International Journal of Biomedicine 12(4) (2022) 560-566
http://dx.doi.org/10.21103/Article12(4)_OA6

ORIGINAL ARTICLE

Radiology

INTERNATIONAL JOURNAL OF BIOMEDICINE

Response of Human Malignant Glioma Cells to Asymmetric Bipolar Electrical Impulses

Amr A. Abd-Elghany[*1,2], A. M. M. Yousef[1,3]

[1]*Radiology and Medical Imaging Department, College of Applied Medical Sciences, Prince Sattam Bin Abdul-Aziz University, Alkharj 11942, Saudi Arabia*
[2]*Biophysics Department, Faculty of Science, Cairo University, Egypt*
[3]*Physics Department, Faculty of Science, South Valley University, Kena, Egypt*

Abstract

Electric and electromagnetic pulses have been shown to enhance the endocytosis rate, with all-or-nothing responses beyond a field strength threshold and linear responses as a function of field strength and treatment duration utilizing bipolar symmetrical and monopolar pulses, respectively. Malignant glioma (MG) is resistant to chemotherapy. The present study looked for a new electrical impulse that can aid electrochemotherapy to deliver anticancer drugs while using less electrical energy. Bipolar asymmetric electric pulses were applied to U251MG cells suspended in physiologically conductive media in the presence of molecular probes, including Bleomycin. The delivered electric pulses with a pulse duration range of 180-500 µs and a frequency range of 100-400 Hz had a low field intensity ranging from 1.5 V/cm to 7.3 V/cm. Spectrophotometric and spectrofluorometric measurements were used to investigate the impact of these variables on cancer cell survival and the molecular probe uptake induced by the electric pulses. An all-or-nothing response was observed above a specified threshold of electric field intensity of 4 V/cm. This threshold was unaffected by changes in repetition frequency or pulse duration. It was not a temperature effect that caused the molecular probe uptake to increase. When bipolar asymmetric electric pulses were applied just before electroporation, the effectiveness of the cytotoxic impact of bleomycin was increased from 80%, when employing electroporation pulses alone, to 100%.**(International Journal of Biomedicine. 2022;12(4):560-566.)**.

Keywords: electroendocytosis • asymmetric bipolar electrical impulses • spectrofluorometry • U251MG cells

For citation: Abd-Elghany AA, Yousef AMM. Response of Human Malignant Glioma Cells to Asymmetric Bipolar Electrical Impulses. International Journal of Biomedicine. 2022;12(4):560-566. doi:10.21103/Article12(4)_OA6

Abbreviations

BSA, bovine serum albumin; **BLM,** Bleomycin; **FITC**, fluorescein isothiocyanate; **MG**, malignant glioma; **PEF**, pulsed electric field; **EP,** electric pulse.

Introduction

One of the most challenging aspects of drug delivery is transporting exogenous materials into the cell's cytoplasm. Various approaches to incorporating macromolecules into cells have been explored over the last few decades.[1-3] An interesting procedure is based on an electrically driven process called electroporation, in which cells are subjected to a high-intensity pulsed electric field (PEF) for micro- to milliseconds duration. The membrane undergoes short-term permeability alterations as a result of this exposure due to transient defects in membrane structure termed "electropores," allowing small molecules to diffuse through the membrane along their electrochemical gradients or the uptake of large molecules (e.g., electrically mediated gene delivery) through mechanisms, including electrophoresis.[4-6] Electroendocytosis, i.e., electric field endocytosis, is a method for incorporating macromolecules into cells that involves exposing cells to a

***Corresponding author:** Amr A. Abd-Elghany. Radiology and Medical Imaging Department, College of Applied Medical Sciences, Prince Sattam Bin Abdul-Aziz University, Alkharj 11942, Saudi Arabia. Biophysics Department, Faculty of Science, Cairo University, Egypt. E-mail: amrabdelghany25@gmail.com*

pulsed low electric field. It is arising as a complementary method to electroporation.[7] Electroendocytosis, unlike electroporation, which causes nanometer-sized electropores to develop on the lipid bilayer of the plasma membrane, causes cell membrane internalization and fission via endocytotic vesicles.[8,9] It incorporates either macromolecules that are bound to membrane receptors by receptor-mediated endocytosis[10,11] or any soluble drugs by fluid-phase endocytosis (pinocytosis).[12,13] Exocytosis, a reciprocal mechanism, restores the cellular membrane area.[14]

