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Iontophoretic Transport of High and Low Molecular Weight Plasmids through Durapore as a Model Membrane

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ABSTRACT

Background: The aim of this study was to investigate the impact of plasmid molecular weight, plasmid aggregate size, plasmid concentration and DC current application on the transport of plasmids across Durapore, as a model membrane. ***Methods:*** Two plasmids (pUC19 and pCMVβ) with different molecular weights were amplified in *Escherichia coli* and purified on ion exchange columns (Qiagen). Plasmid integrity was confirmed by agarose gel electrophoresis. The concentrations of DNA were determined by the absorbance at 260 nm. Passive and Iontophoretic (cathodal) permeation experiments were performed through the sclera and the amount of plasmid was assessed using Quant-iT™ PicoGreen probe with fluorimeter. The aggregate size of plasmids in HEPES buffer (pH=7.4) were determined using particle size apparatus. ***Results:*** The passive study results revealed no trans-membrane flux of plasmids. Application of cathodal Iontophoresis at current intensities of 0.5, 1, 2, 3 and 4 $mA.cm^{-2}$ resulted in a higher transport of pUC19 across the membrane in comparison with pCMVβ. The permeation increased as the current or plasmid concentration increased. Electrophoresis studies revealed that use of platinum electrodes can degrade the plasmids. The aggregate size of pUC19 and pCMVβ were 62 ± 4 and 76 ± 7 nm respectively. ***Conclusion:*** Use of platinum electrodes in gene delivery can damage the DNA molecules. Application of Iontophoresis technique could be helpful in gene delivery especially for low molecular weight plasmids. Plasmid aggregates size is a characteristic property of plasmids in gene delivery across membranes.

Introduction

Since many human diseases are associated with genetic disorders and also regarding the advances in the field of molecular biology and human genome decoding, it is expected in the near future that gene therapy play a critical role in the treatment of various diseases, especially genetic disorders. This new treatment method aims to deliver normal genes to the cells containing the defective gene in order to establish normal gene expression and production of natural protein so that to provide proper functioning of cells and the relevant tissues.[1] Thus the importance of developing new methods in gene delivery is clear. Generally, gene delivery methods are classified into two groups: Use of biological carriers such as viruses and application of chemical or physical techniques. The first method has limitations such as risk of viral toxicity, host immune response and difficult formulation.[2,3] Although possessing the advantages of being non-toxic and easily applicable, chemical methods of gene delivery are of low efficiency and end in low gene expression. Therefore, the current physical methods are of great importance due to their high efficiency in gene delivery and no immune response of the body against them.[1]

Plasmid is a double-stranded DNA usually circular molecule capable of replicating and expressing in appropriate conditions.[4] These molecules are naturally seen in bacteria, yeast and plants; however, they can be artificially imported into the animal cells, as well. The plasmids used in genetic engineering and gene delivery are called Vectors.[5] The plasmids are considered more in gene therapy due to higher stability in vitro and in vivo, easier preparation and independent reproducibility and expression in human cells compared with different types of RNA and DNA fragments.[6]

Since the nucleic acids have a negative surface charge, thus, one of the methods with high efficacy in gene

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delivery is Iontophoresis. Iontophoresis is a localized non-invasive technique in drug delivery which is used for transferring ionized drug to various tissues through a weak electrical current.[7] The Iontophoresis machine includes an electrical current power supply which is connected to the electrodes, an anode (positively charged) and a cathode (negatively charged). A direct electrical current constantly exists from the cathode to anode compartment. In drug delivery, the ionized drugs are placed in the compartments with the same charge, so that the negatively charged drugs or macromolecules are placed into the cathode compartment and the positively charged drugs or macromolecules are placed into the anode compartment.[8] Hence, in Iontophoretic gene delivery, the gene is placed in the cathode compartment. It is proved that using electrical current in gene delivery, not only leads in increased plasmid concentration in the target tissue, but also results in greater penetration of the plasmid into the cells and center of the tissue. However, the exact mechanism of this effect is not still clear and it seems that a complex mechanism is involved.[9]

In this study, the simultaneous effect of three factors of plasmid aggregate size, plasmid concentration and Iontophoresis technique on the transport of two different plasmids across Durapore membrane was evaluated. The pCMVβ plasmid with 7164 base pairs and containing a CMV promoter and beta-galactosidase coding region and the pUC19 plasmid with 2686 base pairs were used.

