the current in strokes largely reflects the fact that the polarization between a cloud and the ground resembles a parallel plate capacitor. In such capacitors, the charges are distributed over the plates, and upon formation of a conductive arc in the air separating them, charges first flow along the plates before entering the arc. In contrast, in our setup the charge on the emitting electrode is highly focused in its conical tip; the arc location is thus well defined and

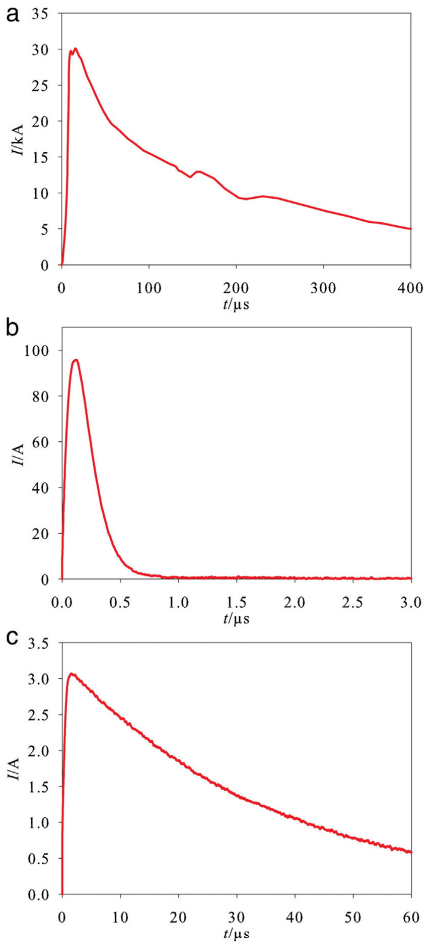

Fig. 5. Time courses of electric current, $I(t)$, from different discharge sources. (a) A natural negative first lightning stroke discharged into the ground, with the median peak current and zero-to-peak risetime as given in Table 1 of Ref. [43], and with the overall time course taken from Fig. 12 of Ref. [45]. (b) Taser gun discharged into an agar plate (details in Section 3.2.1) through a 15 mm arc. (c) A 0.25 μF capacitor discharged into an agar plate, with the emitting electrode in direct contact with the agar. Note that each of the panels features different current and time scales.

controlled, which is essential for reproducible experiments, yet reduces the time required for a complete discharge through the arc.

These differences between the time courses of the current in a stroke and its experimental model could in principle be reduced by replacing the conical emitting electrode in the experimental system by a flat one; together with the flat surface of the sample, such a setup would resemble a parallel plate capacitor. But as in all such capacitors, arcs of the discharges would then form in unpredictably varying locations, making controllable and reproducible experiments very difficult. An alternative is to avoid the arc, applying the discharge by bringing a charged parallel plate capacitor into direct electric contact with the sample. Fig. 5c shows the time course of the current generated as a 0.25 μF capacitor charged to 400 V was discharged through an agar plate as in Fig. 5b, but with the emitting electrode's tip brought into contact with the center of agar surface, and with the precharged capacitor's contacts then connected to the electrodes of the exposure system via a rapid semiconductor switch

(switching time below 0.1 μs), so that the charge did not accumulate in the electrode tip, but only passed through it. In such a setup, the time course of the current is more similar to that of a lightning stroke, with the falltime much longer than the risetime, but at the expense of further significant reduction in the magnitude of the current, which is the consequence of the limitations of a capacitor's design and maximal charging voltage it can sustain.

If one wanted to simulate also the irregularities in the typical time course of the current in strokes, the data describing such a course, suitably downscaled in magnitude, can be uploaded into a programmable function generator, its output signal then amplified by a suitable current amplifier [44] and injected into the sample similarly to the capacitor discharge described in the paragraph above. This same principle can also be applied to simulate any arbitrary current course of interest.

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Generator and Setup for Emulating Exposures of Biological Samples to Lightning Strokes

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Generator and Setup for Emulating Exposures of Biological Samples to Lightning Strokes

Matej Reberšek, Igor Marjanovič, Samo Beguš, Flavien Pillet, Marie-Pierre Rols, Damijan Miklavčič, and Tadej Kotnik*

***Abstract* — Emulation of lightning strokes is widely used in assessment of effects strokes have on devices, but not on living organisms. We developed a setup for controlled exposure of small biological samples to conditions they experience when lightnings strike their habitats. We designed a generator delivering capacitor discharges with zero-to-peak and peak-to-half times similar to natural strokes (~5 and ~75 µs, respectively) and similarly accompanied by acoustic shock waves. We tested the generator with our exposure chamber described previously, where conical emitting electrode delivers the current across an air gap centrally into a disk-shaped sample enclosed by a ring-shaped receiving electrode for radial current dissipation through the sample. Short discharges (<1 µs) proceeded in this manner, while longer discharges tended to short-circuit the electrodes, proceeding entirely through plasmified air above the sample. Recording at 341 thousand frames per second with Vision Research Phantom® v2010, the fastest commercially available camera, we found that also in these cases the initial arc descended vertically into the sample, but then became accompanied by arcs descending increasingly sideways; after 8–12 µs, as the first of these arcs formed direct contact with the receiving electrode, it transformed into a channel of plasmified air, short-circuiting the electrodes. We eliminated this artefact by a Plexiglas® cylinder positioned concentrically between the electrodes and reaching downwards to the sample, thus precluding a short-circuiting between the electrodes. While bacterial spores are highly resistant to electric pulses delivered through direct contact, we show that with discharges delivered as arcs through an air gap and thus accompanied by acoustic shock wave, spore inactivation is readily obtained.**

***Index Terms*—Electroporation, lightning, exposure system, electrotransformation, horizontal gene transfer, evolution**

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I. Introduction

NATURAL lightning strokes are unpredictable, uncontrollable, and impossible to ignore: they turn sand into glassy mineral tubes (fulgurites), damage electrical equipment, and harm living organisms.

A number of emulators and standards have been developed for testing the robustness of devices that can get exposed to lightning strokes in their operating environment [1]–[3]. Realistic testing of such robustness is particularly important in aircraft industry, and modern lightning emulators designed for such testing can reproduce the electric currents of natural lightning strokes, with peak amplitudes of up to 200 kA, and with waveforms emulating those in lightning strokes: zero-to-peak time of several µs and decay time in tens to hundreds of µs [4], [5]. This allows to emulate the effect of the stroke current proceeding in its entirety through the device, which has to withstand this without any loss of functionality.

While lightnings also strike natural habitats of many living organisms, scientific interest in studying the effects of such exposures has until recently been scarce. A likely contributor to this was the prevailing view that a stroke can only harm the organisms, and the mechanisms of inflicted damage – thermal (degradation of biomolecules), electrical (electroporation of cells' membranes and resulting leakage of intracellular material), and mechanical (pressure shock due to the strong acoustic waves accompanying a stroke) – are generally rather well understood.

Cell membrane electroporation caused by high-voltage, short-duration electric pulses can be irreversible, killing the cells, but it can also be reversible, with the exposed cells remaining viable, which is increasingly exploited to introduce into living cells various active substances that cannot permeate an intact membrane, particularly anti-cancer drugs and genetic material [6], [7]. Taking the latter fact into account, there were several studies focusing on – or at least touching upon – the possibility of electroporation-based gene transfer triggered naturally by lightning strokes [8]–[10]. Still, the exposures used in these experiments were produced by electric pulse generators in which neither the current's peak nor its time course parameters (zero-to-peak time, peak-to-half time) were similar to those of lightning strokes. Moreover, the current was always delivered through electrodes in direct contact with the sample, thus lacking the acoustic shock wave characteristic of strokes and more generally of electric arcs.

In the last two decades, sequencing of genomes gradually started revealing that biological evolution, particularly in

unicellular organisms, is influenced significantly by horizontal gene transfer (HGT) – uptake of genetic material from their environment or other organisms, and its integration into the new host's genome [11]. The well-recognized biochemical mechanism of uptake from the environment is termed *natural competence* (or *natural transformation*), and it was recently demonstrated that this mechanism functions even with highly fragmented and damaged DNA [12]. Still, as natural competence is based on a complex system of proteins [13], it must itself have developed during a certain stage of evolution, and how DNA uptake from the environment could have proceeded before this is an open question.

