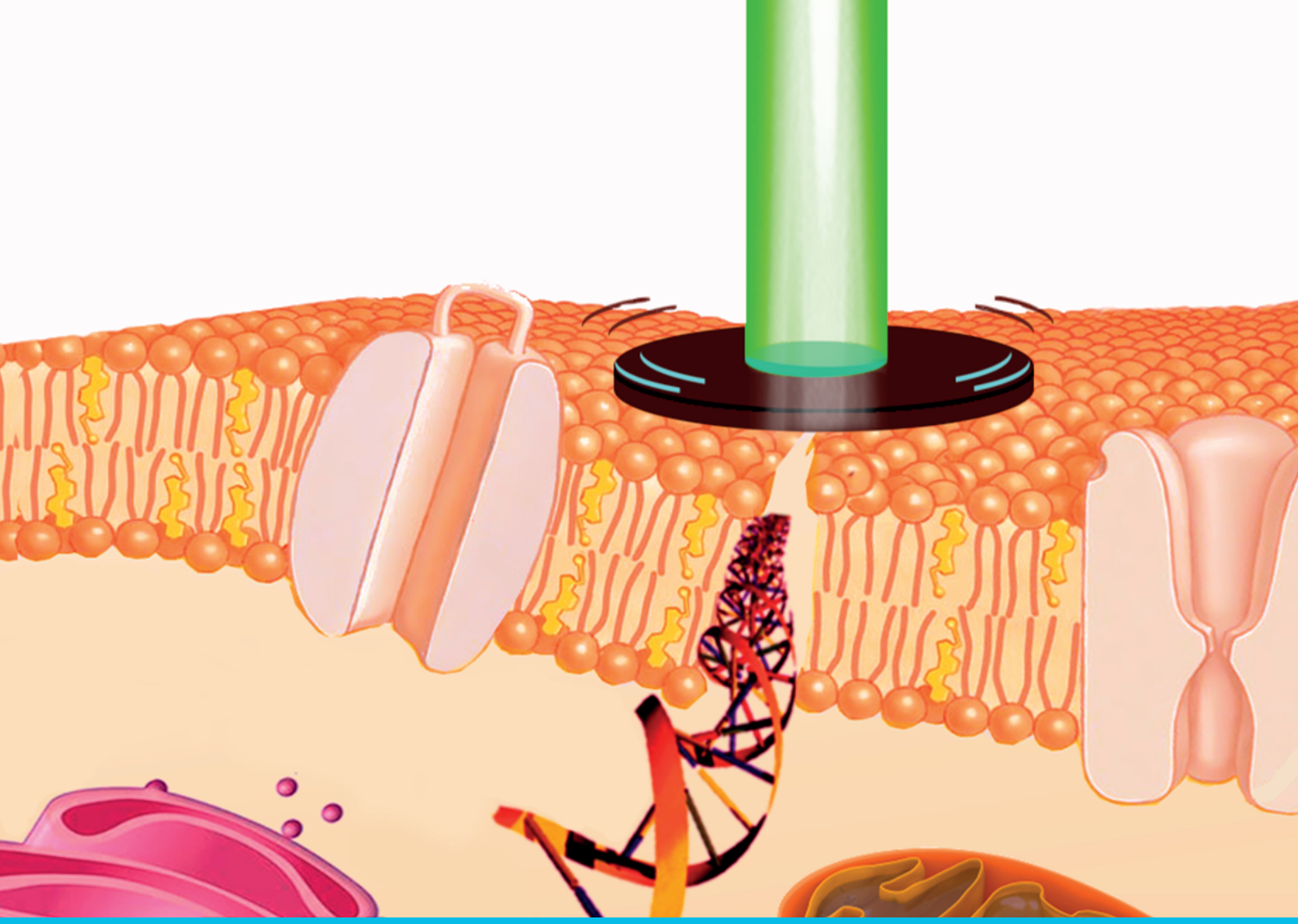

Alexandre Duarte Silva

PHOTOACOUSTIC WAVES FOR GENE THERAPY

Master in Medicinal Chemistry

Departamento de Química

FCTUC

September, 2015

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Universidade de Coimbra

Alexandre Duarte Silva

PHOTOACOUSTIC WAVES FOR GENE THERAPY

Dissertation presented as evaluation for the Master in Medicinal Chemistry

Supervisor: Prof. Carlos Serpa Soares

Co-Supervisor: Prof. Luís G. Arnaut

September, 2015

University of Coimbra

Aos meus pais e avós

Os meus pilares inderrubáveis

À minha irmã

O meu modelo de dedicação e esforço

À Mariana

Pelo amor, coragem e determinação

Em memória do meu avô Luís.

"Sei que vou guardar
Na alma um segredo.
Um estudante a cantar
E uma guitarra ao peito

Dizia:
Nos teus braços vejo o Mundo,
Sem pressa de embarcar.
Agora que chega a hora
Surge a memória
Do que vivi aqui.
Coimbra, ai quem me dera
Parar o tempo e ficar...

Quem neste largo escuta
Sente o medo e a tristeza:
Ir embora, ir à luta,
Não mais ver tua beleza

Coimbra:
Nos teus braços vejo o Mundo,
Sem pressa de embarcar.
Agora que chega a hora
Surge a memória
Do que vivi aqui.
Coimbra, ai quem me dera
Parar o tempo e ficar...
Nos teus braços vejo o Mundo,
Sem pressa de embarcar"

Balada da Despedida de 2015,
Grupo de Fado In Illo Tempore.

Agradecimentos

Embora a finalização de um ciclo de estudos e elaboração de uma tese, seja, pela sua vertente académica, um trabalho individual, este é, em bom rigor, um desafio que é objeto de vários contributos que não podem nem devem deixar de ser realçados.

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Um sincero agradecimento a todos.

"Tudo o que um sonho precisa para ser realizado…
…é alguém que acredite que ele possa ser realizado."

Roberto Shinyashiki

Lista de Comunicações Científicas

Comunicações Orais

"Ondas fotoacústicas aplicadas à transfecção de genes in vitro"
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Golden Prize – Melhor Comunicação Oral

Comunicações em Painel

"Non-Invasive Optical Diagnosis: An Opportunity In The Infrared"
II Simposio de Jóvenes Investigadores de la SEQT (Madrid, Maio, 2015).

"Near-Infrared Dyes For Non-Invasive Optical Diagnosis"
XXIV Encontro Nacional da Sociedade Portuguesa de Química (Coimbra, Julho, 2015).

"Photoacoustic Waves applied to gene transfection in vitro"
European Society for Photobiology Congress (Aveiro, Setembro, 2015).

Resumo

O sucesso da terapia genética depende do desenvolvimento de métodos seguros e eficazes na entrega de ADN externo às células alvo. A transfecção genética mediada por ondas de pressão induzida por luz laser, um método de transfecção não-viral, tem recebido especial atenção devido à sua segurança e ao alto controlo espacial dos pulsos laser. Pulsos de laser de curta duração (30 picosegundos e 8 nanosegundos), absorvidos por materiais piezofotónicos com elevadas eficiências de conversão de luz em pressão, originam transientes de ultra-frequências, ondas fotoacústicas, pela expansão termoelástica do material conversor. A característica distinta destas ondas fotoacústicas é a geração de gradientes de pressão repentinos (> 1 bar / ns) com fluências de luz laser baixas (menores que 100 mJ/cm^2), que são inofensivos, seguros (permitem uma viabilidade celular superior a 90% mesmo depois de vários minutos de exposição) e podem ser gerados repetidas vezes para permeabilizar a membrana celular bem como outras barreiras biológicas. Os resultados demonstraram o potencial das ondas fotoacústicas na terapia génica, com uma eficiência de transfecção superior a 1% em células da linha celular COS-7. O laser com pulso de duração de 8 nanosegundos provou ser menos eficiente do que o laser de picosegundos para as mesmas condições o que realça a importância da presença de maiores pressões bem como distribuições de frequências mais elevadas, e assim maiores gradientes de pressão, para o efeito de permeabilização da membrana celular. No futuro, depois de um conjunto de otimizações, será possível introduzir o método na prática clínica através da utilização de fibras óticas instaladas em cateteres tornando esta técnica uma das mais específicas e flexíveis em terapia génica.