Materials and Methods

Materials

Durapore® membrane with pores of 100 nm (Millipore, UK), buffered powder HEPES, hydroxyethylpiperazine ethane sulfonic acid (Sigma Aldrich, USA), buffered powder TAE, Tris acetate EDTA buffer (Sigma Aldrich, USA), double-stranded DNA-specific kit and fluorescent reagent, Quant-iT ™ PicoGreen®, (InvitrogenTM, UK), platinum electrode (Aldrich, USA), Silver electrode (Aldrich, USA), agar powder (Merck, Germany), ethidium bromide (Sigma Aldrich, USA), λDNA marker 2 (Sigma Aldrich, USA), DNA marker agar color (Sigma Aldrich, USA), plasmid purification Giga kit (QIAGEN Plasmid Giga Kit), ampicillin (Sigma Aldrich, USA), bacterial culture medium LB (Merck, Germany), E. coli colonies containing pCMVβ plasmid, and colonies of E. coli containing pUC19 plasmid (Pharmaceutical Research Laboratory, University of Helsinki), Nafaion membrane (Millipore, UK), fresh cow eyes (purchased from Helsinki slaughterhouse, Finland); the rest of the materials were used in pure and biological grade.

Devices and Equipments

Side by side diffusion cell (Logan, UK), DC electrical current generating device (Shanghai Sute Electrical Co Ltd), fluorimeter device (Perkin-Elmer, USA), electrophoresis device (Thermo, USA), centrifuge machine equipped with refrigerator (Beckman, USA), freeze dryer machine (Christ, Germany), pH meter (Metrohm, Switzerland), Zeta sizer machine (Malvern, UK), analytical balance with an accuracy of 0.00001 gram (Sartorius, Germany), auto-sampler (Gilson, USA), shaker incubator (Kühner, Switzerland), UV spectrophotometer (Shimadzu, Japan).

Methods

PCMVB Plasmid Replication and Extraction

E. coli bacteria were used for replication. The plasmid was transformed earlier into the bacteria in Pharmaceutical Research Laboratory, University of Helsinki, Finland. Initially, a loop of transformed bacteria was added into 50 ml of LB culture medium containing ampicillin and incubated in 37 ºC and 300 rpm for 8 hours. The resulted cloudy suspension was divided into other five sterile containers and the final volume increased to 2 liters using fresh LB culture medium containing ampicillin. The bacterial solutions were incubated for one night under the same condition. After isolation of the bacteria by centrifugation in 8000 rpm for 15 minutes, the replicated plasmids were extracted and purified using QIAGEN Giga kits according to relevant guidelines.[10] After lyophilisation, the plasmids were dissolved in 3 ml of TAE buffer and the concentration and purity was evaluated using UV spectrophotometer at wavelengths of 260 and 280 nm. In order to ensure the extraction of the plasmid, it was digested with restriction enzymes and gel electrophoresis testing was performed in the presence of lambda DNA markers.

Extraction and Amplification of PUC19 Plasmid

The replication and extraction method of this plasmid was just like the previous plasmid and was conducted using E. coli bacteria transformed by pUC19.

Stability Test of Plasmid Solution in the Presence of Platinum and Silver Electrodes

To investigate the stability of the plasmids in the presence of the electrodes of Iontophoresis, the testing was conducted in the presence of two types of conventional platinum and silver electrodes. This test was only conducted on the pCMVβ plasmid as a model and its results can be generalized to all types of plasmids. In the case of platinum electrodes, the cathode and anode electrodes of the Iontophoresis machine were directly placed in plasmid solutions. In the case of silver electrode, due to the necessity of using sodium chloride salt in the presence of this electrode, the electrodes placed in salt solution and separated from the plasmid solution using NafaionR membrane. Direct currents of 0.5, 2.5 and 5 mA were applied separately to each solution for 2 hours. Each experiment was repeated three times and sampling was performed at 0 and 2 hours. In order to determine the plasmid concentration, the Quant-iT ™ PicoGreen fluorescence kit and the reagent was used according to

the instructions. Absorption of the fluorescence-labeled samples was determined by fluorimeter and the plasmid concentration was calculated using calibration curve. For further study on the effect of the electric current on the plasmid, gel electrophoresis test was also performed on the samples.