Furthermore, natural competence is found in some bacteria and in rare archaea [14], but there are no known naturally competent eukaryotes (organisms whose cells' genetic material is contained in a nucleus), yet during the last five years the scientists are increasingly acknowledging the evolutionary importance of HGT also in eukaryotes [15]. The currently recognized mechanisms of HGT in eukaryotes are gene transfer from their intracellular symbionts [16] and from infecting viruses [17], but it has been demonstrated that the most important genes transferred into eukaryotes by HGT stem from archaea [18], and whether these can all be attributed to transfer from endosymbionts (which are overwhelmingly bacteria, not archaea) or viruses is highly questionable.

We have recently posited that a possible answer to both early-life and modern gene transfer into organisms lacking natural competence lies in physical (as opposed to biochemical) mechanisms of DNA release and uptake, which could have functioned from the very beginning of cell-based life, and which also function in eukaryotes. We've argued that a particularly promising such mechanism, with both theoretical and indirect empirical support, is *lightning-triggered HGT* – DNA transfer enabled by lighting-induced cell membrane electroporation, and possibly augmented by lightning-driven electrophoretic motion of DNA [19].

For realistic experimental studies of the feasibility of lightning-triggered HGT, the conditions have to be as close to natural ones as possible. Organisms exposed should be chosen among those living in habitats accessible to lightning strokes, the lab exposure environment should emulate that habitat (e.g., marine bacteria should be exposed in a medium resembling seawater), and only natural DNA should be used (in biotechnological applications, DNA molecules are often artificially modified to increase their stability and transferability). At least as important is a realistic emulation of the lightning stroke; the current should be delivered through an actual electric arc proceeding from the emitting electrode through air into the sample, and the time course of the current should resemble that of a typical lightning stroke.

With tests of aircraft robustness, where the whole lightning stroke current can proceed through one of its devices, both the current amplitude and its waveform delivered in testing have to equal those of actual strokes. Partly in contrast, in exposure systems designed for studying lightning-triggered HGT, the electric current waveform should also equal that of a stroke with respect to the time scale (i.e., it should have similar zero-to-peak and decay times), yet its amplitude can be downscaled without any essential loss of emulation consistency. Namely, upon its entry from the air into the ground, the lightning stroke's current always dissipates, and thus a downscaling of current amplitude in exposure systems merely reduces the size of concentric areas subjected to various ranges of current density and resulting effects on exposed sample. Obviously, the very highest current densities of lightning strokes are absent in downscaled exposure systems, but those are lethal to all living organisms, while the conditions of reversible electroporation, and thus favourable for HGT, are reached even with downscaling of the stroke current by a factor of 10^4 [20].

We have previously reported on our design of an exposure system that provides easy sample insertion and removal, protection from airborne particles, observability during the exposure, accurate discharge positioning and delivery into the sample through an arc with an adjustable air gap [21]. In its initial testing, we connected the system to a taser gun, confirming that for discharges lasting ~0.5 μs, the conical emitting electrode delivers the current across an air gap centrally downwards into a disk-shaped sample, and that the ring-shaped receiving electrode ensures radial current dissipation through the sample [21]. Here, we describe our design and testing of a generator delivering discharges similar to those of natural strokes both in its duration and its waveform of the electric current (zero-to-peak time of ~5 μs, peak-to-half time of ~75 μs), and also similarly accompanied by an acoustic shock wave.

We first describe the design of the generator circuit for delivery of arc discharges. We then show how with the exposure system outlined above, a transition from short discharges (up to several μs) to those with durations characteristic of lightning strokes (tens or hundreds of μs) alters the current flow radically, with eventual short-circuiting between the electrodes proceeding through the air plasmified by the discharge current. We present the details of this artefact through photographs taken at 341 thousand frames per second with Vision Research Phantom® v2010, the fastest currently commercially available camera, and we describe a modification of the exposure system that eliminates this artefact. We tested our generator and setup by exposing the spores of *Bacillus pumilus* to the discharges, attaining consistent and reproducible irreversible electroporation of spores; in contrast, no such effect was obtained with classical-type exposure in which the discharges were delivered through electrodes in direct contact with the sample, so that an arc and the accompanying acoustic shock wave were absent.

II. Materials And Methods

A. Generator Design and Functioning

The schematic diagram of the pulse generator we developed is shown in Fig. 1. We opted for a capacitor discharge circuit [22] as the waveform of its output current is very similar to those of lightning strokes [20]. For safety reasons and to minimize leakage current, we isolated the output of the generator from the grid voltage by an isolation transformer

(T; 18 V, 60 VA, ELMA, Slovenia). A high-voltage direct current converter (HV DC-DC; RB60-6P, Matsusada, Japan) was used to supply the output stage of the generator. The output voltage of the converter was set by a potentiometer (P) in the range from 0 to 5 kV, and monitored by a voltmeter (V). We used resistors with a high impedance (R) to separate the converter from the output. The capacitor (C; MOP105-5MN, Condenser Products, USA) stored the energy for the pulse during the charging phase, and the high-voltage relay (S; RL 42, SPS electronic, Germany) released this energy into the load during discharge phase. As the impedance of the exposure system was approximately 100 Ω, the 1 μF capacitance of the capacitor C was chosen, so that its discharge generated an arc current with a time constant of exponential decay of ~100 μs, similar to those of natural lightning strokes.

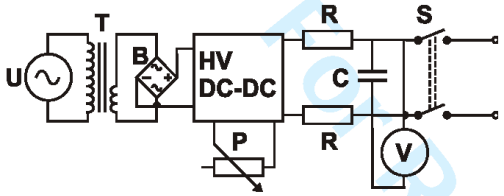

Fig. 1 Schematic diagram of the pulse generator for lightning emulation. U: grid power supply. T: isolation transformer. B: bridge rectifier. HV DC-DC: high-voltage DC-DC converter. P: linear potentiometer. R: high-impedance resistors. C: capacitor (1μF, 5 kV). S: high-voltage relay. V: digital voltmeter.

During the first batch of our experiments, the discharge generator was connected to the exposure system as described previously [21]. Later, as we discovered that discharges longer than 8–12 μs gradually deviate from a vertical path into a diagonal one and short-circuit the electrodes (see Section III.A), we modified the setup by enclosing the emitting electrode with an inner concentric Plexiglas® cylinder reaching down to the sample surface, thus forcing the discharge current to flow through the sample and thus avoiding the short-circuiting.

Capacitor C was charged to 5 kV and discharged through the high-voltage relay S, proceeding from the conical emitting electrode vertically downwards into the sample, and hence horizontally radially through the sample into the ring-shaped receiving electrode, thus emulating the exposure of the sample to a lightning stroke.

B. Monitoring and Measurements

Evolution of the discharge arc was imaged by a high-speed camera (Phantom® v2010, Vision Research, USA) with fixed focal length lens (EF 50 mm f/1.8, Cannon, Japan), either with or without a welding protection glass (90x110 mm Shade 11, Technolit, Germany) placed between the arc and the camera lens. During the experiment, the image frames were being acquired continuously by the high-speed camera with 256×128 resolution and 12 bits per pixel at 341 thousand frames per second (2.93 μs per frame, consisting of 2.55 μs exposure time and 0.38 μs inter-frame delay). Acquisition was triggered by the oscilloscope as it detected the onset of the electric current, with the first frame marked as time zero (0 μs).

Time courses of voltage and electric current of the discharge were measured by a voltage probe, a current probe, and an oscilloscope (PPE6kV, AP015, and WavePro 7300A; all from LeCroy, USA) isolated from the grid voltage by an isolation transformer KDVP-23499 (Elma TT, Slovenia). In cases of short-circuiting between the electrodes, current was measured by CWT 150B Mini current probe (Powertek, UK).

Acoustic shock wave was measured with a sound pressure level (SPL) meter with an attenuator (2230 and ZF 0020; both from Brüel & Kjær, Denmark), and by a microphone with a preamplifier and an amplifier (4191, 2669, and Nexus; all from Brüel & Kjær, Denmark), and a sound card (E-MU 0404 USB, Creative, USA). Measurement system was calibrated with a pistophone (124 dB). The measurements were taken at 1 m distance from the exposure system, both with and without the outer Plexiglas® cylinder enclosing the exposure system, and both with and without the inner Plexiglas® cylinder used to prevent short-circuiting between the electrodes. The frequency spectrum of the acoustic shock wave was computed using the fast Fourier transform in MATLAB R2014 (MathWorks, Natick, USA).