Two main types of electric pulses have reported endocytosis induced by pulsed low electric fields.[7,10,11,13-15] The first pulse type used was bipolar symmetrical square pulses with field intensity ranging from 1.2 V/cm to 8 V/cm, pulse durations ranging from 75 µs to 580µs, frequencies ranging from 50 to 400 Hz, and total exposure times ranging from 5 to 90 minutes. A bipolar pulse was adopted to limit unidirectional electrophoresis and to reduce electrochemical deposits at the surface of the electrodes. On three separate cell lines, exposures lasting more than 10 minutes resulted in a ~50% improvement in fluid-phase endocytosis, compared to controls with the same exposure time but no electric pulses. This improvement was defined as an all-or-nothing incident that occurs at electric field intensity thresholds ranging from 1.6 V/cm to 2.6 V/cm, depending on the cell type. The cell response to the PEF was unaffected by changes in repetition frequency or pulse duration.[12] As the second pulse type, we used unipolar rectangular pulses with field strengths ranging from 2.5 V/cm to 20 V/cm, pulse durations ranging from 50 µs to 250 µs, at temperatures ranging from 4 to 37°C, and conductivities ranging from 6.4 mS/cm to 18.6 mS/cm. In all experiments, the total exposure time was 1 minute. The electrically induced increase in BSA-FITC uptake by two separate cell types showed a linear dependency with no sign of a field strength cut-off value. At 20 V/cm, maximum uptake could be up to 7.5 times higher than in controls. The uptake was also linearly dependent on the pulse duration and medium conductivity.[7,10,11] These two sets of data were markedly different.

MG is a form of brain tumor with a high death rate in humans and is resistant to typical cancer therapies like surgery, chemotherapy, and radiotherapy. The blood-brain barrier in the areas surrounding the tumor is reversibly damaged when electroporation is administered to the brain with specific treatment parameters, which are greatly helpful in treating the infiltrating cells with maximum delivered chemotherapeutic doses.[16-19] The response of U251MG cells to asymmetric bipolar electric pulses was investigated using various electrical parameters. Increased receptor-mediated endocytosis was detected in U251MG cells, which were characterized by an all-or-nothing response at field strength threshold just after electric pulse exposure. Such a low PEF can be used as an adjuvant with electrochemotherapy to enhance MG treatment with lower electrical parameters and lower doses of anticancer drugs.

Materials and Methods

Cell culture

Modified Eagle's medium (MEM, Sigma-Aldrich, Germany) enriched with L-glutamine, 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), streptomycin (125 µg/ml) and penicillin (100 units/ml) were used to culture human MG cells U251MG driven from malignant astrocytic tumors. The cells were grown in number by adding 1 ml of MEM(1x) containing ~10^6 cells with 9 ml of MEM(1x) free of cells in culture flasks incubated at 37°C for 3 days in a CO_2 incubator (5±1% CO_2 and 95 % relative humidity). The attached cells were collected by trypsin-EDTA (0.05%, Sigma-Aldrich, Germany). After centrifugation (210 g, 5min, room temperature), the culture medium MEM(1x) was removed as a supernatant, and cells were resuspended (4-8×10^6 cells/ml) in the exposure medium S-MEM (product 21385, Invitrogen), a calcium-depleted modification of EMEM, which has a physiological conductivity (10 mS/cm). Exposure media were subjected to degassing at 23±2°C for 10min using a vacuum pump (Thermo Scientific, Germany).