Evaluation of Plasmid Aggregate Size in HEPES Buffer Solution

Solutions of pUC19 and pCMVβ plasmids in similar concentration with the solutions that used in the test of plasmids transferring through Durapore membrane were used. Thus, solutions with 20 µg/ml plasmid concentration in 25 mM of HEPES buffer with pH=7.4 were prepared from the stock solution of the two plasmids. The aggregate size of both plasmids was determined using Zeta sizer device which is a common device in determining the particle size and zeta potential of the particles, according to the instructions. This device can simultaneously calculate the polydispersity index of the particles.

Plasmid Transport through Durapore Membrane Using Iontophoresis

This test was designed to study the impact of Iontophoresis technique on transfer of pCMVβ and pUC19 plasmids through Durapore membrane with 100 nm pore size. After cutting the membrane, a piece of membrane was placed in each pair of diffusion cells and 1.5 ml of plasmid solution with 20 µg / ml plasmid concentration in 25 mM of HEPES buffer with pH=7.4 was poured in one cell (donor compartment) and on the other side of the membrane (receptor compartment), 1.5 ml of HEPES buffer was placed. The cathode and anode electrodes were respectively placed in the donor and receiver compartments; the Durapore membrane area located between the two compartments was 1cm^2. The test duration was 4 hours and in the time intervals of 0, 30, 60, 120, 180 and 240 minutes, 10 µL was removed from each donor compartment and replaced by the same volume of fresh HEPES buffer. In order to evaluate the effect of the electrical current intensity on the rate of plasmid transfer through the membrane, direct currents of 0.5, 1, 2, 3 and 4 mA were used. Also, in order to evaluate and compare the effect of plasmid concentration in the receptor compartment on the rate of plasmid transfer, the test was also conducted using pCMVβ plasmid with 60 µg/ml concentration. In order to show the effect of Iontophoresis on plasmid transfer through sclera, the test was also conducted on this tissue. For this purpose, the bovine eye sclera tissue was first removed by a scalpel and fixed between the two compartments of the testing cells. It should be mentioned that the surface area between the two compartments was 1 cm^2. In this test, 4 mA/cm^2 cathodic and anodic current and pUC19 which had smaller aggregate size with a concentration of 60 µg/ml were used. This technique was previously used for transfer of acetaminophen and dextran across sclera.[11]

Determining the Amount of Transferred Plasmid with Fluorescence Reagent

In order to determine the amount of samples with fluorescence reagent, the samples were initially diluted with TE buffer and three replicates of each sample were placed in three wells of plates with 96 cells (100 µL in each well). Then 100 µL of freshly prepared reagent of Quant-iT ™ PicoGreen was added to each well and 5 minutes after placing the samples in the dark, the fluorescence absorption was determined by a fluorimeter. According to the protocol, the excitation wavelength of DNA complex with reagent is 480 nm and the emission wavelength is 520 nm; the fluorimeter was adjusted in these wavelengths. Also, in each plate specific concentrations of standard DNA was poured to draw a calibration curve. Therefore, using the calibration and fluorescence absorption curves of each sample, the plasmid concentration and plasmid transfer rate were determined in the samples.[12]

Results

Plasmid Replication and Extraction

Gel electrophoresis testing showed that the plasmids were correctly replicate, extracted and the final plasmid solution has appropriate concentration, as well.