C. Experiments

Sporulation of *Bacillus pumilus* (ATCC 27142) was performed for 5 days at 37°C in Difco Sporulation Medium (DSM) [23]. The residual vegetative bacteria were removed by heat shock (80°C for 20 min) and lysozyme digestion (50 μg/ml of lysozyme in 50 mM Tris-HCl at pH 6.2; for 1h at 37°C). The purification of spores was achieved by centrifugation (5 min, 10000 g), and cleaning in 0.05% SDS and then three times in deionized water. Spore concentration was evaluated by colony counting after incubation overnight at 37°C. The inactivation rate was evaluated by comparison between the number of colonies in the area of interest before and after exposure to electric arcs.

III. Results and Discussion

A. Design and Testing of the Generator and Setup

The most challenging choice we had to make in the design of the generator was that of the most appropriate output switch. The options considered were an insulated-gate bipolar transistor (IGBT), a spark gap, and a relay [24], [25]. All these options are commercially available for 5 kV as the maximum output voltage we opted for, but IGBTs are rather limited in their maximal current and their rise-times, prone to irreparable damage, and furthermore they are the most expensive of the considered options. We thus opted for a commercial relay, with the air gap between the emitting electrode and the sample in our exposure system forming the spark gap to which this relay delivers a discharge.

In our initial tests, we connected the generator shown in Fig. 1 to the exposure chamber that we have previously described and tested with short discharges (~0.5 μs) delivered by a taser gun [21]. For such short discharges, the arc of the discharge always descends roughly vertically from the tip of the conical emitting electrode into the sample, and then

dissipates through the sample radially towards the ring-shaped receiving electrode (see e.g. Figs. 3a and 4a in Ref. [21]).

In contrast, as shown in Fig. 2, when we delivered a discharge designed to decay exponentially with a time constant of ~100 µs, during the first ~0.5 µs the discharge and its arc behaved as predicted, with the electric current rising to ~50 A and voltage gradually decreasing from the initial ~5 kV, but then the current kept rising, first at a decreasing rate, yet after ~8 µs increasing very rapidly to almost 1400 A, thus almost instantaneously discharging the capacitor, followed by further ~35 µs of strong current and voltage oscillations, likely attributable to the inductance of the wires connected to the generator's rapidly discharging capacitor.

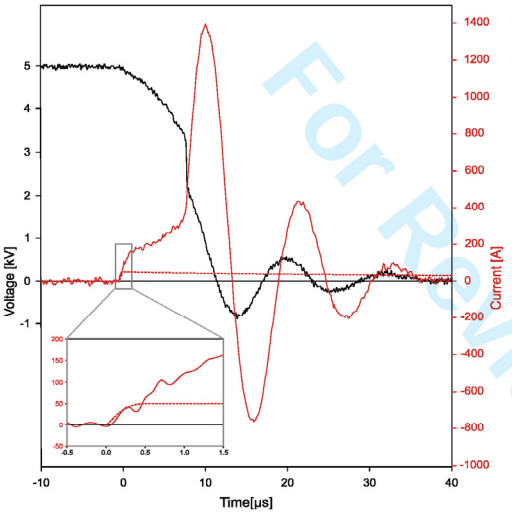

Fig. 2 Time course of voltage (black) and current (red) of the discharge delivered by the generator shown in Fig. 1 connected to our exposure chamber described and illustrated in detail in Ref. [21]. The dashed curve sketches the time course of the electric current expected of a discharge without short-circuiting between the electrodes (see Fig. 5).

Visually, we observed a very bright arc formed along the shortest path between the tip of the conical emitting electrode and the ring-shaped receiving electrode, proceeding entirely through plasmified air, thus short-circuiting the electrodes and evading the sample. Still, it was clear that due to the extreme brightness of this arc, the transient phenomenon preceding this short-circuiting was concealed from the naked eye. To investigate this transient phenomenon, we utilized the Vision Research Phantom® v2010, the fastest currently commercially available camera kindly provided by Vision Research Europe, and recorded the events at 341 thousand FPS (one frame per 2.93 µs), with the relevant frames shown in Fig. 3.

As Fig. 3 shows, the arc first descended vertically into the sample. Within ~3 µs, the vertical arc became accompanied by several radial arcs proceeding partly through the sample, but gradually ascending into the plasmified air above it. After ~9 µs (in this case, and generally 8–12 µs), a significant initial segment of the radial arcs was already above the sample, and

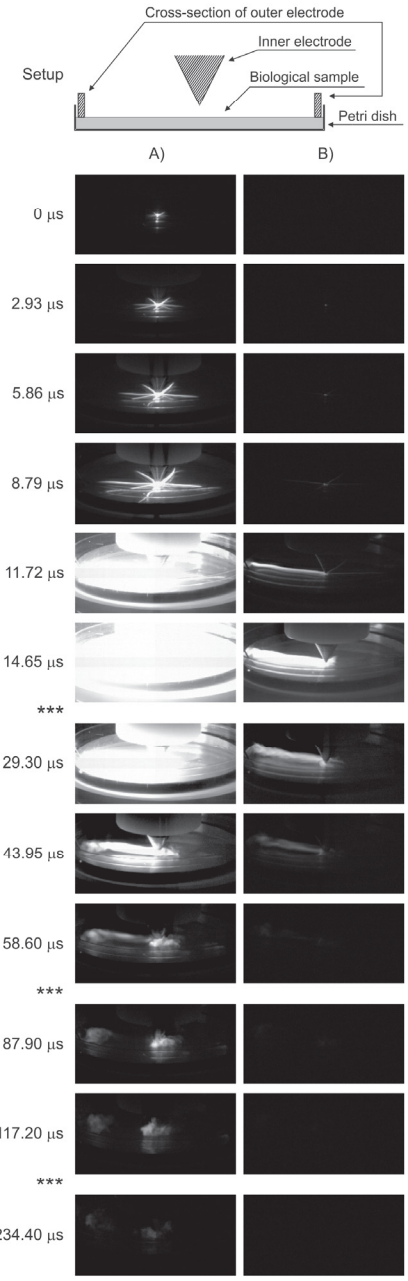

Fig. 3 Evolution of the discharge arc delivered by the generator shown in Fig. 1 connected to our exposure chamber described and illustrated in Ref. [21]; the frame timing is at the left, with the arc formation serving as the trigger. (A) frames taken without a filter; (B) frames taken through a welding protection glass. The three asterisks (***) indicate skipped frames.

their visible endpoints, still in the sample, were rapidly approaching the receiving electrode at sample's outer edge. As one of these arcs came into direct contact with the receiving electrode, it transformed into a channel of plasmified air short-circuiting the electrodes, drawing almost all current of the discharge through this channel and thus enhancing its plasmification, glowing so bright as to saturate the frames unless they were photographed through a welding protection glass (see the frames in Fig. 3 from 11.72 µs onwards). The frames at the bottom of the left row of Fig. 3 show that even after the electric current ceased, the plasmified air continued to glow visibly for further ~200 µs.

In natural lightning strokes, there is no equivalent to the discrete receiving electrode of our exposure system, so there is also no counterpart to the short-circuiting effect described and depicted above, which thus represents an artefact resulting from system's design. As long as the current dissipates roughly radially through the sample towards the receiving electrode, this is an adequate emulation of the stroke current's dissipation in the ground, but once the radial arcs emerge largely into the air above the sample, and particularly as one of these arcs short-circuits the metallic electrodes, such an "emulation" loses all resemblance to lightning strokes.

To eliminate this artefact, we modified the exposure system, incorporating an additional, inner Plexiglas® cylinder placed concentrically between the emitting electrode and the receiving electrode, so as to form a tight contact with the surface of the sample, thus precluding the discharge from evading the sample from above and short-circuiting the electrodes. We first tested this solution with a cylinder almost as wide as the sample (inner and outer radius of 35 mm and 40 mm, respectively, while the receiving electrode had an inner radius of 41 mm; see the top of Fig. 5). As Fig. 4 shows, this resulted in the discharge current evolving largely as initially envisaged, reaching the peak of ~50 A in ~5 µs and then decaying exponentially with a peak-to-half time of ~65 µs. As revealed by Fig. 5, the radial rays still evolved sideways, but more gradually and only until reaching the inner edge of the newly incorporated insulating cylinder.