Molecular probes

Receptor-mediated endocytosis was detected using either BSA (66 kDa) that has been conjugated to fluorescein isothiocyanate (5-FITC, Sigma-Aldrich, Germany) (1:4 v/v) to get a final concentration of 6.8 µM or BLM (1.5 kDa) as an anticancer drug (Laboratoire Roger Belon, France) at concentrations ranging from 30 nM to 30 µM.[10,11,13,15]

Low-intensity bipolar asymmetric electrical impulses

Suspended cells (100µl of medium containing 0.5 to 1.0×10^6 cells) were subjected to a low-intensity train of bipolar, asymmetric, rectangular voltage pulses with the area of the positive part of the pulse (above the baseline) nearly equal to the area of the negative part of the pulse (below the baseline) by employing a 50 MHz pulse generator (Model 801, Wavetek, San Diego, USA) whether or not a molecular probe was present (BSA or BLM). The exposure was performed in a vertical position by two stainless-steel electrodes with an interdistance of 0.2 cm, resulting in a medium to the electrode contact area of 0.5 cm^2 and a quasi-uniform electric field. The exposure was done in S-MEM (a physiologically conductive medium, 10 mS/cm). A digital storage oscilloscope (HITACHI, Japan) was used to monitor the electric field characteristics online. The electrical parameters comprised electric field strengths ranging from 1.5 to 7.3 V/cm, pulse individual durations from 180 to 500 µs, and pulse repetition rates from 100 to 400 Hz. All trials were carried out at 23°C, 4°C, and 37°C, with no significant changes noted between the temperatures (data not shown). A digital thermometer was used to measure the samples' temperature at the end of the exposure. For each set of exposures, a control (molecular probe only, no electric pulses) sample was placed between the same electrodes.

Application of electroporation pulses

U251MG cells previously exposed to bipolar asymmetric electric pulses (6.9 V/cm, 200 Hz repetition frequency, 400 µs pulse duration, and 2 min exposure time) in the presence of 30 µM BLM were transferred aseptically to an electroporation cuvette (Al electrodes) with a gap width of 0.2 cm and subjected to 8 square unipolar electroporation pulses of 600 ms pulse duration and 1000 V/cm field intensity using BTX Harvard electroporator (Model No. 620, BTX Harvard Apparatus, USA).

Determination of the fluorescent probe uptake using spectrofluorometry

The collected samples were centrifuged for 250 RCF for 10 min twice after washing them with PBS. After the supernatant was discarded, pellets were suspended in a one-milliliter buffer (Lysis reagent buffer), then diluted 100 times by H_2O and mixed by the vortex. Total fluorescence was spectrofluorometric (at 494 & 520 nm for BSA-FITC, Kontron SFM 25- England) evaluated to calculate released fluorescence molecules from the ruptured cell. Molecule numbers/cells were calculated according to FSC (standard curve of fluorescence). The fluorescent probe uptake by cells subjected to electric pulses was calculated and compared to the control samples treated with BSA-FITC only (no electric pulses).

Determination of the number of cells using spectrophotometry

The colorimetric detection and quantification of total protein, corresponding to the number of cells, in dilute aqueous solutions were done using Micro BCA™ Protein Assay Kit (Pierce, Rockford, USA). The water-soluble complex exhibited a strong absorbance at 562 nm, which is linear with increasing protein concentration. To avoid fluctuations in cell counts eventually resulting in cell damage caused by the electric pulses, and to determine cell content in each of the treated samples, the total protein content was measured in all the samples. Protein standards were prepared by diluting 2.0 mg/ml BSA stock standard with water to set the standard curve. The working reagent was prepared according to the manufacturer's instructions; 40 µl of cell lysate for each sample (including unknown samples treated with different electrical parameters and untreated samples of the known number of cells) were added to 110ml of water in the appropriate microwell plate wells. Blank wells only contained water (150 µl). Then 150ml of the working reagent was added to each well. The plate was covered and incubated at 37°C for 2 hours. After incubation, the plate was cooled at room temperature, and the absorbance was measured at 562 nm on a plate reader. The reading for each standard or unknown sample was deducted from the average reading for the blanks. The average blank-corrected reading for each BSA standard was plotted against its concentration in g/ml to create a standard curve. Another standard curve was created by graphing the protein concentration of the unknown untreated samples vs. cell numbers. The standard curve was used to calculate the number of cells in each electrically treated sample when the molecular probe was present.