Evaluation of The Stability of Plasmid Solution in the Presence of Platinum and Silver Electrodes

Measuring the concentration using fluorescence reagents and kits showed that applying electrical current to the pCMVβ plasmid solution using a platinum electrode reduces the plasmid concentration. The greater the current strength, the greater plasmid degradation observed. In a current of 5 mA, the plasmid concentration reaches zero which indicates plasmid degradation in the presence of platinum electrode. In the case of using silver electrode, no change in concentration was observed after 2 hours of electrical current application. The image of gel electrophoresis for samples to which the current was applied with platinum or silver electrodes is shown in figure 1. In this image, the Lambda DNA marker is shown in part A for comparison which is the standard DNA and no current is applied to it.

Plasmid Aggregate Size in HEPES Buffer Solution

The average aggregate size for PCMVβ was 76 ± 7 nm with polydispersity index of 0.31 and the mean aggregate size for pUC19 was 62±4 nm with polydispersity index of 0.28.

Plasmid Transfer through Durapore Membrane Using Iontophoresis

The impact of Iontophoresis on the transfer rate of pCMVβ and pUC19 plasmids is shown in figures 2 and 3 respectively. As is shown in these figures, use of Iontophoresis results in increased plasmid transfer through Durapore membrane and with increasing the intensity of electrical current, more plasmid is passed

through the membrane. The more increased transmission was seen for pUC19.

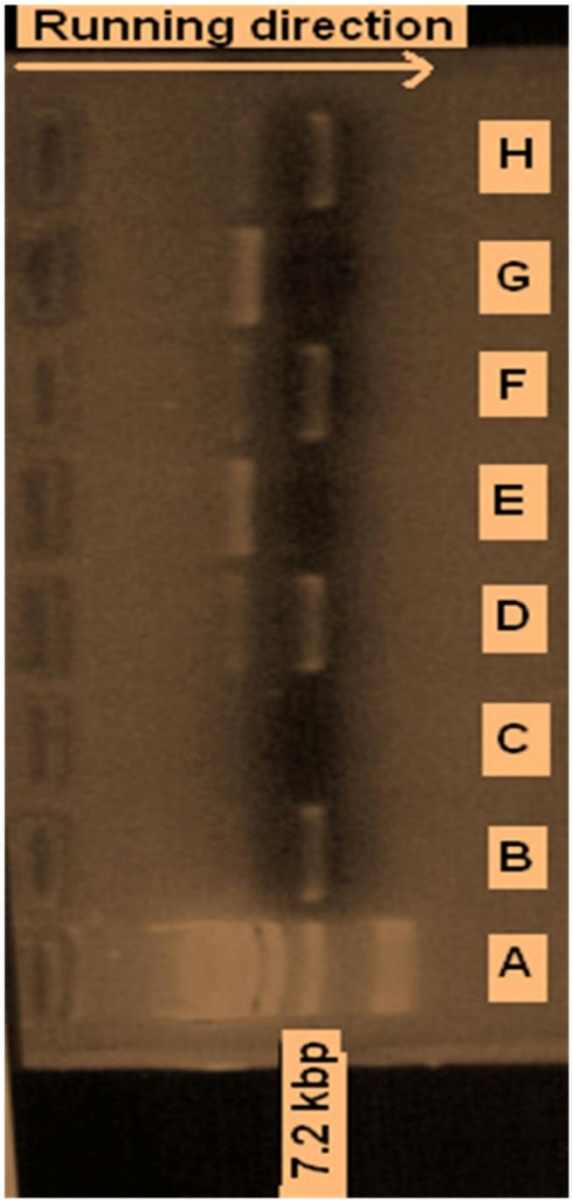

Figure 1. The image of gel electrophoresis for pCMVβ plasmid in the presence of silver or platinum electrodes and applying various intensities of electrical current; A: Lambda DNA marker without applying current, B: plasmid in the presence of silver electrode with 5 mA current, C: plasmid in the presence of platinum electrode with 5 mA current, D: plasmid in the presence of silver electrode with 2.5 mA current, E: plasmid in the presence of platinum electrode with 2.5 mA current, F: plasmid in the presence of silver electrode with 5 mA current, G: plasmid in the presence of platinum electrode with 5 mA current, H: plasmid sample after 2 hours without applying electric current. 7.2 Kbp indicates the number of base pairs of pCMVβ plasmid and the arrow shows movement direction of the DNA molecules from the negatively charged electrode towards the positively charged electrode.