Photoacoustic Waves for Gene Therapy

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KEYWORDS: Gene Therapy, Non-viral vector, Laser-based gene transfection, Photoacoustic waves, Biological barriers permeabilization, GFP

ABSTRACT: The success of gene therapy depends on the development of safe and effective methods to deliver foreign DNA to target cells. Gene transfection mediated by laser induced pressure waves, a non-viral method, has received attention because of its safety and the high spatial control of laser light. Laser pulses with short time widths (30 picoseconds and 8 nanoseconds), absorbed by piezophotonic materials with very high light-to-pressure conversion efficiency, give rise to ultra-high frequency pressure transients (photoacoustic waves) by thermoelastic expansion of the light-to-pressure material. The distinct feature of photoacoustic waves is the generation of very steep pressure gradients (>1 bar/ns) at low laser fluences (below 100 mJ/cm^2) which are harmless, safe (cellular viability above 90%) and can be generated repeatedly to permeabilize cellular membrane. The results demonstrated the ability of photoacoustic waves to enable gene therapy, with transfection efficiency of 1% in COS-7 cell line using plasmid DNA gWizGFP. The nanosecond laser has proved to be less efficient than the picosecond laser for the same conditions which shows the importance of higher pressures and higher frequencies in the cell membrane permeabilization effect. In the future, after a set of optimizations, will be possible to introduce the method in clinical practice through the use of optical fibres installed in catheters making this technique one of the most specific and flexible in gene therapy.

ABBREVIATIONS

DMEM, Dulbeccos's Modified Eagle's medium; PA wave, Photoacoustic wave; GFP, Green Fluorescence Protein; DNA, Deoxyribonucleic acid; LISW, Laser Induced Stress Waves; PAC, Photoacoustic Calorimetry; FACS, Fluorescence Activated Cell Sorting

INTRODUCTION

In the last years there has been a rapid development of molecular technology allowing the identification of hundreds of incorrect genes and genetic diseases. The prevention and treatment of many of these anomalies, such as cancer, hereditary diseases or infectious diseases, is possible by the introduction of DNA inside the affected cells in order to correct the protein expression errors which often leads to these diseases and body malfunctions, a process known as gene therapy [1].

The success of gene therapy and its introduction in clinical practice depends on the development of a safe method that can efficiently deliver DNA in human cells. There are two main classes of methods used in this field: methods which use viral vectors and methods that make use of non-viral vectors [2]. Viral vectors methods use viruses that effectively bind to host cells and introduce their genetic content in these cells operating as delivery vehicles of therapeutic DNA. Despite their efficiency, these methods have many constraints in terms of safety, particularly mutagenesis, unexpected immune responses [3] or lack of specificity in targeting [4], and also difficulties in producing large amounts of pure virus. In order to overcome the intrinsic safety problems raised from viral vectors, non-viral methods, that possess a larger scale production capacity, emerged as a research focus in recent years [5]. Electroporation [6, 7], ultrasound [8 - 10], microinjection or laser-induced stress waves (LISW) [11 - 19] are just some of the physical non-viral techniques currently studied to transiently increase the permeability of cell membranes.

Gene transfection mediated by laser light, and more specifically by pressure waves induced by laser light, has received special attention in recent years due to the advantages that this technique presents. Notable advantages are: the high spatial control of laser energy, the flexibility and the possibility of introduction into the clinical practice through the installation of an optical fiber across a catheter or endoscopically [20].

The laser induced stress-waves can permeabilize biological barriers, such as the cellular membranes, with a reversibility that enables cell viability thanks to the rapid recovery of the barrier function of membranes [21 - 23]. As an example, plasmatic membrane of mouse breast sarcoma cell line, EMT-6, recovers its protective functions 80 seconds after being hit by a 300 bar stress wave [23]. The use of this type of waves allows acting simultaneously on many cells**.** Another major advantage of the method is that such waves can be used for treatments in deep tissues due of its efficient propagation in human tissues. [17]. The application of LISW waves for gene transfection has been successfully applied both *in vitro* [11 - 12, 15] and *in vivo* [13 - 14, 16 - 18]. In all of the reported experiments, the transfected area corresponds to the area where the stress waves were applied demonstrating a very high spatial control of the technique.

Laser irradiation of a proper target material can produce two main types of stress waves: shock waves and photoacoustic waves (PA waves) [24]. Shock waves are generated by dielectric breakdown and material ablation (when the optical power density is above thermal threshold of the target material) and these waves are characterized by an abrupt discontinuous change in the material properties that propagates at a supersonic speed and induces a high increase in the temperature and pressure of the traversed medium [24]. Thermoelastic expansion generates the PA waves which propagates into the material at the speed of sound [17] with a moderate rise in system pressure. Thermoelastic expansion involves the transient heating of the material surface, and in the absence of a change of state, is the dominant mechanism of acoustic generation [24]. Stress waves generated by laser light are characterized by short rise times of the wave, high amplitude pressure transients and broader wave bandwidths [25, 26].

Doukas and co-workers [27, 28] explored the principles of transdermal and intracellular drug delivery with pressure waves generated by laser pulses, and showed the critical relevance of generating a pressure transient with high peak pressure and stress gradient (4 bar/ns in that case). However, their low energy-conversion efficiency required nanosecond laser fluences up to 5 J/cm^2 to affect biological barriers, which is unfeasible in the clinical environment due to the peak's pressures generated by ablation (p_{max} > 500 bars) that may damage the tissues.

More recently, Terakawa *et al* [15] successfully achieved gene transfer into mammalian cells promoted by nanosecond lasers induced pressure waves. They suggest that the transfection efficiency depends on the pressure applied integrated over time (impulse) rather than on the stress gradient (defined as peak pressure divided by wave rise time), a hypothesis also stated by Kodama *et al.* [29].

Studies related to gene transfer using PA waves (generated by thermoelastic effect) have been published by Visuri *et al* [30], where they use a diode-pumped frequency-doubled Q-switched Nd:YAG laser, which emits pulses of 532 nm light with 90 ns of duration, with a laser energy ranged from 100 to 350 μJ with repetition rate of the laser variable up to 5 kHz into a 1% solution (wt/vol) of Amaranth red dye solubilized in distilled water. They were able to increase to 14 times the incorporation of dyes with molecular weights up to 40,000 Da, in cells. Further developments of this methodology are not expected because their acoustic conversion is very low (use of exceptionally low laser energies): tensile part of the waves and rise times below the feasible minimum for disrupting biological barriers (0.1 bar/ns < 1 bar/ns) [27].

More recently we showed that 100 MHz PA waves with peak pressures $p_{max} \approx 12$ bar safely and painlessly perturb the skin barrier function and allow for the transdermal delivery of large molecules and proteins [31]. The distinct feature of PA waves is the generation of very steep pressure gradients (>2 bar/ns) at low laser fluences (below 50 mJ/cm^2) which are harmless, painless and can be generated repeatedly [26, 31]. The unique properties of these waves make them excellent candidates for permeabilization of cell membranes, balancing gene transfection efficiency with unprecedented safety [26]. The use of this technique was shown to be safe in Human Osteosarcoma cell line (MNNG/HOS) with cell viability above 90% [26], even when the cells are exposed to hundreds of PA waves in a short period of time when compared to the reduced number usually used in similar experiences, [12, 15].