Determination of BLM cytotoxicity and electroendocytosis

Another confirmatory method (cloning efficacy test) was used to determine the cytotoxic effect of the anticancer drug BLM in the presence or absence of the electric pulses. Half an hour after exposing them to an electric field, every 100 cells were placed in 1mL of culture medium, then 4mL of the cell suspension were sucked out, placed in a Petri dish (60 mm), and incubated for 5 days to form colonies. At the end of the incubation time, the colonies were fixed in Petri dishes with 4% formaldehyde (Sigma-Aldrich, Germany) and stained in 1% crystal violet (Biochrom, Berlin, Germany). By counting clone colonies and comparing them to the control group, the proportion of cells that survived after being exposed to electric pulses with or without BLM was calculated.

Cell observation with Inverted Light Microscope

A portion of the cell suspension that was exposed to different types of electrical pulses in the presence of BLM (30 µM) was re-cultured and incubated at 37°C in a CO_2 incubator (5±1% CO_2 and 95 % relative humidity). After 3 days the attached cells were observed under the inverted light microscope (Olympus, Tokyo, Japan) with a magnification of 100x. A negative control group represents cells with no BLM and no electric pulses.

Statistical analysis

In Figures 2-6, each value in the data indicates the mean±standard error of 3 separate experiments, each of which was repeated three times. For each Figure, the average control value and SD were determined using the individual control values from all the relevant independent experiments. Inter-group comparisons were performed using unpaired Student's t-test. A probability value of $P<0.05$ was considered statistically significant.

Results

Effect of field intensity

The influence of field intensity was studied at room temperature by exposing U251MG cells suspended in the physiologically conductive medium (10 mS/cm) with the molecular probe to pulsed bipolar asymmetric trains (200 Hz repetition frequency and 400 µs pulse duration) of different electric field strengths (1.5-7.3 V/cm) for 2 min, followed by 8 min of incubation without pulses. There was no significant response to any tested values below 3 V/cm. A significant increase in the fluorescence probe uptake was detected only for electric pulses higher than the threshold intensity of 4 V/cm (by ~38-110%) (Figure 1).

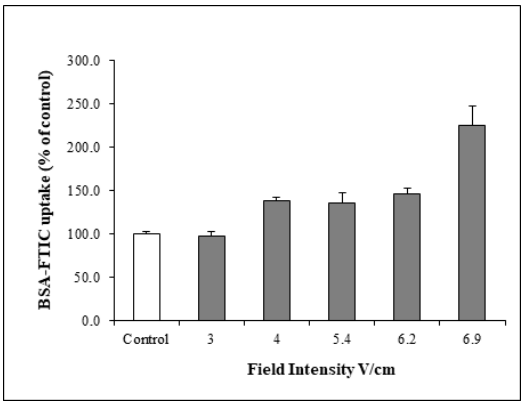

Fig. 1. *BSA-FTIC uptake by U251MG cells suspended in S-MEM (10mS/cm) as a function of field strengths of bipolar asymmetric electric pulses at 200 Hz, 400 µs, 2 min in the presence of BSA-FITC followed by exposure of 8 min to the BSA-FITC without electric pulses. (□) Control cells treated were with 6.8 µM BSA-FITC only. 100% represents the uptake of control samples in 10 min. Statistical significance: P<0.001.*

Due to the electrochemical reactions at the stainless-steel electrode surfaces, field intensities higher than 7.3 V/cm couldn't be tested. There was no cell loss at all the tested values of field intensities and no temperature increase.

Effect of frequency

To evaluate the effect of frequency on the uptake of fluorescent probes, cells were exposed in a physiologically conductive medium to bipolar asymmetric electric pulses using 4 V/cm field intensity (the threshold intensity) and 400 µs pulse duration. The exposure time was 2 min (electric pulses + BSA-FITC), followed by 8 min incubation in the presence of BSA-FTIC without pulses. The controls were incubated for 10 min with BSA-FITC only. In this group of experiments, a significant increase (P<0.001) in BSA-FITC uptake was found at frequencies higher than 100 Hz. The increase was almost identical for all the repetition frequencies tested, at ~40% higher than the unexposed controls (Figure 2). These results confirmed those obtained by changing the field intensity. No change in the number of cells was found using different repetition frequencies.