In figure 4, the effect of plasmid concentration on the pCMVβ plasmid transfer rate is shown. As is evident, with increasing concentration from 20 to 60 µg/ml, the effect of electrical current on plasmid transfer from the membrane increased.

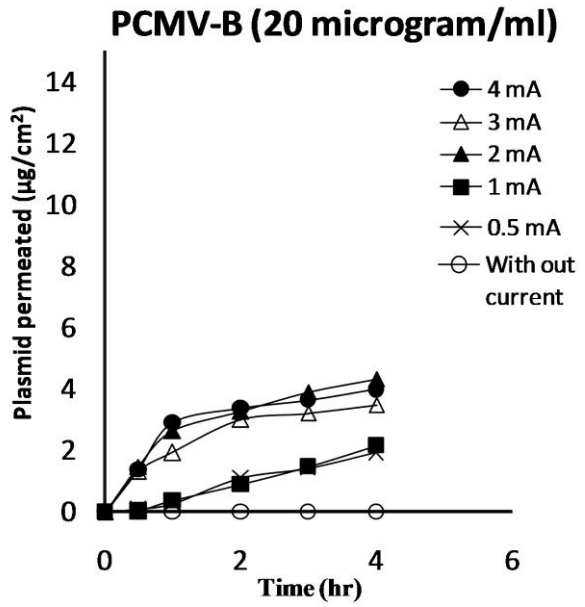

Figure 2. The impact of Iontophoresis with various intensities of electrical currents on transfer of pCMVβ from Durapore membrane model; the vertical lines represent SD (Standard Deviation) and the mean values are for three replicates for each sample.

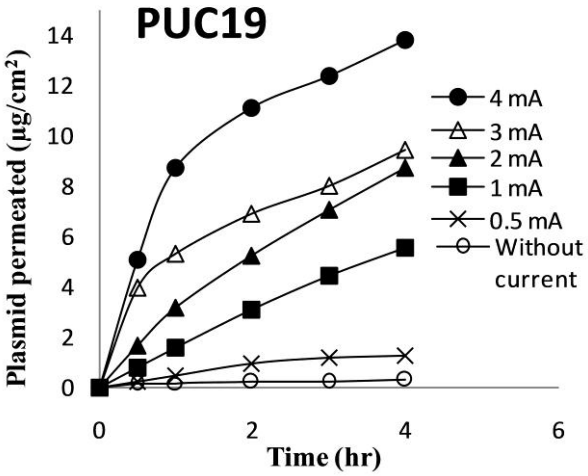

Figure 3. The impact of Iontophoresis with various intensities of electrical currents on transfer of pUC19 from Durapore membrane model; the vertical lines represent SD (Standard Deviation) and the mean values are for three replicates for each sample.

In the Exvivo test of pUC19 transfer through bovine eye sclera, no plasmid passed through the membrane after 4 hours with 4 mA/cm^2 current.

Discussion

As indicated in figure 1, use of platinum electrodes can cause damage to the plasmids; however, the plasmids stability was not affected by silver electrodes. The cause of this degradation can be explained by

comparing the reactions taking place in the anode and cathode of both electrode types. In the platinum electrode, the reaction is as follows:

Anodic reaction: $2H_2O \rightarrow 4H^+ + O_2\uparrow + 4e^-$

Cathodic reaction: $4H_2O + 4e^- \rightarrow 4OH^- + 2H_2\uparrow$

The overall reaction: $2H_2O \rightarrow 2H_2 + O_2\uparrow$

The oxygen produced in the reaction can cause oxidation of the sensitive plasmid molecules in the presence of the anode. It is previously reported that the platinum electrodes can cause oxidation of molecules much more stable than DNA.[13] The reaction in the silver electrode is as follows:

Anodic reaction: $Ag \rightarrow Ag^+ + e^-$

Cathodic reaction: $Ag^+ + e^- \rightarrow Ag$

In this type of electrode no oxygen is produced to cause oxidation of the plasmid.