These photoacoustic waves are generated by absorption of laser light pulses by piezophotonic materials that convert light into pressure. Efficient piezophotonic materials must have high linear absorption coefficients (μ_a), ultrafast radiationless transitions and high thermal expansions [26]. When such materials are confined by rigid boundaries and irradiated with short laser pulses, they launch intense PA waves [32]. The peak pressure of PA wave (p_{max}) generated is proportional to the energy absorbed (ΔH_{th}) and inversely proportional to the thickness (h) of the material [25, 33], meaning that the PA waves require thin films that absorb a large portion of incident light. The PA wave spectral band is that of the laser pulse when the expansion time of the heated volume — $\tau_s = 1/(\mu_a c_s)$, where c_s is the speed of sound in the medium — is shorter than the laser pulse duration, $\tau_s < \tau_L$ [34, 35]. "Piezophotonic" (light-to-pressure) transduction occurs when the laser pulse energy is absorbed and converted into heat in a lifetime τ_S shorter than τ_L. In other words, thermalization of the energy in the irradiated volume is faster than the laser pulse.

The sudden increase in local temperature produces a thermoelastic expansion of the transducer material and launches a broadband and high frequency ultrasonic wave (PA wave) that can interfere with biological membranes through volume and enthalpy fluctuations. The cell permeabilization can be mediated by physical interactions including heat, cavitation or mechanical stress [36].

This work highlights the generation of acoustic waves by pulsed laser irradiation of solids, which can be applied to the permeabilization of cellular membranes. Photoacoustic waves with moderate peak pressures and reasonable impulses (pressure over time) but high stress gradient (peak pressure divided by wave rise time) were used to overcome the barrier function of plasmatic membrane. The impact of the characteristics of PA waves and the time and method of exposure of cells to PA waves were evaluated for the transfection efficiency of plasmid coding for green fluorescent protein (GFP).

EXPERIMENTAL SECTION

Cell Culture

Immortalized cell line COS-7, monkey fibroblast cell line, was grown as monolayers in humidified atmosphere with 5% CO_2 at 37 °C. The cells were culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% antibiotics (penicillin and streptomycin) (Invitrogen). MilliQ water was deionized with a Millipore Milli-Q water purification system.

Piezophotonic materials, photoacoustic waves measurements and pressure measurements

Light-to-pressure transducer films, using either Mn^{III} 5,10,15,20-tetraphenylporphyrinate (Mn-TPP) [31] or Epolight 1178 (tetrakis amminium structure undisclosed and commercially available from Epolin Inc.) homogeneously incorporated in a polystyrene matrix was kindly provided by LaserLeap Technologies, S.A. (Coimbra, Portugal). The thicknesses of the films were measured with a digital micrometer and the absorbances were recorded in Cary 5000 Series UV-Vis-NIR Spectrophotometer (Agilent Technologies).

The photoacoustic waves measurements were performed using a front face irradiation set-up developed by our group [33]. This design is known to provide the maximum sensitivity of the very fast heat depositions of short-lived acoustic transients [25]. The samples of piezophotonic materials were irradiated through a quartz window. The photoexcitation of the Mn-TPP samples employed the second harmonic (532 nm) of Nd:YAG lasers (EKSPLA PL 2143A, with pulse duration of 30 ps, and Spectra Physics Quanta Ray GCR-130, with laser pulses of 8 ns) and the Epolight 1178 samples were excited with the fundamental wavelength (1064 nm) of the previous lasers. The photoacoustic signals were detected by Panametrics/Olympus contact piezoelectric transducers (100 MHz and 225 MHz) connected to a DPO7254 Tektronix digital oscilloscope (2.5 GHz bandwidth). Procedures for data collection and analysis were the same as for time-resolved PAC [34].

Pressure measurements were performed with a needle hydrophone calibrated for the 1 MHz to 20 MHz range (model MH28 from Force Technologies), connected to the previously mentioned oscilloscope, with two different setups (Figure 1A and 1B). Only the Mn-TPP piezophotonic film and the respective lasers were used in the experiments with the hydrophone. The light-to-pressure material was confined between an optical window (1.0 mm thick) and a mirror (0.6 mm thick) using silicone as acoustic coupler. The mirror reflected the light back into the piezophotoacoustic material and protected the hydrophone from the laser light. These three interfaces were tightly connected in a device manufactured by our group. The interfaces remain coupled even several laser pulses, in contrast to other experiments reported in the literature [37]. The pressure waves were detected immediately after the mirror in setup A or after a plastic interface placed over the mirror, also coupled with silicone, in setup B (Figures 1A and 1B). The signals were calibrated on the basis of a sensitivity of 167.08 nV/Pa (0.016708 V/bar), which was provided by the manufacturer.

The thermoelastic expansion that gives rise to PA waves is, in principle, non-destructive. In practice, signs of material fatigue are apparent after thousands of shots. This is visually characterized by a change in colour and a decrease in opacity of the films. The films used in the experiments described above were regularly replaced. The laser pulse energy was measured with a Power Meter (Newport Model 1918-C)

Generation of photoacoustic (PA) waves

As in previous pressure waves measurements, the generation of PA waves for subsequent studies in cells was used only Mn-TPP film as piezophotonic material as well as the same experimental design including the same lasers (Figure 1A and 1B).

In the “indirect method” the photoacoustic waves were transmitted to the cells by physical contact between the back of the mirror and the bottom of the well with the acoustic coupling being improved with a thin silicone layer. In the “contact method” the device that generate pressure waves was dipped in medium culture and placed in close contact with the cells. In these case, the mirror protected the cells from de laser light.

Gene transfection with photoacoustic waves

Cells in culture with confluence levels above 80% were detached with trypsin and subsequently seeded in 24-well plates at a density of 30.000 cells/well and in 12-well plates at a density of 40.000 cells/well in 1 mL and 2 mL of growth medium respectively. After 24 hours of incubation, and immediately before irradiation, the culture medium was removed and it was added 300 µL of plasmid solution in culture medium without serum and antibiotics. The used plasmid DNA encoding Green Fluorescent Protein, GFP, was the gWizGFP (Aldevron).

The "contact method” was tested on 12-well plates where the irradiation was carried out in direct contact with the cells. Alternatively, the "indirect method" was tested on 24-well plates, where the irradiation was carried out using the contact between the bottom of the culture plate and the photoacoustic wave generating device. To allow a better coupling was used silicone. Different exposure times to the photoacoustic waves, different plasmid concentrations and various laser fluences were investigated.

Immediately after the irradiation were added 700 µL and 1700 µL of culture medium supplemented with serum and antibiotics to the plates of 24 and 12 wells respectively.

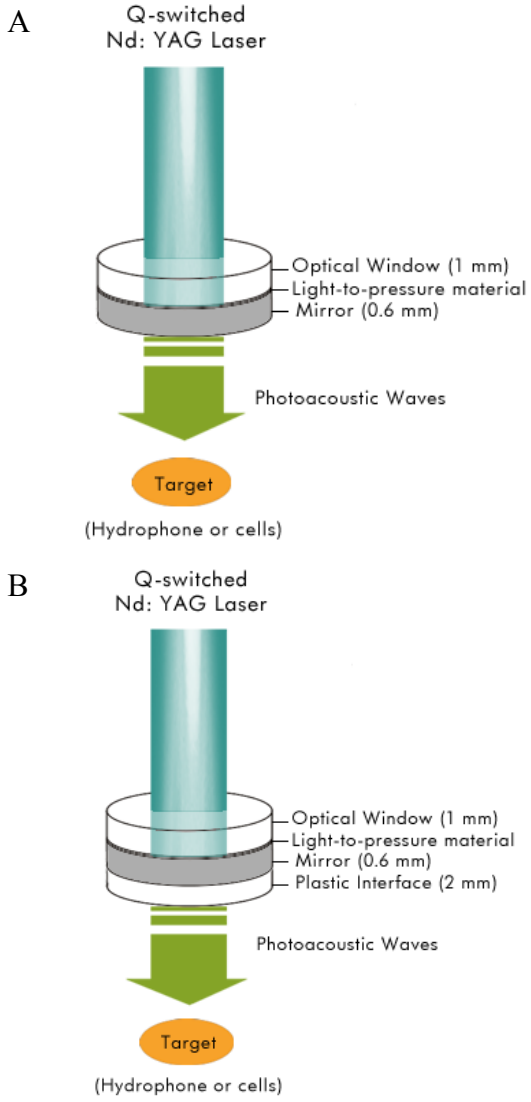


Figure 1. Experimental setup for generation of PA waves. Figure 1A represents “contact method” while Figure 1B shows the “indirect method”