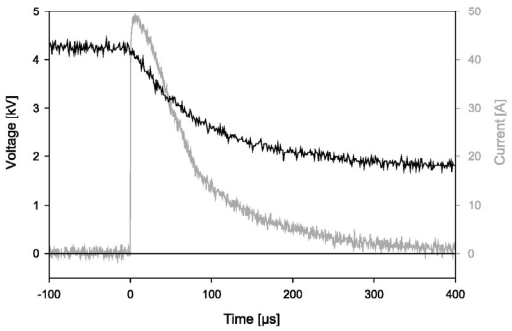

Fig. 4 Time course of current (black) and voltage (grey) of the discharge delivered by the generator shown in Fig. 1 connected to our exposure chamber modified by addition of an inner insulating cylinder preventing the short-circuiting and formation of a plasma channel between the electrodes.

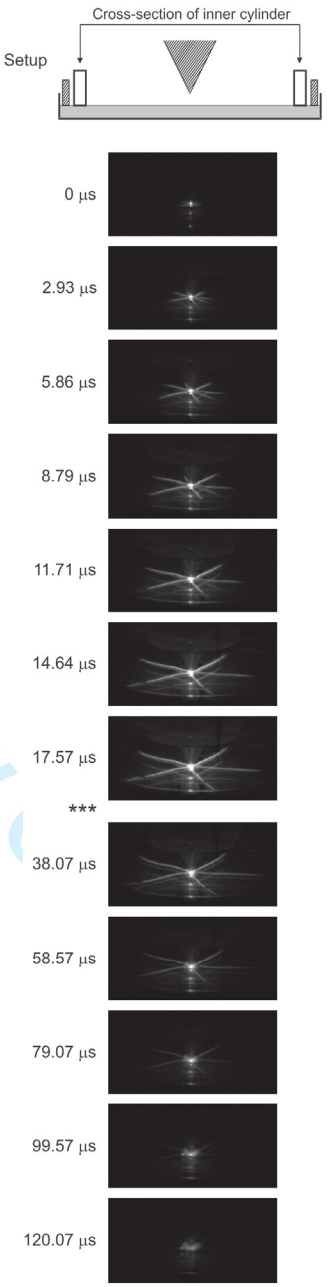

Fig. 5 Evolution of the discharge arc delivered and recorded as in Fig. 3A, but with an inner insulating cylinder preventing the short-circuiting and formation of a plasma channel between the electrodes. Frame timing is at the left, and three asterisks (***) indicate skipped frames.

Inside the cylinder, the rays still gradually emerged into the air above the sample, reaching their maximal luminosity after 15-25 μs and then gradually fading, with only a central glow persisting for further ~100 μs (in later experiments, we used narrower cylinders to force the discharge current to flow almost entirely through the sample see e.g. Fig. 8).

Here, we should address a possible concern regarding the considerable downscaling in the electric current of a discharge delivered by our exposure system (e.g., as shown in Fig. 4) with respect to lightning strokes; while their waveforms are very similar, the amplitudes differ by a factor of ~600 (peak current of ~50 A as opposed to ~30 kA). Yet both with the lightning stroke and with the arc discharge delivered by our system, once the electric current enters from the air into a conductive medium, it dissipates through this medium roughly radially away from its point of entry. Thus, in both cases, as the distance r from the point of discharge's entry increases, the current density J and the electric field E it induces decrease in a monotonic and continuous manner. As a consequence, the same values of J and E caused in a given medium by a lightning stroke's current at a given r_1 are also caused in this same medium by a discharge current downscaled in amplitude at some $r_2 > r_1$. For example, for a lightning stroke current of 30 kA dissipating three-dimensionally in the ground, $J = 50$ A/cm^2 is reached at $r = 9.77$ cm, while for a discharge with a peak current of 50 A dissipating two-dimensionally in a disk-shaped sample 5 mm thick, the same value of J (and, if the medium is the same, also the same value of E) is reached at $r = 3.18$ mm.

It is true that the very highest values of J and E caused by lightning strokes do not occur in downscaled exposure systems, yet under such extreme conditions, all living matter is damaged irreversibly and lethally, and through mechanisms of heating and/or electrical breakdown that are rather elementary and well understood.

These considerations show that the setup as described above necessarily with an inner cylinder installed as to prevent short-circuiting between the electrodes can emulate the electrical conditions of an exposure to a lightning stroke current quite adequately. Lightning strokes are accompanied by thunder, and the arc discharges delivered by our system were also accompanied by a loud sound, so we analysed the similarities of the acoustic shock waves generating them.

A high-pressure shock wave is formed in plasmified air during the lightning stroke, which travels faster than the speed of sound [26]. The shock wave expands roughly cylindrically away from typical plasma channels of lightning strokes, while for downscaled laboratory arc discharges the expansion is roughly spherical [27]. With expansion, the shock wave slows down, and upon reaching the speed of sound, it transforms into an acoustic wave (known as thunder in lightning strokes) with a characteristic frequency spectrum. The distance at which this occurs is referred to as the relaxation radius, r_R, of the shock wave, and for typical lightning strokes it is at ~40 cm, where the peak sound pressure is ~200 kPa (200 dB) [26]. At $r > r_R$, the acoustic wave of a stroke (the thunder) has a distinctive frequency spectrum with two most prominent acoustic frequencies at ~200 and ~800 Hz. Close to r_R, the two peaks are of similar magnitude, but the higher frequency is attenuated more rapidly, so that farther away the frequency at ~200 Hz prevails in the spectrum, and it characterizes the sound heard most dominantly in a typical thunder [28].

The arc discharge delivered by our setup is also consistently accompanied by a shock wave. To estimate its strength and relaxation radius, we measured its actual sound pressure (Fig. 6) and frequency spectrum (Fig. 7) with the outer Plexiglas® cylinder of our system removed, as it would attenuate the pressure and distort the spectrum. Measured at 1 m from the arc, the peak sound pressure was 255 Pa (142 dB) and the peak frequency ~3400 Hz when the short-circuiting plasma channel was formed (i.e., with also the inner Plexiglas® cylinder absent), and 63 Pa (130 dB) with peak frequency of ~1800 Hz when short-circuiting was prevented by adding the inner cylinder. Using the equations from [26] and [29], the relaxation radius of our arc discharge without short-circuiting is at ~15 cm, where the peak sound pressure is in the range between ~420 Pa (146 dB) and ~2.8 kPa (163 dB), with the lower bound corresponding to cylindrical, and the upper bound to spherical expansion of the shock wave.

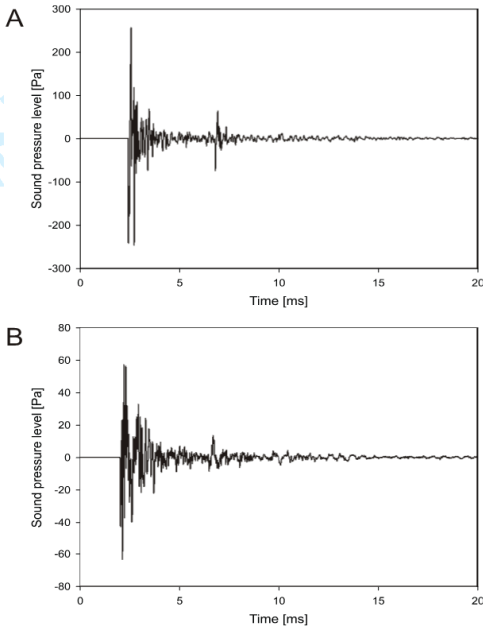

Fig. 6 The acoustic shock wave (time course of the sound pressure level) accompanying the discharge arc delivered by our setup, as measured 1 m from the arc, without the Plexiglas® cylinder enclosing the exposure system. A: without the inner Plexiglas® cylinder added to prevent short-circuiting between the electrodes; B: with this inner cylinder.

With the outer cylinder reinstalled, the sound pressure level at 1 m was attenuated to 23.8 Pa (121.5 dB) and 6.47 Pa (110.2 dB) with the inner cylinder absent and present, respectively.