Study of the aggregate size of both plasmids revealed their aggregation in HEPES buffer solution. The size of these aggregations is very important in passage of the plasmids through biological membranes. So that if the membrane pores size is smaller than the aggregate size of the plasmids, the plasmids cannot pass through the membrane. In the present study, the transfer of plasmids through bovine eye sclera was also evaluated; however, no plasmid was transferred through sclera tissue in different currents and the reason was the smaller scleral pores diameters in comparison with the size of plasmid aggregates. It is previously reported that by increasing the diameter of the penetrating molecule, the transfer rate from the scleral highly decreases so that if the diameter of the molecule reaches to more than 8.25 nm the transfer would hardly takes place.[14,15]

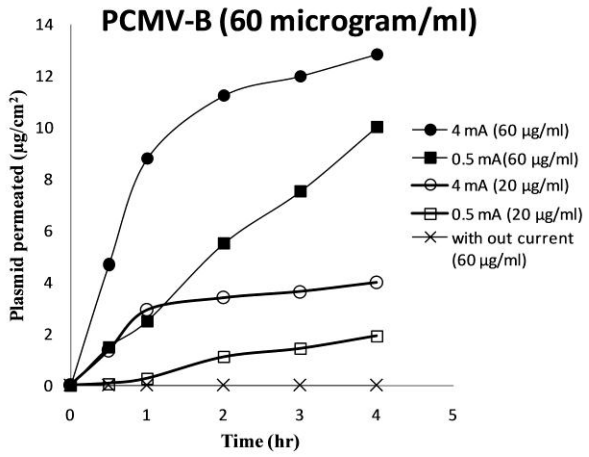

Figure 4. The effect of pCMVβ concentration on plasmid transfer rate from Durapore membrane. The vertical lines represent SD (Standard Deviation) and the mean values are for three replicates for each sample.

The effects of applying electrical current with increasing intensities on transfer of both types of plasmids from the Durapore membrane are shown in figures 2 and 3. As is shown in these figures, in the absence of electrical current, no pCMVβ plasmids passed through the membrane and only a small amount (0.4 ± 0.8 micrograms per square centimeter) of the pUC19 plasmid passed through the membrane within 4 hours. It was expected because of the plasmid aggregate size. By applying electrical current, the transfer rate increases so that in 4 mA/cm^2 current intensity the maximum transmission occurs. The effect of electrical current on the transfer of pUC19 is more than pCMVβ so that with this current, at the end of 4 hours, 13.78 ± 0.35 µg of pUC19 and 3.97 ± 0.2 µg of pCMVβ is transferred through the membrane. It is previously reported that the effect of Iontophoresis on molecule transfer through the biologic membranes is highly affected by the molecular weight and size of the molecule.[11] In figure 3 the effect of PCMVβ concentration in the donor compartment on transfer of this plasmid through membrane is shown. With increasing of plasmid concentration from 20 µg / ml to 60 µg / ml, the total amount of plasmid passing through increased from 2 ± 0.2 µg to 10 ± 0.3 in 0.5 mA/cm^2 current and from 4 ± 0.4 to 13 ± 0.4 in 4 mA/cm^2 current respectively, which indicates the dramatic effect of concentration on plasmid transfer through the membrane. This is proved that the effect of the drug molecule concentration on its transfer by Iontophoresis is highly important.[8]

Conclusion

It was the first time that the effect of Iontophoresis, molecule aggregate size and plasmid concentration on transfer rate of these molecules through a model membrane was evaluated. The results show that using Iontophoresis for plasmid transfer through biologic membranes, platinum electrodes can damage the plasmid molecules. Considering the results from the plasmid aggregate size, the plasmid aggregate size in different solutions must be determined before delivering the plasmids using Iontophoresis and the solution with least aggregate size should be selected. Increased intensity of electrical current and also increased plasmid concentration leads to increased transfer of these molecules through membrane.

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