Gene transfection with Lipofectamine

Lipofectamine 2000 (Invitrogen™), a cationic lipid used regularly in “in vitro” transfections, was used as a positive

control [38]. Transfection was performed in 12-well plates with the cells adhered to the well surface forming a monolayer with confluence levels close to 80-90%. DNA and Lipofectamine lipoplexes were prepared in growth medium (DMEM) without serum and antibiotics by addition of Lipofectamine 2000 (4 µL per well) with plasmid DNA at a concentration of 10 µg/mL. After 20 minutes of incubation, the lipoplexes were added to the cells in a final volume of 300 µL. Four hours after incubation was added 1700 µL of culture medium supplemented with serum and antibiotic.

Cell Viability and Transfection Efficiency

Cell viability was measured using the Alamar Blue ® assay, 24 hours after the exposure to the appropriate transfection method (PA waves or Lipoplexes) [39]. In this experiment has been added a volume of medium culture of 400 µL and 600 µL for 24-well plates and 12-well plates respectively, with 10% Resazurin. Fluorescence was analysed on a Microplate Reader (Synergy HT™ from BioTek ®) with excitation at 530 nm and emission observed at 590 nm.

The viability is given by the expression:

$$Cell\ Viability\ (\%)\ \frac{Fl\ 590\ nm\ treat\ cells}{Fl\ 590\ nm\ control\ untreat\ cells}\ x\ 100$$

Twenty-four hours after exposing cells to the photoacoustic waves or lipoplexes (DNA + Lipofectamine) GFP expression in the cells was evaluated. Initially this evaluation was performed using an inverted microscope (Olympus CKX41SF-5) coupled to a fluorescence system (Olympus U-RFLT50). GFP was excited using the filter BP460-490 nm and the emitted fluorescence was observed through a filter BA520IF. In a second phase, the percentage of cells expressing GFP was determined, i.e. the percentage of transfected cells, by flow cytometry. Flow cytometry measures the fluorescence intensity of individual cells. This allows the rapid fluorescence measurement of a large number of cells while simultaneously measuring the size and shape of the cells passing through the instrument. The flow cytometer (BD FACSCanto™ II, BD Biosciences) was set to collect data on 5000 cells using an excitation wavelength of 488 nm and detecting fluorescence at green portion of visible spectrum. The same number of cells were observed from each sample, correcting for any difference in cell density.

RESULTS

Piezophotonic materials, photoacoustic waves measurements and pressure measurements

Table 1 shows the properties of piezophotonic materials made with Epolight 1178 and Mn-TPP (a photoacoustic calorimetry reference) dispersed in polystyrene.

Film	Material	Dye	Thickness (µm)	λ_{ex} nm	Abs.	μ_a cm^{-1}
A	Polystyrene	Epolight 1178	70	1064	2.05	293
B	Polystyrene	Mn-TPP	70	532	1.23	178
C	Polystyrene	Mn-TPP	50	532	1.02	204

Table 1. Properties of representative piezophotonic materials used in this work.

Characterization of the photoacoustic waves (PA) generated by these piezophotonic materials, distribution of frequencies and peak pressure was performed and the results are shown in Figures 2, 3, 4 and 5.

The photoacoustic experiments for the films A and B are shown in Figure 2. For both lasers, 8 nanoseconds pulse (Figure 2A) and 30 picoseconds pulse (Figure 2B) the intensity of PA waves were higher for the film B (Mn-TPP) for the same laser fluences. This difference is more apparent for the laser with pulse duration of 8 ns.

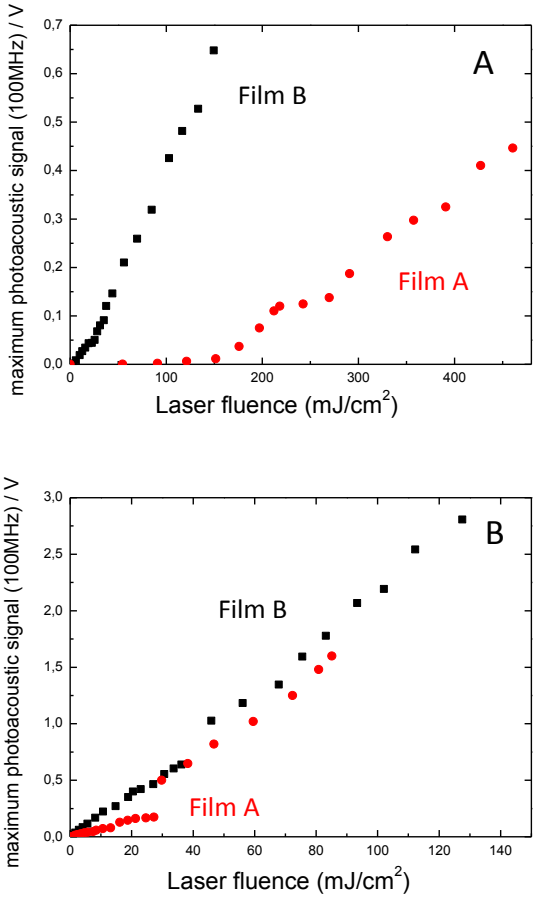


Figure 2. Maximum photoacoustic signal produced by Film A (red dots) and Film B (black square) upon pulsed laser excitation at 1064 nm and 532 nm, respectively, measured by a 100 MHz contact transducer at various fluences. Figure 2A shows the results for 8 ns laser pulse and Figure 2B for 30 ps laser pulse.

The representative Fast Fourier Transform (FFT) of PA wave produced by films A and B are reproduced in Figure 3 with excitation using laser pulses with 8 ns and 30 ps durations and detection with a 225 MHz transducer.

For both materials there is the occurrence of higher frequency components when using the shorter duration laser pulses. For the film A, the distribution of frequencies of PA waves, generated by it, is centred at 19 MHz for both lasers. The film B (Mn-TPP) when irradiated by 8 ns pulses originates PA waves focused at 30 MHz and when excited by pulses of 30 ps duration generates waves with distribution of frequencies centred at 55 MHz.

The response of this transducer is within −6 dB (i.e., power reduction by a factor of 4) of its maximum in the frequency range between 98.6 and 335 MHz, which means that the decrease in the signal by −30 dB (power reduction by a factor

of 1000) from the principal frequency and the last components of high-frequencies is not due to the transducer.