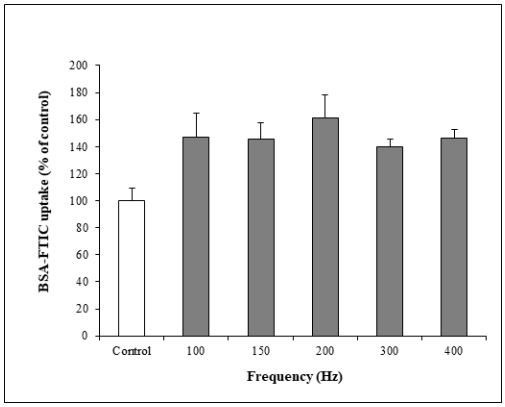

Fig. 2. *BSA-FTIC uptake by U251MGis suspended in S-MEM (10mS/cm) as a function of repetition frequency for bipolar asymmetric EP at 4 V/cm, 400 µs, 2 min, and BSA-FITC followed by exposure of 8 min to the BSA-FITC only. (□) Control cells were treated with 6.8µM BSA-FITC only. 100% represents the uptake of control samples in 10min. Statistical significance: P<0.001.*

Effect of pulse duration

To study the effect of pulse duration BSA-FITC uptake, cells were exposed to the physiologically conductive medium to electric pulses using 4 V/cm field intensity and 200 Hz repetition frequency. The exposure time was 2 min (electric pulses + BSA-FITC), followed by an 8 min incubation with BSA-FTIC only. The controls were incubated for 10 min with BSA-FITC only. A significant increase (P<0.001) in BSA-FITC uptake was found at pulse durations higher than 180 µs. The increase was almost identical for all the pulse durations tested, at a value approximately 40% higher than the unexposed controls (Figure 3). No change in cell survival was found using different pulse durations.

Electroendocytosis arises mainly during EP exposure

In this experiment, there was no BSA-FITC in the medium at the time of the electric pulses delivery medium. Thus, just after U251MG cells' exposure to electric pulses of 6.9 V/cm field strength, 200 Hz repetition frequency, and 400 µs pulse duration in physiologically conductive medium cells were incubated at room temperature (~23°C) for various durations in the presence of 6.8µM BSA-FITC. The exposed cells showed no enhanced dye uptake compared to the control cells (Figure 4). Compared to the controls, there was no cell loss in the exposed samples. One hundred percent corresponds to control cells that were only exposed to BSA-FITC for 2 minutes.

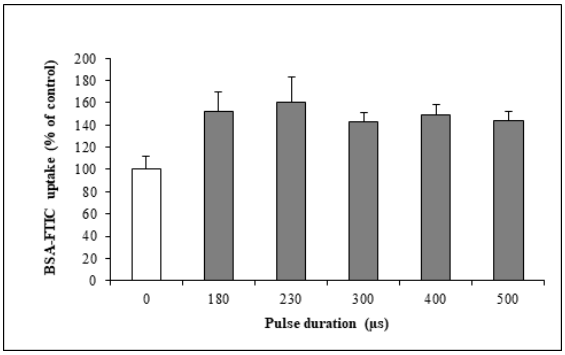

Fig. 3. *BSA-FTIC uptake by U251MG cells suspended in S-MEM (10 mS/cm) as a function of pulse duration for bipolar asymmetric electric impulses at 4 V/cm, 200 Hz, 2min in the presence of BSA-FITC followed by exposure of 8min to the BSA-FITC only. (□) Control cells were treated with 6.8µM BSA-FITC only. 100% represents the uptake of control samples in 10min. Statistical significance: P<0.001.*