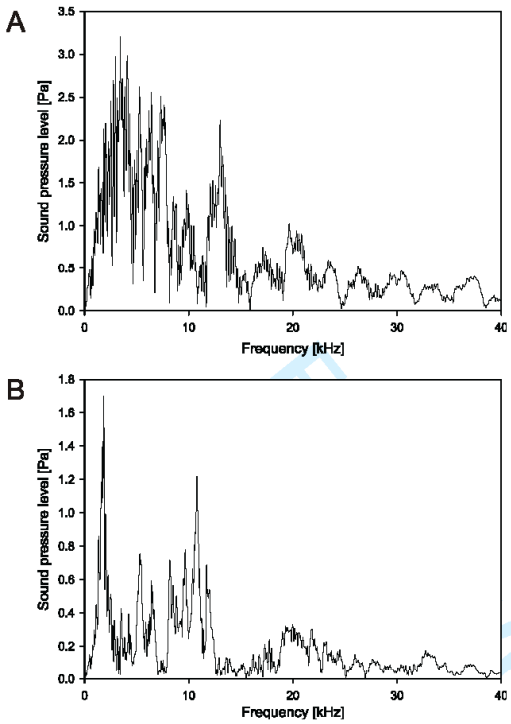

Fig. 7 The frequency spectrum of the acoustic shock wave shown in Fig. 6. A: without the inner Plexiglas® cylinder; B: with this inner cylinder.

B. *Application in Biological Experiments*

We tested the use of our generator and setup by delivering various discharges to a sample of *Bacillus pumilus* spores. Bacterial spores are known to be highly resistant to "classical" electroporation protocols in which the discharges are delivered through electrodes in direct contact with the sample, where the electric arc does not proceed through the air, and the accompanying acoustic shock wave is thus absent [30]. In Fig. 8, we demonstrate successful killing of these spores by discharges delivered into the spore-containing sample as as arcs through an air gap and thus accompanied by an acoustic shock wave. Panels B and C clearly illustrate the difference between the effect of pulses short enough (0.5 μs) for the discharge arc to remain vertically descending, and pulses long enough (20 μs) for the arc to start and complete its migration from the vertical path to the diagonal path between the electrodes, finally evading the sample. Comparison of panels D and E shows that consecutive discharges tend to form plasma channels in directions proximate to the direction of the first discharge, yet gradually scattering. Panel F demonstrates that spore killing is also significant when short-circuiting between the electrodes and thus plasmification of the air above the spores is prevented.

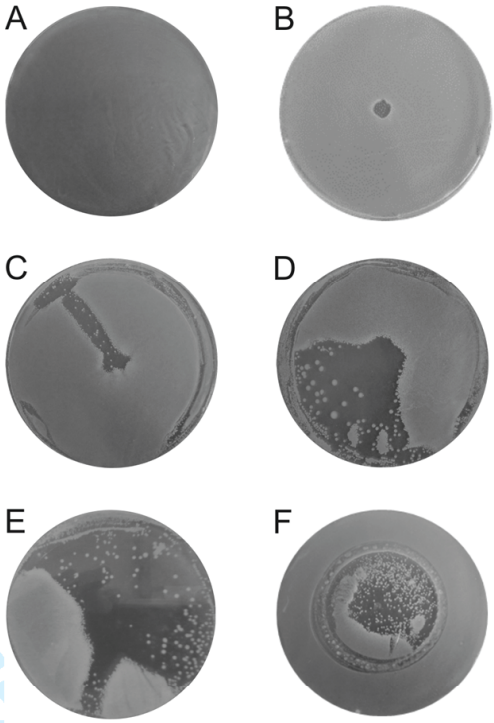

Fig. 8 Areas of *Bacillus pumilus* spore killing with various number, type and duration of arc discharges delivered with our setup. A: control. B: 200 discharges, 12 kV, 0.5 μs. C: 1 discharge, 5 kV, 20 μs. D: 20 discharges, 5 kV, 20 μs. E: 50 discharges, 5 kV, 20 μs. F: 50 discharges, 5 kV, 100 μs time constant, with an inner Plexiglas® cylinder (inner radius 22 mm, outer radius 26 mm) preventing a short-circuiting between the electrodes.

IV. Conclusion

We have developed and tested a discharge generator that can be used with the previously described exposure system to expose biological samples to controlled and reproducible arc discharges, thus allowing to study the effects of such discharges on living organisms. In particular, we have demonstrated that such arcs, in which the electric field is accompanied by an acoustic shock wave, can cause killing of the generally extremely resilient bacterial spores.

Moreover, as outlined in the Introduction, there is ample motivation for systematic studies of lightning-triggered horizontal gene transfer (HGT) among microorganisms, with potential implications for their evolution both early and modern. The tests of our generator and the exposure system described here, together with the calculated estimates presented, show that this setup is adequate for emulation of lightning strokes entering natural habitats, and should thus act as an essential piece of equipment for realistic experimental studies of the feasibility of lightning-triggered HGT.

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Abstract: In the context of spore contamination involved in bio-terrorism and food preservation, the development of new techniques for spore inactivation is an important challenge. Here, we report a successful application of electric arc discharges resulting in spore death. We compared two types of electric arcs, different with respect to their durations. The discharges with 0.5 µs duration induced a small inactivation area around their point of entry into the sample, while those with 20 µs duration induced a much larger inactivation area roughly proportional to the number of discharges delivered. In particular, 50 discharges of 20 µs duration induced inactivation in more than 55% of surface treated at an inactivation rate above 3.5 log10. These preliminary results are promising and warrant developing electric arcing as a novel method for spore inactivation.

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Cover Letter

Dear Editors,

We are hereby submitting what is to our knowledge the first report on successful application of electric arc discharges for inactivation of spores of *Bacillus pumilus*, a non-pathogen model of *Bacillus anthracis*. This inactivation was performed by delivering electric arc discharges through an air gap into the sample of spores deposited on agar in a petri dish. As we describe, with 50 electric arcs of 20 µs duration, 55% of the 90-mm petri dish area was subject to inactivation, at about 3.5 log10 inactivation rate.

We are sure these results are promising and warrant further investigation, perhaps leading to electric arcing being developed into an established method for spore inactivation. We outline the steps that will have to be made for this.

In the hope you will find the manuscript of sufficient quality and interest for publication in Bioelectrochemistry, we remain.

Sincerely Yours,

Marie-Pierre Rols and Tadej Kotnik, on behalf of all the authors

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Inactivation of spores by electric arcs

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Abstract

In the context of spore contamination involved in bio-terrorism and food preservation, the development of new techniques for spore inactivation is an important challenge. Here, we report a successful application of electric arc discharges resulting in spore death. We compared two types of electric arcs, different with respect to their durations. The discharges with 0.5 µs duration induced a small inactivation area around their point of entry into the sample, while those with 20 µs duration induced a much larger inactivation area roughly proportional to the number of discharges delivered. In particular, 50 discharges of 20 µs duration induced inactivation in more than 55% of surface treated at an inactivation rate above 3.5 log10. These preliminary results are promising and warrant developing electric arcing as a novel method for spore inactivation.

1. INTRODUCTION

Bacterial spores are one of the most resilient life forms known, exceptionally resistant to chemical, environmental and physical stresses [1]. Spores can survive in dormant phase in extreme conditions, even in space, [2] and can hence cause contamination during interplanetary missions [3]. Furthermore, spores can remain viable even during geological time spans; for instance, spores of *Bacillus subtilis* were recovered and revived from abdominal contents of extinct bees trapped in amber fossilized 25 to 40 million years ago [4]. Such resilience and endurance of bacterial spores is explained by a highly dehydrated structure of their core that includes their genomic material and ribosomes, and protection of this core by a multilayer envelope, consisting of a highly impermeable inner membrane, a temperature resistant peptidoglycan cortex, an outer membrane, and chemically resistant protein coat [5]. This resilience poses considerable obstacles in inactivation of pathogenic spores, which are related to large number of different diseases. Thus, bacterial spores can cause respiratory infection (e.g. *Bacillus anthracis* acting as the etiologic agent of anthrax [6]), food contamination (e.g. by *Clostridium botulinum* causing botulism [7]), and fatal paralytic illness (e.g. *Clostridium difficile* involved in infectious diarrhea [8]).

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Within this context of high noxiousness and resistance to extreme conditions, efficient methods for inactivation of pathogenic spores are of utmost importance. Sterilization by heating above 100°C is classically used and efficient for food preservation [9], but cannot be used for decontamination of thermally sensitive materials. Two alternatives are gamma irradiation [10] and exposure to ethylene oxide [11], which are efficient, but expensive and often also harmful to the matter being decontaminated.

Another potential approach is to expose the material to electric pulses. While this approach, based primarily on irreversible electroporation, is efficient in inactivation of many bacteria in their vegetative state, bacterial spores are known to be much more resistant to pulses delivered through electrodes in direct contact with the sample [12]. An alternative is to deliver the electric pulses through an air gap, thus generating an arc discharge; in this case, there are several effects acting simultaneously, as in addition to the electric field, the sample is exposed to ultraviolet light [13], as well as to an acoustic shockwave [14], in

which the brief surge of mechanical pressure can also cause cavitation [15]. This multiple-effect approach was first described in 1962 for inactivation of bacteria in water in their vegetative state [16], but it has apparently not yet been tested on their much more resilient spores.