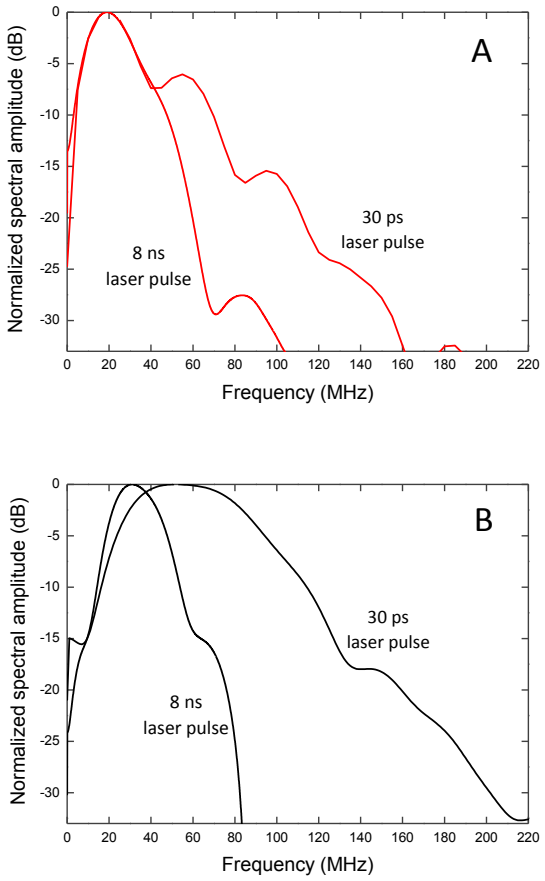


Figure 3. Representative FFT of the PA waves produced by Film A (Figure 3A) and Film B (Figure 3B) upon pulsed laser excitation at 1064 nm and 532 nm, respectively, measured by a 225 MHz contact transducer with 8 ns laser pulse and 30 ps laser pulse.

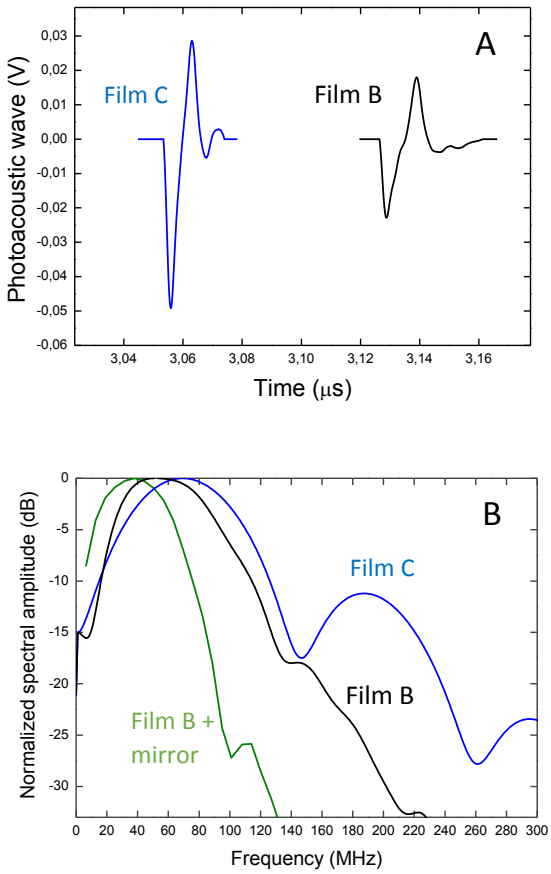


Figure 4A Pressure wave produced by Film B (black line) and Film C (blue line) upon pulsed laser excitation at 532 nm with 30 ps pulse duration as measured by a 225 MHz contact transducer. Figure 4B show FFT of the PA waves of Figure 4A (with the same colours) and present a FFT for the PA waves generated by Film B (green line) with a mirror (0.6 mm thick) between the film and transducer when excited in the same conditions.

Figure 4A shows, for the same laser fluence with pulses of 30 ps duration, the PA waves produced by Films B and C. Both films are made of Mn-TPP dispersed in polystyrene but have different thicknesses. It can be seen that while the same laser fluence, the Film C presents a PA wave more intense than the Film B and also presents a shorter temporal profile. The distribution of frequencies of these two waves are shown in Figure 4B. As previously mentioned, the PA wave produced by Film B has its frequencies centred at 55 MHz. The photoacoustic waves generated by Film C have substantially higher frequencies being centred at 70 MHz wherein the frequencies near 200 MHz are relatively intense in contrast to the waves produced by Film B.

Figure 4B also shows the distribution of frequencies of the waves generated by Film B when exist a mirror with a thickness of 0.6 mm between the film and the transducer (green line). In this case the PA wave detected immediately after the mirror presents a distribution of frequencies focused at 38 MHz with the lack of high frequencies.

This latest wave reflects the experiences performed over the remaining work, more precisely in the absolute pressure measurements and in the *in vitro* assays.

Film B immersed in water was irradiated with 100 mJ/cm^2 laser fluence using 8 ns pulse (I_L = 12.5MW/cm^2) and 30 ps pulse (I_L = 3333 MW/cm^2) at 532 nm and the PA waves produced are shown in Figure 5A and 5B respectively. The pressure measurements were performed for two experimental setups, the black lines represents PA waves when using the "Contact Method" (Figure 1A) whereas grey lines show the waves by using "Indirect Method" (Figure 1B) where there is a plastic (2 mm thick) coupled to the mirror, measured with a 20 MHz needle hydrophone.

Table 2 shows pressure parameters of the four PA waves represented in Figure 5, i.e. peak pressure Pmax +, Pmax - and the rise time of the first positive half-cycle, tr, defined as the time from 10% to 90% of the peak pressure.

Gene transfection with Lipofectamine

Lipofectamine 2000 ® is a synthetic cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell [38].

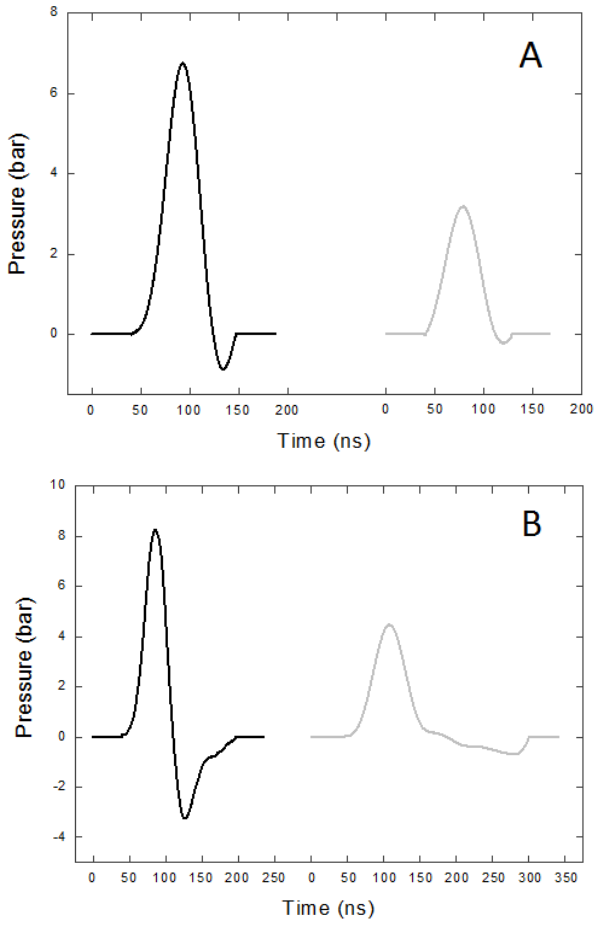


Figure 5. Pressure waves produced by Film B as a result of absorption of a 100 mJ/cm^2 at 532 nm and measured by a 20 MHz needle hydrophone. Figure 5A and 5B shows the pressure waves when the sample is excited with the laser of nanoseconds and picoseconds respectively. The PA waves represented with black lines were performed using the experimental design 1A while the pressure waves expressed as grey lines were obtained with the setup 1B.

PA Wave	Pulse duration	Setup	P_+ (bar)	P_- (bar)	t_r (ns)
A_1	8 ns	Contact	6.8	0.9	27
A_2	8 ns	Indirect	3.2	0.2	25
B_1	30 ps	Contact	8.3	3.2	23
B_2	30 ps	Indirect	4.5	0.7	33

Table 2. Photoacoustic waves parameters of Figure 5 measured by a 20 MHz Needle Hydrophone. Values are representative of several experiments.