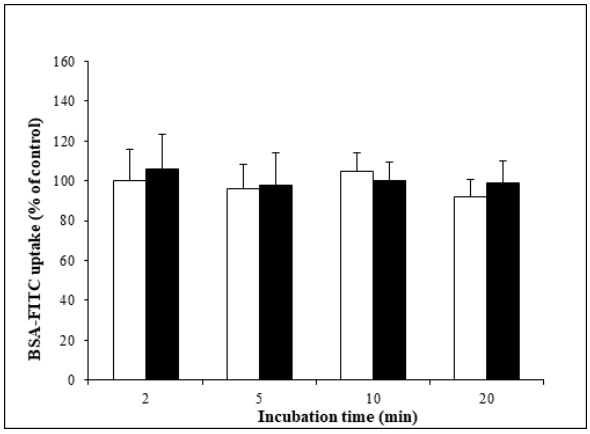

Fig. 4. *Uptake of the fluorescent probe by U251MG cells, using different incubation times, S-MEM (10 mS/cm) exposure medium, 6.9 V/cm field intensity, 200Hz repetition frequency, 400µs pulse duration, and 2min exposure time to the EP only. (□) Incubation in the presence of BSA-FITC without EP. (■) Incubation in the existence of BSA-FITC just after the exposure to EPs.*

BLM cytotoxicity triggered by low-intensity bipolar asymmetric EPs

After exposure to low-intensity bipolar asymmetric electric pulses (6.9 V/cm, 200 Hz, 400 µs, 120-sec total exposure, BLM at concentrations of 3 µM and higher), increased levels of cellular death rates were observed, which was a good predictor for the occurrence of electroendocytosis. For cells treated with bipolar asymmetric EP and BLM at various concentrations (30 nM - 30 µM), colonies were counted and standardized to the positive control (BLM alone, no electric pulses). There was no significant cell loss for cells treated with BLM at different concentrations, although survival was reduced considerably by ~18% in the absence of electric pulses at 30 µM BLM. The survival was significantly lowered (P<0.001) by ~17% and ~40% at 3 µM and 30 µM, respectively (Figure 5), in the presence of bipolar asymmetric electric pulses. Negative control represents samples without any treatment (drug-free, no EPs).

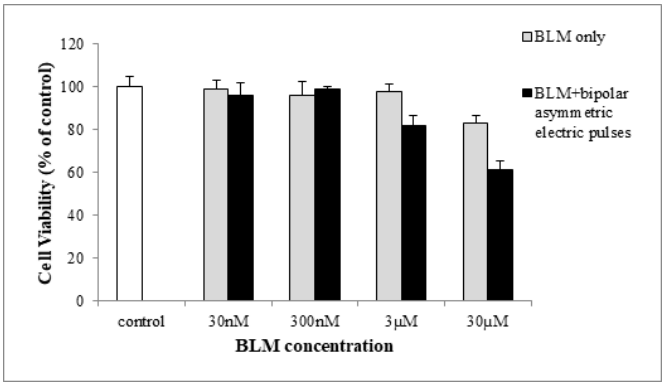

Fig. 5. *Cell survival of U251MG cells exposed to 6.9 V/cm, 200 Hz repetition frequency, 400 µs pulse duration, and 2 min exposure time in the presence of BLM. □ Control represents drug-free, and no EPs samples.*

Morphological and cell viability changes

Using an inverted light microscope, the efficacy of PEF to generate deformation and cell survival change was investigated. U251MG healthy control cells are spindle-shaped (Figure 6a). Cells in monolayer exponential growth became deformed and decreased significantly in number (Figures 6 b-d) because of the electric pulse application. Cells treated by electroporation pulses (1000 V/cm, 8 square unipolar pulses, and 600 µs pulse duration) after bipolar asymmetric bipolar PEF (6.9 V/cm, 200 Hz repetition frequency, 400 µs pulse duration, and 2 min exposure time) in the presence of BLM disappeared from the dishes, which indicates a ~100% mortality rate (Figure 6d).