Here, we describe for the first time a successful application of electric arc discharges resulting in inactivation of spores of *Bacillus pumilus*, a non-pathogen model of *Bacillus anthracis*. This inactivation was performed by delivering an arc discharge through an air gap into the sample of spores deposited on agar, with the exposure system developed and described previously by Marjanovič and Kotnik [17], yet with a more powerful discharge generator developed subsequently. We compared the spore inactivation caused by two types of electrical discharges: 0.5 µs and 20 µs electric arcs, of which particulatly the latter were accompanied by an intense light emission and acoustic shockwave. The best results were obtained with 20 µs electric arcs leading to a 55% of the sample area subject to inactivation, at about 3.5 log10 inactivation rate.

2. Materials And Methods

2.1 Preparation of spores

Spores of *Bacillus pumilus* (ATCC 27142) were produced after 5 days culture at 37°C in Difco sporulation medium (DSM), as previously described by Schaeffer *et al* [18]. The remaining vegetative bacteria were inactivated by pasteurization (80°C for 20 min) and lysed by 1h exposure at 37°C in a lysozyme solution (50 µg/ml of lysozyme in 50 mM Tris-HCl pH6.2). The spores were centrifuged (5 min, 10000 g) and washed 1 time in deionized water, 1 time in SDS 0.02% and 3 times in deionized water. For each experiment, 1 ml of spore solution containing about 2.10^7 spores/ml was added and spread in a petri dish with a 90 mm diameter (Sterilin plate, Thermo Scientific, UK). The overflow was removed (900 µl) and the petri dish was dried 15 min prior to experiment. The number of spores estimate was 2.10^6 by petri dish.

2.2 Exposure to electric arcs

The exposure system is shown schematically in Figure 1, and was previously described in detail by Marjanovič and Kotnik in 2013 [17]. Before exposure, the ring electrode was placed in contact at the periphery of the petri dish. Electric arcs were generated between the emitting tip electrode and the ring electrode. Two generators were used with different time of electric arc exposure: a Taser gun (Great Power 750000, Great Power Co., South Korea) delivering ~0.5 µs electric arcs of ~12 kV at ~100 A, and a custom-made generator built at the Faculty of Electrical Engineering, University of Ljubljana delivering 20 µs electric arcs of ~5 kV at ~50 A [19].

Here Figure 1

2.3 Quantification of inactivation

The spore density in control condition was evaluated by colony counting from successive dilutions of spores spread and incubated overnight at 37°C (Supplementary data1). First, the inactivation areas were measured with the software ImageJ 1.46r (National Institutes of Health, USA). Then, the inactivation rates in the areas exposed to electric arcs were calculated as the ratio between the spore density under control conditions and the spore density in the areas of exposure. The inactivation area and the inactivation rate were calculated from 3 independent experiments.

3. RESULTS

3.1 Inactivation of spores by 0.5 µs electric arcs

In Figure 2, we show the effects of delivering ~20 electric arc discharges, each with an ~0.5 µs duration, delivered in intervals of ~300 ms (the durations of the discharges and the intervals between them as delivered by the Taser gun, see Section 2.2, were neither adjustable nor highly reproducible, but the monitoring of the exposures on the oscilloscope assured that in no experiment their actual values differed from those stated above by more than 10%).

In these exposures, the arc always descended from the tip of the conical emitting electrode downwards and entered centrally into the sample, with the electric current then dissipating roughly radially outwards through the sample to the ring-shaped receiving electrode (for a photographic example, see e.g. Fig. 3 in Marjanovič and Kotnik [17]). In this manner, the current density and induced electric field decreased roughly inversely to the distance from the point of the arc entry into the sample. As a consequence, the area of spore inactivation caused by the exposure was roughly centered at the petri dish midpoint, and it was rather small, as only ~0.65% of the total petri dish surface was subject to inactivation, but inactivation there was complete, with no detectable colony within this area (Figure 2).

Here Figure 2

3.2 Inactivation of spores by 20 µs electric arcs

In Figure 3, we show the effects of delivering a varying number of electric arc discharges, from 1 to 50, each with a 20 µs duration, delivered in intervals of ~3 s.

In these exposures, the arc also initially descended from the tip of the conical emitting electrode downwards into the sample, but then tended to tilt gradually but increasingly sideways, so that after 8-12 µs, it created a direct diagonal connection between the tip of the conical emitting electrode and the ring-shaped receiving electrode, thus largely proceeding above the sample.

For a single 20 µs electric arc, the path of the arc from its initial almost vertical descent into the sample, along its gradual lateral shift towards the receiving electrode at the sample's edge, to its formation of a

direct connection with this electrode, is clearly visible in Figure 3a, with the area of inactivation formed along this path and its immediate vicinity.

Here Figure 3

With an increasing number of arc discharges delivered, consecutive arcs tended to form plasma channels in directions proximate to the direction of the first discharge (as seen in Figure 3b for the case of 10 discharges), yet they gradually scattered, eventually covering most of the sample (as seen in Figure 3c for the case of 50 discharges). Thus, the inactivation area gradually increased with the number of discharges delivered, reaching 7% for 1 arc, 27% for 10 arcs and 55% for 50 arcs as outlined by the black curve in the three panels of Figure 3, and stated quantitatively in Table I. However, the inactivation rate was similar in the area inactivated with a spore inactivation about 3.5 log10. Outside this area, there was no detectable inactivation.

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Here Table 1

4. Discussion

The results presented above demonstrate that electric arc discharges can cause substantial inactivation of spores of *Bacillus pumilus*. As efficient methods for spore inactivation are lacking, yet of utmost importance, these results are promising and warrant further investigation. Still, we are aware that much further work is needed before electric arcing could become established as a method for spore inactivation.

First, the results with 20 µs arcs were affected, as explained in Section 3.2, by gradual tilting of the arc's path of descent from the emitting electrode into the petri dish. If this tilting were more limited, e.g. by

reducing the radius of the internal container b in Fig. 1, the inactivation area would be more restricted to the central area of the dish, but how quickly this area would increase with the number of arcs delivered remains to be evaluated with various container radii. Nonetheless, also the tilting of the arc's path could be exploited as an approach to increase the inactivation area, e.g. by constructing the receiving electrode from segments insulated from each other, and connecting consecutive segments, one by one, to the ground as to force the tilting of each individual arc to proceed towards the particular segment grounded at the time.

Second, our experiments have been performed in 90-mm petri dishes, while for applications of spore inactivation, the interest both industrially and clinically lies on much larger areas. Testing of electric arcing's efficiency on such scales will require correspondingly upscaled exposure systems and generators, which also raises the problem of operating safety, which at the scale used here is much less acute.

Finally, as mentioned in the introduction, unlike contact discharges, where exposure is to electric field alone, arc discharges also expose the sample to ultraviolet light and a mechanical (acoustic) pressure wave; very close to the arc's point of entry from air into the sample, perhaps in the closest few square millimeters at the sample's very surface, also the highly elevated temperature of the locally plasmified air certainly plays a role. The relative contributions of these effects to the final rate of inactivation have not been evaluated here, and for practical applications of inactivation are also not of particular relevance, yet for fundamental understanding of the basic mechanisms involved they will have to be investigated. This will likely require intricate modifications and upgrades to the experimental apparatus used here, but we are certain that such a reductionistic study is to a large extent feasible.

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FIGURE LEGENDS

Figure 1 : Schematic representation of the exposure system developed by Marjanovič and Kotnik [17]. The system is enclosed by a transparent plexi-glass tube (a). An internal container also made of plexi glass (b) prevents the electric arc from short-circuiting the conical emitting electrode (c) and the ring-shaped receiving electrode (d). The emitting electrode is in the air above the sample, while the receiving electrode is in direct contact with the outer edge of the disk-shaped sample. An electric arc is illustrated (e) as exiting downwards from the emitting electrode into the sample containing the spores (f) deposited on the petri dish (g).

Figure 2: Spore inactivation with ~20 electric arc discharges of ~0.5 µs duration. The inactivation area is outlined by the black curve.

Figure 3: Spore inactivation with 1 (a), 10 (b), and 50 (c) electric arc discharges of 20 µs duration. The inactivation area is outlined by the black curve.