The cationic lipid molecule is often formulated with a neutral co-lipid (helper lipid). The positive charge on the surface of the liposome generates an electrostatic interaction with phosphate groups of nucleic acids and facilitates contact with the negatively charged cell membrane. The neutral co-lipid mediates fusion of the liposome with the cell membrane effecting entry of the nucleic acid [38]. These lipoplexes are used as positive control allowing a comparison between a traditional method of in vitro transfection and the method under study, PA waves.

The negative control, performed only in presence of plasmid, showed no transfected cells (Supplementary Information) showing that high molecular weight molecules, such as plasmids, cannot cross the cell membrane without the presence of a delivery system.

Figure 6 shows GFP expression by cell line COS-7 24 hours after transfection with Lipofectamine 2000 ®. The transfection efficiency can be evaluated by either the number of cells expressing GFP and by the intensity of this fluorescence.

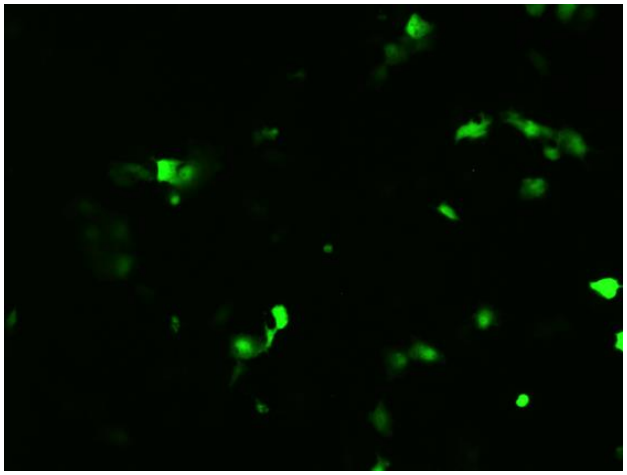


Figure 6. Representative photo of GFP expression by cell line COS-7 24 hours after transfection with Lipofectamine using plasmid gWizGFP [10 μg/mL].

Gene transfection with PA waves – "Indirect Method"

Cells when exposed to pressure waves undergo permeabilization of the membrane and, therefore, is expected an increase of the delivery of high molecular weight molecules, such as plasmids [11, 12, 15]. Figure 7 illustrates some cells expressing GFP, i.e., transfected cells, 24 hours after having been subjected to PA waves for 10 minutes in the presence of a plasmid concentration of 100 μg/mL by the "Indirect Method" with 100 mJ/cm^2 of laser fluence (30 ps pulse duration)

Different times of exposure to PA waves than as described were tested, in particular 5 minutes, as well as the 8 ns laser pulse, but there were fewer transfected cells. (Supplementary Information).

Gene transfection with PA waves – "Contact Method"

As seen above, PA waves can permeabilize the cell membrane in a way that large materials present in the extracellular medium enter into the intracellular environment.

Despite this, the transfection levels obtained were very low. Thus, in an attempt to improve the efficiency of the "indirect method", was used a different protocol, referred as "contact method" where the PA waves were applied directly on the cells, dipping the generating device in the culture medium.

Different laser light fluences, DNA plasmid concentrations and different exposure times were tested to understand the influence of each parameter on transfection efficiency.

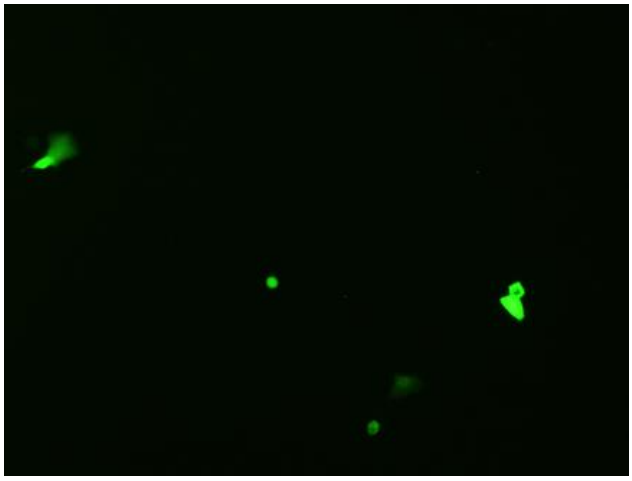


Figure 7. Representative image of gene transfection with gWizGFP [100 µg/mL] 24 hours after the application of PA waves with the "Indirect Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence (30 ps pulse duration).

Figures 8 and 9 show the results of transfection, 24 hours after application of PA waves for 10 minutes by the "Contact Method" with 100 mJ/cm^2 of laser fluence (30 ps pulse duration) in the presence of a plasmid concentration of 100 µg/mL and 500 µg/mL respectively. In both figures is clear that the results for this method were significantly higher compared to the previous method.

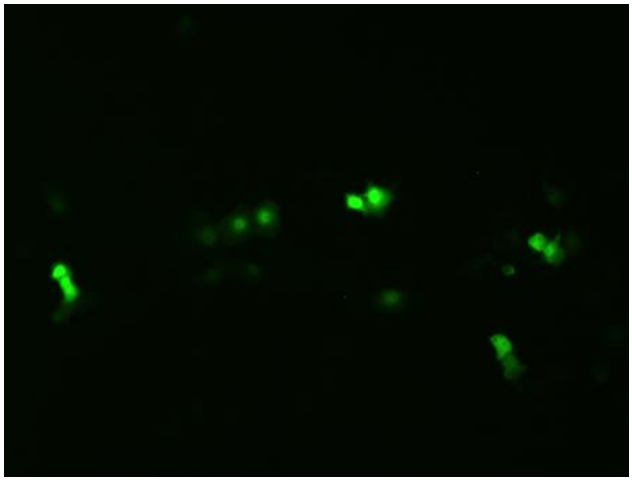


Figure 8. Representative image of gene transfection with gWizGFP [100 µg/mL] 24 hours after the application of PA waves with the "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence (30 ps pulse duration).

Different laser fluences than as described were tested, namely 33 mJ/cm^2 and 66 mJ/cm^2 well as various times of exposure to PA waves, in particular 3, 5 and 15 minutes, and the other laser pulse (8 ns of duration) but also there were fewer cells expressing GFP. (Supplementary Information).

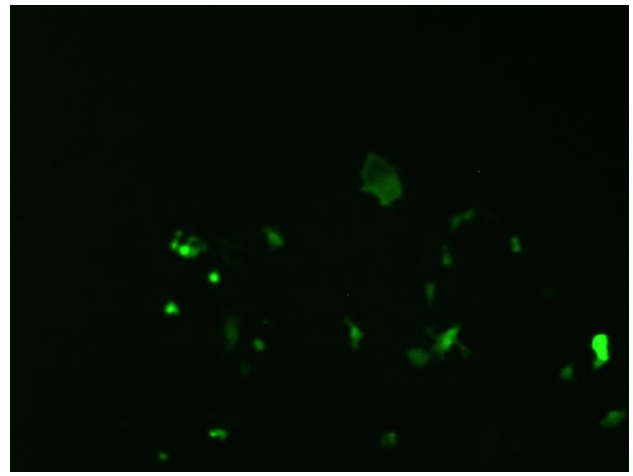
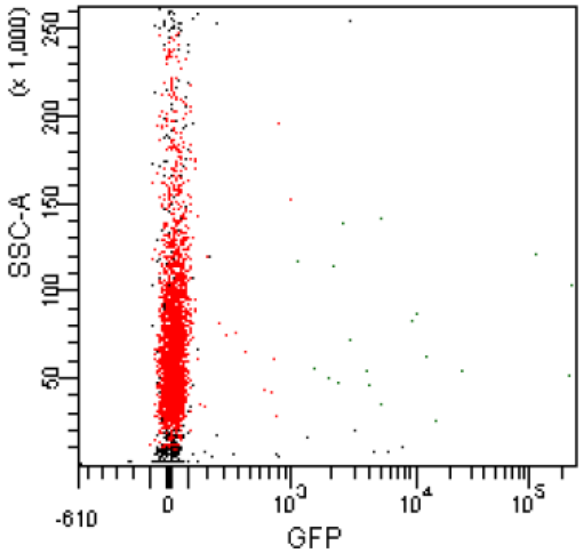


Figure 9. Representative image of gene transfection with gWizGFP [500 µg/mL] 24 hours after the application of PA waves with the "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence (30 ps pulse duration).