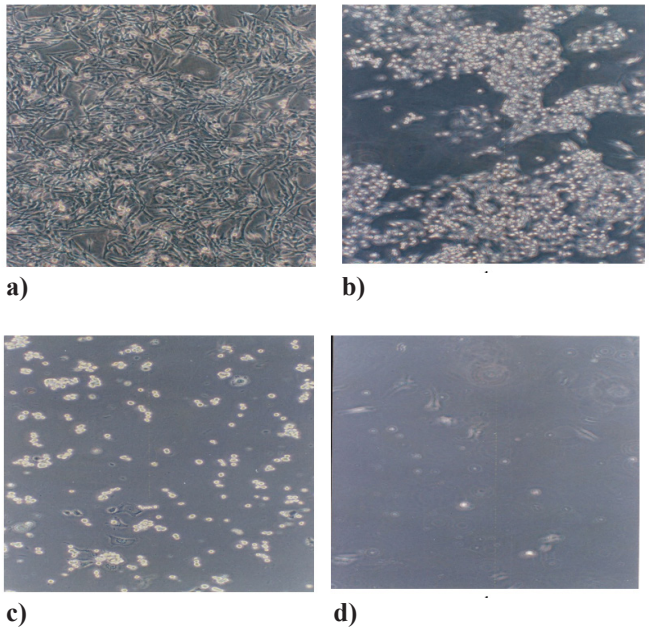

Fig. 6. *Monolayer exponential growth of U251MG cells under inverted light microscope. (a.) Negative control (healthy human malignant glioma cells without neither BLM nor electric pulses). (b.) Cells exposed to bipolar asymmetric electric pulses in the presence of 30 µM BLM. (c.) Cells exposed to electroporative pulses in the presence of 30 µM BLM. (d.) Cells exposed to bipolar asymmetric electric pulses then electroporative pulses.*

Discussion

The application of a low PEF was found to modify the cell surface, resulting in increased adsorption and subsequent uptake of macromolecules such as DNA, dextran, and BSA. [7,10,11] The electrophoretic segregation of charged moveable protein and lipid molecules in the plane of the cell membrane was attributed to this surface change. This phenomenon has previously been demonstrated both theoretically and practically. [20-24] Subsequent researchers proposed that these modifications are directly responsible for improved adsorptions and motivated uptake by a change in plasma membrane curvature that promotes endocytotic vesicle formation.[7.9.11]

A bipolar asymmetric signal with the area of the positive part of the pulse (above the baseline) nearly equal to the area of the negative part of the pulse (below the baseline) was chosen to perform the present study. The tested signal has a sort of "net" pulse in one direction (one short and relatively intense pulse "above" the threshold in one polarity and one long and much less intense pulse "below" the threshold in the other polarity). It was an attempt to approach the unipolar condition (pseudo-monopolar) of Rafi Korenstein's lab[11,13] with minimization of potential electrophoresis and electrochemical reactions at the electrodes (because the product of the pulse duration multiplied by field strength is similar between the positive and the negative part of this bipolar asymmetrical pulse).

The experimental results showed a statistically significant increase of ~38%-110% in non-permeant fluorescent probe uptake when exposed to bipolar asymmetric low-intensity square pulses for 2 minutes total exposure time using a physiologically conductive medium (10 mS/cm). This rise took place above a field intensity threshold value of 4 V/cm that was independent of the pulse duration, frequency, and temperature during the exposure. These results agreed with those obtained by Mahrour et al.[12] and disagreed with those obtained by Antov et al. in terms of cell viability and linear dependence. There was no significant uptake change using the low conductive medium (sucrose 0.25 M, 1 mS/cm) (data not shown). Conductivities of 1mS/cm were not tested in the previous studies.. The effect of conductivity may suggest that the increase in uptake is reliant on the electric current running through the cell's suspension and the resistance of the exposure medium. The resistance of the physiologically conductive medium (10mS/cm) was 40 Ω, while the resistance of the low conductive medium (1 mS/cm) was 400 Ω. The calculated current flowing through the cells suspended in S-MEM (10 mS/cm) was 20 mA at 4 V/cm compared to 2mA with that obtained by the low conductive medium (1mS/cm) using the same field intensity. The number of Coulombs liberated in the conductive medium using 4 V/cm, 200 Hz, 400 µs, and 2 min total exposure time was 0.192 Coulombs, compared to 0.0192 Coulombs liberated in the low conductive medium. The results obtained by changing either the repetition frequency or the pulse duration demonstrated an increase in BSA-FITC uptake at 0.0432 Coulomb, which corresponded to 4 V/cm, 100 Hz, 180 µs, and 2 min total exposure time in the S-MEM. Thus, the number of Coulombs (and the current intensity) in the low conductivity medium is not the limiting factor. We can therefore exclude a direct effect of

the current passing through the cell suspension. This conclusion is reinforced by the presence of a clear plateau for frequencies above 100Hz (Figure 3) or pulse durations longer than 180μs (Figure 4) at 4V/cm as the number of Coulombs is much less, but the cell response is similar.