Table I. Calculation of spore inactivation with 20 µs electrics arcs. The inactivation area was estimated in percentage and the inactivation rate was indicated in log10.

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Table 1

	Inactivation area	Inactivation rate
1 arc	7.1 ± 0.63	3.43 ± 0.14
10 arcs	27.3 ± 4.49	3.41 ± 0.32
50 arcs	55.2 ± 12.00	3.57 ± 0.36

Table I. Calculation of spore inactivation with 20 µs electrics arcs. The inactivation area was estimated in percentage and the inactivation rate was indicated in log10.

Figure 1
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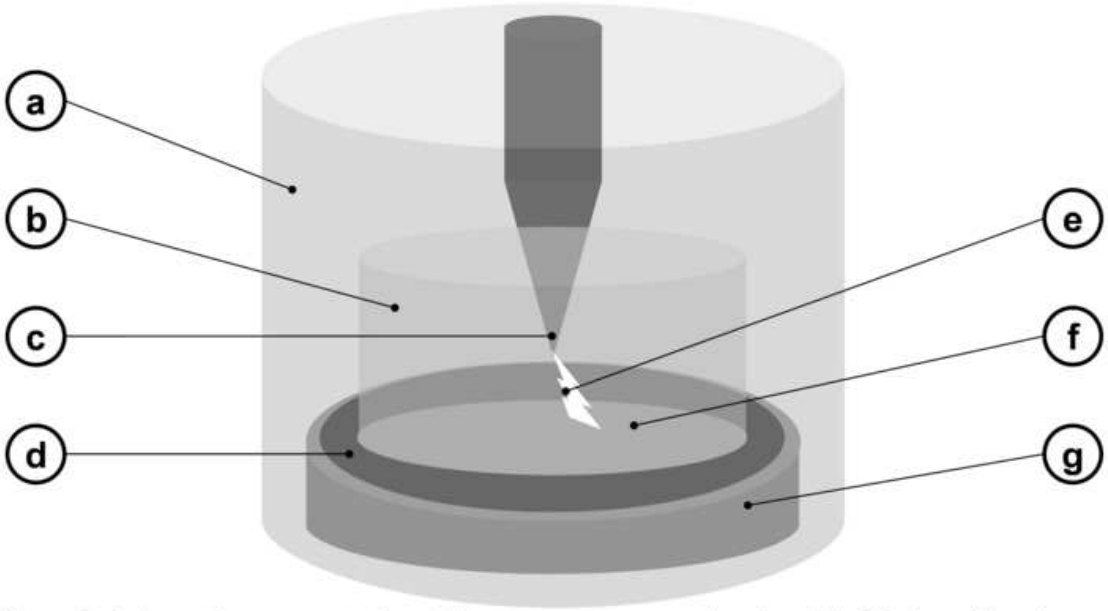

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Figure 2
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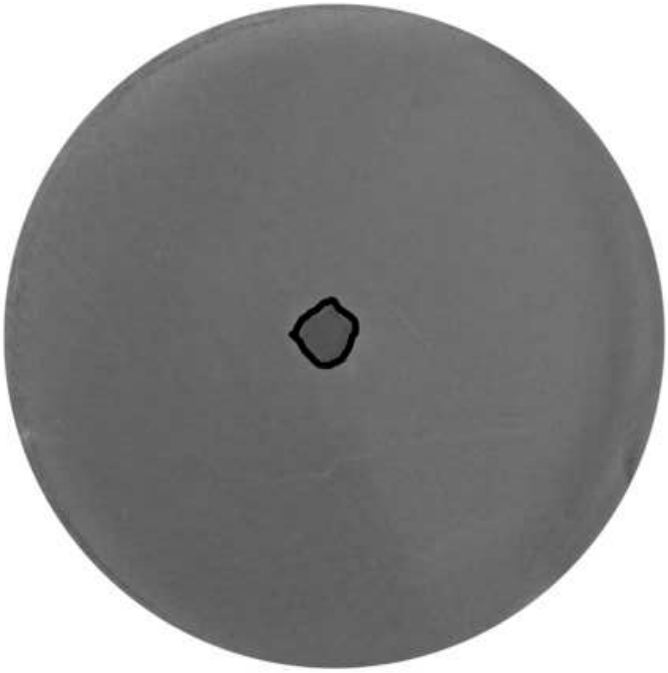

Figure 2: Spore inactivation with ~20 electric arc discharges of ~0.5 µs duration. The inactivation area is outlined by the black curve.

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Figure 3
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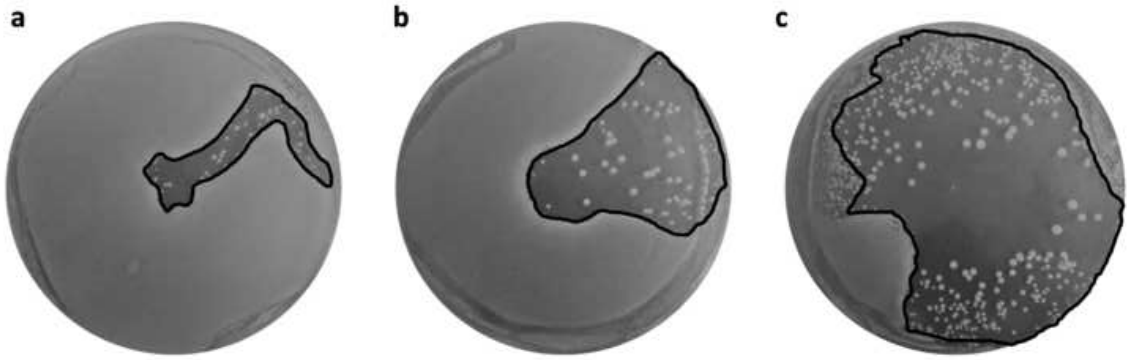

Figure 3: Spore inactivation with 1 (a), 10 (b), and 50 (c) electric arc discharges of 20 µs duration. The inactivation area is outlined by the black curve.

4 IZVIRNA PRISPEVKA K ZNANOSTI

4.1 Razvoj sistema za elektrotransformacijo bakterij v naravnim podobnih pogojih

Reverzibilna elektroporacija je široko uporabljana metoda za vnos DNA v mikroorganizme (elektrotransformacijo), vendar so laboratorijski protokoli usmerjeni v doseganje čim višje učinkovitosti – organizme izpostavimo homogenemu električnemu polju natančno izbrane jakosti in trajanja, vnešene molekule DNA pa so pogosto tudi umetno modificirane z namenom povečanja obstojnosti, prehodnosti v mikroorganizme in izražanja v njih. To laboratorijske pogoje močno razlikuje od naravnih, ki nastanejo ob udaru električne strele v habitat mikroorganizmov, s čimer prav tako lahko pride do elektroporacije in elektrotransformacije – električno polje je tako krajevno kot časovno močno spremenljivo, DNA v mikroorganizmih in njihovem habitatu pa ne vsebuje umetnih modifikacij. Zato smo za raziskovanje elektroporacije v pogojih, podobnih naravnim ob udaru strele, razvili sistem ZEVS, ki omogoča nadzorovano in ponovljivo izpostavitev bioloških vzorcev (prokariotskih celic, evkariotskih celic ali tkiv) elektrostatičnim razelektritvam v kontroliranem okolju (točno določena dolžina obloka razelektritve, merjenje časovnega poteka električnega toka, ki teče skozi vzorec, snemanje poteka poskusov s hitro kamero). Za sistem smo razvili še ustrezne elektrode (konično emisijsko elektrodo in več različnih ozemljitvenih elektrod), nato pa smo ga nadgradili še z razvojem generatorja ZEVS, ki omogoča ponovljivo generiranje elektrostatičnih razelektritev s časovnim potekom električnega toka, podobnim tistemu pri udaru medianske naravne strele (prvega povratnega udara negativne navzdol usmerjene strele tipa CG), le z manjšo amplitudo toka: maksimalnim tokom ~50 A, dvižnim časom toka do maksimalne vrednosti ~5 µs in razpolovnim upadnim časom ~65 µs (časovno konstanto eksponentnega upadanja ~100 µs).

Z opravljenimi poskusi smo pokazali, da je sistem ZEVS primeren za preučevanje vplivov elektrostatičnih razelektritev na prokariotske in evkariotske celice ter da z njim lahko povzročimo tako ireverzibilno elektroporacijo, katere posledica je iztekanje DNA iz celic, kot reverzibilno elektroporacijo, ki privede do vnosa DNA v celice in njeno izražanje.