The negative control in both methods showed again that, alone, the plasmid cannot cross de plasma membrane, as demonstrated by the absence of any cell expressing GFP (Supplementary Information).

Flow Cytometry Results and Transfection Efficiency

After an initial optimization stage using the fluorescence microscope to determine the efficacy of the method qualitatively, flow cytometry with FACS methodology (Fluorescence Activated Cell Sorting) was used for the conditions optimized in the previous studies, in order to obtain the efficiency of transfection quantitatively. Figures 10 and 11 show the results for gene transfection with gWizGFP [100 µg/ml and 500 µg/mL, respectively] 24 hours after the application of PA waves with the "contact method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence.



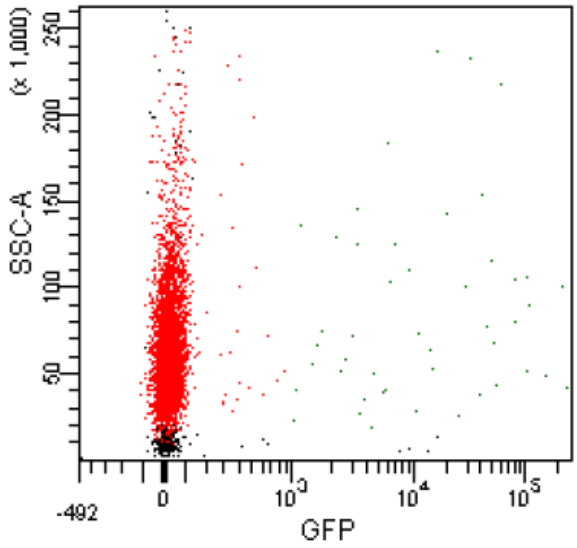


Figure 10 (up) and 11 (down). GFP expression by the cells (green dots) in relation to their side-scattered light (SSC) 24 hours after the application of PA waves with the "contact method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with gWizGFP [100 μg/ml and 500 μg/mL, respectively].

The negative control showed no transfected cells (Supplementary Information). The transfection using lipofectamine reached 23% of transfected cells (Supplementary Information). Figure 9 shows a percentage of 0.6% of cells expressing GFP while Figure 10, for the highest plasmids concentration, achieved 1% of transfected cells.

Cell Viability Assays

The viability of transfected cells using Lipofectamine or PA waves (directly and indirectly at fluence laser of 100 mJ/cm^2) is shown in Figure 12. The use of Lipofectamine proved to produce more damage to cells, leading to a cell viability in the order of 80%. The PA waves exposed in direct contact to cells reduce the cell viability by around 5%, whereas the application through the plastic of culture plate yielded a cell viability indistinguishable from the control.

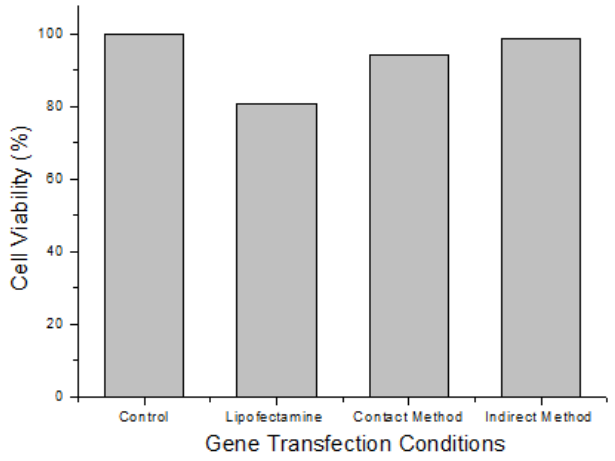


Figure 12. Cell viability for the cell line COS-7 over different conditions of transfection. "Contact Method" and "Indirect Method" were performed at a fluence laser of 100 mJ/cm^2 with pulse duration of 30 ps.

DISCUSSION

Materials presented in Table 1 present some properties that can make them good piezophotonic converters. The Mn-TPP was chosen because it is a known photoacoustic reference compound (species that convert all the absorbed light into heat in a time shorter than the laser pulse) and associated with polystyrene meets the necessary requirements for piezophotonic materials. For the dye Epolight 1178 a complete photoacoustic characterization is not fully done. Although we do not know the full structure of this dye, its basic structure qualifies this dye has a "molecular heater" [40, 41]. Using this dye in a future piezophotonic material can have enormous advantages due to the high absorption at 1064 nm, the fundamental wavelength of Nd:YAG lasers. In order to understand their photoacoustic behaviours, several photoacoustic measurements were carried out under the same conditions (except as shown in Table 1, the excitation wavelength) to compare Mn-TPP and Epolight 1178 in polymer matrix films.

Figure 2 shows that both materials are able to generate pressure waves mainly through thermoelastic expansion mechanism due to the absence of a change of state. For any of the four cases presented in Figure 2, the progressive use of higher fluences, i.e., more energy deposition in the irradiated material, produced PA waves of higher intensities, following equation Arnaut et al [25]:

$$\Delta p = \Delta H_{th}\, \gamma / h \quad (1)$$

where ΔH_{th} is the thermal energy released per unit area, γ the Grüneisen coefficient that characterizes the thermoelastic behaviour of materials and h the thickness of the material.

The maximum pressure amplitude generated by thermoelastic expansion after absorption of a laser pulse with the optical power density I_L is given by [24]:

$$P_{max} = I_L\, \gamma / c_s \quad (2)$$

This equation explains that for identical fluences of the two lasers, the intensity of the PA waves is higher for the laser with 30 picoseconds pulses. Its higher optical power density (I_L) leads to a higher peak pressure.

A comparison of the film A and B, with Epolight 1178 and Mn-TPP dyes, respectively, showed that for identical fluences of laser light, the Mn-TPP film generates photoacoustic waves of higher intensity regardless of the laser pulse duration used. This demonstrates that the Epolight cannot reach such good results as a photoacoustic reference possibly due to other concurrent photochemical processes.

Regarding the distribution of frequencies of the PA waves presented in Figure 3, the excitation with picosecond laser pulses generates waves with higher frequencies, which can be seen for both materials. The Mn-TPP film also showed higher frequencies than the Epolight film for the same laser. In any of the films, the distribution of frequencies is identical for all fluences used, which suggests that, even for higher fluences, the predominant mechanism of generation of pressure waves is the thermoelastic expansion.

As mentioned in the introduction, when the acoustic relaxation time τ_s is shorter than the laser pulse, $\tau_s < \tau_L$, where $\tau_s = 1/(\mu_a\, c_s)$, the spectral band of the laser pulse will determine the spectral band of the PA wave [34,35]. Thus, short laser pulses gives ultrahigh frequency waves. For example, in this case, a pulse with 8 ns of duration would provide a distribution of frequencies near of 125 MHz.

Both films, due to their properties, have acoustic relaxation times higher than the laser pulses used, 14 ns for film A and

24 ns film B, reason why the frequencies of the photoacoustic wave are limited by this factor. The Mn-TPP meets the criteria of a photoacoustic reference and due to this, its results are closer to the expected contrary to Epolight that does not seem to behave as a photoacoustic reference.