Moreover, this "plateau" confirms that this increase is an all-or-nothing response independent of the energy liberated in the medium, as reported by Mahrour et al. The absence of cell response in the low conductivity medium can simply result from the fact that, contrary to the S-MEM, a cell culture medium at 10mS/cm, the low conductive medium at 1mS/cm is not a "physiological" medium: osmolarity is preserved by the presence of 250mM sucrose, and ion content (Na^+ and Cl^-) is very low. Thus, to increase their endocytosis rate, cells must be in "physiological" conditions.

The results also demonstrated that the increase in receptor-mediated endocytosis in PEF-exposed cells was the consequence of a rapid change: it was apparent immediately after the start of PEF exposure and was not detectable if the fluorescent probe was introduced soon after the end of PEF delivery.

The transmembrane potential was determined by the Schwan equation[25]:

$\Delta\Psi_i = 1.5\ r\ E\ \cos\theta$

where r is the cell radius (a suspended cell is a sphere), E is the field strength in the region where the cell is located, and θ is the polar angle measured from the center of the cell concerning the direction of the field.

According to this formula, the highest voltage is induced at the places where the electric field is perpendicular to the membrane, i.e., at $\theta = 0°$ and $\theta=180°$. Thus, exposing a glioma cell with a radius of 20 μm [26] to an electric field with positive pulse strengths ranging from 4 to 6.9 V/cm resulted in an induced potential differential of 12-20.7 mV across the plasma membrane. As a result, the membrane region facing the anode was hyperpolarized by 12-20.7 mV, while the membrane region facing the cathode was depolarized by the same amount using either unipolar electric pulses, as used by Antov et al.,[11] or bipolar asymmetric electric pulses, as used in the current study. It is well understood that the calculated changes in transmembrane potential do not result in electroporation.

Fluorescence aggregates were produced with the cell exposed to field intensity higher than 7.3V/cm despite degassing the medium before exposure to electric pulses due to the electrochemical reactions at the electrode surface. The generation of the aggregates limits the study at higher field intensities.

Human MG cells were exposed to bipolar asymmetric electric pulses in the presence of the anticancer drug bleomycin to investigate the ability of the drug to provoke electroendocytosis. BLM enters cells by receptor-mediated endocytosis, but in small quantities that are not enough to kill cancer cells,[27-29] so electroporation was used to increase the permeability of the anticancer drug and thus increase its cytotoxicity.[30] The pulse under research was able to destroy a reasonable number of cells at 3μM, reinforcing the idea of employing it as an auxiliary pulse before the electroporation process to allow the use of low doses of chemotherapy.

Conclusion

The features of the tested electrical impulses, as well as the outcomes of the experimentations, suggested that electroendocytosis was triggered only during the application of electric pulses at threshold intensity, with a quick "switch off" of the molecular probe uptake rise after the exposure to electric pulses was terminated. This result allows us to exclude the mechanisms that require more than a few minutes to be stimulated, such as gene expression regulation mechanisms. The meaning of this observation is that for drug delivery by electroendocytosis into MG cells, the drug must be administered before electric pulses. The bipolar asymmetric nature of the electric pulses, as well as the brief exposure time, significantly diminish the potential mechanisms involving electrophoresis of membrane proteins over long distances within the cell membrane; the absence of any relationship with the number of Coulombs or the electric current intensity also limits the influence of electrophoretic effects. The possibility of thermal effects can be ruled out.

Acknowledgments

The present work was supported by the Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University, Al-Kharj, KSA.

Competing Interests

No potential conflict of interest was reported by the authors.

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