4.2 Inaktivacija bakterijskih spor z obločnimi razelektritvami

Bakterijske spore so ena izmed najbolj trdoživih oblik živih organizmov, z zelo visoko odpornostjo na toplotne, kemične in mehanske obremenitve. Patogene spore so prisotne pri velikem številu bolezni in njihova odpornost predstavlja velik problem tudi v kliničnih okoljih. Ena izmed potencialnih metod za inaktivacijo spor je elektroporacija, ki pa se je v več poskusih, izvedenih s kontaktnim dovajanjem razelektritev, izkazala za neučinkovito. S sistemom ZEVS, razvitim v okviru prvega prispevka, smo preizkusili, ali je inaktivacijo bakterijskih spor mogoče doseči z obločnimi razelektritvami, kjer izpostavitev poleg električnega polja obsega še ultravijolično svetlobo in tlačni val, ki lahko povzroči tudi kavitacijo. S serijo poskusov na sporah bakterije *Bacillus pumilus* smo pokazali, da je

inaktivacijo dejansko mogoče doseči in da je dokaj ponovljiva: pri sporah, nasajenih na agarju v petrijevki s premerom 90 mm, smo z eno razelektritvijo trajanja 0.5 µs dosegli inaktivacijo spor na ~0.65% celotne površine, z razelektritvami trajanja 20 µs pa inaktivacijo 7% površine pri eni razelektritvi, 27% pri desetih in 55% pri petdesetih razelektritvah.

4.3 Elektroporacija bi lahko bila četrti naravni mehanizem horizontalnega prenosa DNA

Poskusa ireverzibilne elektroporacije na celicah *E. coli* in genske transfekcije na celicah CHO nakazujeta, da bi bila elektroporacija dejansko lahko četrti mehanizem prenosa HGT v naravi, vendar pa bo za zanesljivejši in kvantitativno relevanten odgovor potrebno s sistemom ZEVS opraviti dodatne poskuse na organizmih, katerih naravno okolje je dosegljivo nevihtnim strelam (denimo na bakterijah, ki naseljujejo površinske morske in sladke vode). Poleg tega pa bo potrebno namesto modelnih laboratorijskih molekul DNA, kot so tiste z genom za GFP ali odpornostjo na antibiotike, uporabiti naravno DNA brez kakršnihkoli modifikacij, s katerimi umetno povečamo njihovo stabilnost ter verjetnost vnosa in izražanja.

5 ZAKLJUČEK

V tej doktorski disertaciji so opisani načrtovanje, izdelava in testiranje modularnega sistema za izpostavitev bioloških vzorcev obločnim razelektritvam kot emulacijam električne strele (sistem ZEVS) ter pripadajočega visokonapetostnega generatorja razelektritev (generator ZEVS). Sistem ZEVS nam omogoča, da biološke vzorce (celice ali tkiva) v kontroliranem okolju (točno določena dolžina obloka razelektritve, merjenje časovnega poteka električnega toka, ki teče skozi vzorec, snemanje poteka poskusov s hitro kamero) izpostavimo elektrostatični razelektritvi z nastavljivo amplitudo električnega toka. To predstavlja ponovljivo emulacijo elektrostatične razelektritve, kakršna poteka pri udaru naravne strele.

Sistem smo zasnovali tako, da ga je mogoče hitro sestaviti in razstaviti, kar omogoča preprosto transportiranje in izvajanje poskusov v različnih laboratorijih. Pozorni smo bili tudi, da je mogoče sistem enostavno in temeljito očistiti, kar bistveno zmanjša tveganje kontaminacije, hkrati pa omogoča ponovljivost poskusov. Pri izbiri materiala smo za dele, ki zahtevajo električno izolativnost, uporabili polietilen, če je bila poleg izolativnosti sestavnega dela potrebna tudi njegova prozornost, pa pleksi steklo. Elektrode smo prvotno izdelali iz bakra, a se je izkazalo, da razelektritve povzročajo njihovo korozijo, zato smo baker v nadaljevanju razvoja nadomestili z nerjavečim jeklom, ki se je izkazalo za bolj odporno proti razelektritveni koroziji. Pri ozemljitveni elektrodi, ki je v neposrednim stikom z biološkim vzorcem, je nerjaveče jeklo ustreznejše tudi zato, ker se v primerjavi z bakrom precej manj elektrolitsko raztaplja in tako manj kontaminira vzorec.

Prve biološke poskuse smo opravili na bakterijah *Escherichia coli*, nasajenih na agarju v petrijevkah, razelektritve pa smo generirali s komercialno dosegljivim električnim paralizatorjem. Pri poskusih smo v krožnem področju do radija 5 mm od središča petrijevke dobili območje skoraj popolnoma brez kolonij *E. coli*. Izračunana jakost električnega polja na tej radialni razdalji je bila ~8 kV/cm. Iz opisanih eksperimentalnih rezultatov in izračunov sledi, da je bilo osrednje območje brez živih bakterij verjetno zaradi njihove ireverzibilne elektroporacije.

Drugi sklop bioloških poskusov smo opravili na evkariotskih celicah CHO. Tudi pri teh poskusih smo za generator razelektritev še uporabljali električni paralizator. Celicam CHO smo tik pred izpostavitvijo razelektritvam dodali plazmidno DNA, z izražanjem katere nastaja zeleno fluorescirajoči protein (GFP). V krožnem pasu na razdalji od 3 do 15 mm od središča petrijevke smo zaznali fluorescenco GFP, torej je bilo to območje reverzibilne elektroporacije. Z izračunom smo ocenili jakost električnega polja na razdalji 15 mm od središča petrijevke na 1.11 kV/cm, na razdalji 3 mm od središča pa na 5.54 kV/cm, kar nakazuje, da so bile celice v osrednjem območju, kjer nismo zaznali fluorescence GFP, mrtve zaradi ireverzibilne elektroporacije, v zunanjem območju, kjer prav tako ni bilo zaznavne fluorescence GFP, pa niso bile elektroporirane, zaradi česar ni prišlo do vnosa DNA.

V tretjem sklopu poskusov smo preučevali ireverzibilno elektroporacijo spor bakterije *Bacillus pumilus*. V preteklosti so se raziskovalci neuspešno trudili doseči ireverzibilno elektroporacijo spor, vendar so pri vseh poskusih dovajali pulze v vzorec kontaktno, torej

preko elektrod v neposrednem stiku z vzorcem, ki je vseboval spore. Pri razelektritvah s sistemom ZEVS pa smo tok v vzorec dovedli preko obloka skozi zrak ter tako vzorec izpostavili še tlačnemu valu in ultravijolični svetlobi. Za napetostni generator smo v tem sklopu uporabili tako električni paralizator kot generator ZEVS. Z obema generatorjema smo dosegli ponovljivo inaktivacijo spor. Pri poskusih s paralizatorjem smo z 20 razelektritvami dobili inaktivacijo na 0.65% celotne površine petrijevke, medtem ko je območje inakativacije pri uporabi generatorja ZEVS pokrivalo 7% celotne petrijevke pri eni razelektritvi, 27% pri desetih in 55% pri petdesetih razelektritvah.

Opravljeni poskusi so pokazali, da je sistem ZEVS primeren za preučevanje vplivov elektrostatičnih (obločnih) razelektritev na prokariotske ali evkariotske celice ter da z njim lahko povzročimo tako ireverzibilno elektroporacijo, katere posledica je iztekanje DNA, kot reverzibilno elektroporacijo, ki privede do vnosa DNA in njeno izražanje.

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Sistem ZEVS je zgrajen tako, da se ga da še dodatno nadgaraditi in ga v veliki meri avtomatizirati, kar bi bilo pametno storiti pred izvajanjem večjega števila poskusov. Pri nadgradnji je sistemu potrebno dodati še koračni motor, s katerim se bo lahko hitreje in bolj natančno določala zračna razdalja med konico emisijske elektrode in biološkim vzorcem pod njo. Sistem bi bilo priporočljivo opremiti še z merilcem vlage in temeprature. Poleg tega pa bi bilo potrebno napisati še programsko orodje, ki bi skupaj z generatorjem ZEVS, osciloskopom in hitro kamero avtomatsko zajemal vse relevantne parametre med poskusom, kar bi močno olajšalo kasnejšo obdelavo podatkov.

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7 IZJAVA

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