The better results obtained with film B for both intensity and distribution of frequencies of generated PA waves, and also some technical limitations with Film A, led us to select Film B for pressure measurements studies and subsequently for cell assays. Film B can produce PA waves with higher peak pressure and with acoustic wavelengths commensurate to the size of the cells ($\lambda = c_s / f$).

Figure 4A shows the importance of piezophotonic material thickness in the conversion of light in PA waves when approximately the same amount of energy is absorbed (equation 1), because the thinnest film (Film C) originated the pressure wave of higher intensity. Furthermore is also known that ultra-high frequencies are strongly attenuated in the materials where they propagate and the detected intensities are diminished even when the source is very close to the detector. This justifies the fact that the film C presented a distribution of frequencies considerably more shifted to the high frequencies than the film B (Figure 4B) [42]. This attenuation of the higher frequencies is also evident when a mirror is placed (with a thickness of 0.6 mm) after the film (Figure 4B). The center of the distribution of frequencies is lowered to 38 MHz and high frequencies are absent. This case is representative of the PA waves which generated in the absolute pressure measurements and subsequently in the cell assays (Figure 1A and 1B).

Figure 5 and Table 2 show the photoacoustic waves, and their properties, produced by Film B under different conditions at the same laser fluence, 100 mJ/cm^2. Either in the "contact method" and in the "indirect method" the laser of picoseconds caused a PA wave with higher value of absolute pressure which is explained again by equation 2: higher optical power densities generate stress waves with higher peak pressures. The observation of an absolute pressure value lower for the "indirect method" is assigned to the energy dissipation by the PA wave when it crossed the plastic that separates the mirror and the hydrophone. For any of the four cases the absolute pressure value obtained was proportional to the laser fluence used (data not show). These pressure values are always underestimated due to the technical difficulties that are inherent to the methodology, as lack of spatial accuracy or setup instability. The rise time values shown in Table 2, to the four situations, are limited by the resolution of the hydrophone (50 ns) whereby in either case this value is lower than the obtained.

In order to use the previous PA waves without safety restraints in gene transfection, the viability of cells was evaluated after their exposure to such waves, for both methods of application with the PA waves that achieved higher pressure (B_1 and B_2, Table 2). The results showed more than 94% of cell viability (Graph 1) confirming the safety of the application of PA waves.

These values have correspondence with the results presented by Terakawa and colleagues [12, 15] on their experiences, 88% and 90% cell viabilities were reported. The lowest cell death caused by the "indirect method" should be related to the loss of intensity of PA waves when these waves cross the plastic of the well where the cells were grown, as evidenced by the pressure decrease in pressure measurements (Table 2), as well a loss of high frequencies (data not show). The intensity decrease in the PA waves will lead to less disruption of lipid packaging and, consequently, a lower cell death. Moreover, manipulation of cells in the "contact method" may lead to some cellular damage by the action of mechanical forces extrinsic to the method itself. Other physical methods used in gene transfection, such as electroporation or ultrasound (low frequency), show cell survival values in the range of 70% [6, 8], considerably lower than the values presented by PA waves.

This shows that thermoelastic generation of PA waves through the irradiation of piezophotonic materials with low intensity lasers meets the safety requirements for gene transfection. Once the application of PA waves was validated in terms of safety, and taking into account the physical characteristics of these waves, they were judged capable to successfully promote gene transfection.

Negative controls, in the presence only of the plasmid DNA, were used to confirm that the plasmatic membrane of the cells acts as a barrier to the transport of large molecules (Supplementary Information).

The use of Lipofectamine 2000 ® as mediator of transfection was used as a positive control of the method under study. This is a well-known and efficient method of transfection, and a positive control informs on the good conditions of all reagents and materials involved in this complex process (Figure 6).

Figures 7, 8 and 9 show COS-7 cells, expressing GFP 24 hours after application of PA waves, and highlights the potential use of this technique in gene therapy. These results confirm that the pressure waves can permeabilize not only the cell membrane but also the cell nucleus as observed before [43].

Both the experiments with lipofectamine or with the application of PA waves reveal a higher number of cells expressing GFP, i.e. transfected cells, and a higher GFP fluorescence intensity at higher incubation times (data no show). This result confirms that the cells remain viable even after longer periods of time and are capable of carrying out gene expression of the incorporated plasmid and produce the target protein, after the cell division processes. This further demonstrates that PA waves did not affect the plasmid, which remained unchanged and viable. Although the assessment of transfection efficiency at latter times could favour measurement sensibility, in order to compare our findings with the work of other authors, the results presented here always refer to 24 hours after the transfection.

Figures (7, 8 and 9) show that there are a higher numbers of transfected cells when the PA waves were applied directly on the cells, in the "contact method". The reason for this result is the same as that pointed out for the cell viability results which indicates that the PA waves applied indirectly, "indirect method", are attenuated when crossing plastics and lose their higher frequencies. PA waves with lower intensities and lower frequencies cause less disruption of the cellular membrane and lead to lower levels of transfection. The absence of transfected cell in the negative controls show, once again, that the PA waves are strongly attenuated in such materials because the neighbouring wells of the negative controls were exposed to PA waves. The plasmid concentration seems to be a relevant factor given the higher efficiency of transfection when higher concentrations are used (Figure 8 to 11). The increase in the application time of the PA waves also leads to more efficient transfection (Supplementary Information). Finally, the nanosecond laser proved to be less efficient than the picosecond laser under the same conditions which emphasizes again the importance of higher pressures and higher frequencies in the

permeabilization of cell membranes (Supplementary Information).

Once the critical variables influencing transfection efficiency were understood, flow cytometry was performed as a quantitative method for detecting transfection. This was done with the best conditions obtained with the qualitative fluorescence microscopy evaluation method, namely, the "contact method", 10 minutes of exposure to picosecond laser pulses and higher concentrations. Flow cytometry determined the percentage of transfected cells after the application of PA waves in the whole volume of the wells. We should mention that in our experimental protocol only a fraction of the cell culture area (approximately 20%) is actually exposed to PA waves. In microscopy we see transfected cells only in the area of the well exposed to PA waves, and no transfection outside that area. For the quantitative cytometry analysis, all the "cell area" was used to assess the transfection efficiency and efficiency of approximately 1% was calculated.

Our measured transfection efficiency is close to that achieved by Terakawa *et al* [12] where in the NIH 3T3 cell line they obtained a transfection efficiency of 2 %, at 37 ºC and with 500 μg/mL of plasmid. In another experiment, Terakawa [15] reached 0.6 % at the same temperature but with a plasmid concentration of 100 μg/mL. In both assays the laser fluences were greater than 1.5 J/cm^2 and their pressure waves achieve maximum pressure of 500 bars [12, 15], about 50 times the maximum pressure of our PA waves. Another important factor which should facilitate the transfection efficiency in the work of Terakawa relative to our work, is the molecular weight of transfected plasmid. In fact, that group was able to permeate cell membrane with a plasmid of 4700 bp (3.01 MDa) whereas we transfected cells with a plasmid significantly larger, 5757 bp (3.74 MDa).

Terakawa et al [12, 15] have shown that the transfection efficiency is three times higher for temperatures above 37 °C (i.e., 43 °C and 45 °C) due to the increase in fluidity of plasma membrane. The cells were incubate at 37 °C in our experimental procedure, but, at the time that PA waves were applied the temperature was cooled to 26 °C (room temperature). This decrease may have the opposite effect to that achieved by the temperature increase and make membrane permeabilization more difficult.

A major advantage of our work relative to that of Terakawa *et al* is the fact that our method generates reproducible PA waves and may be used to generate thousands of PA waves, while their technique is limited to a few laser shots with very high energy lasers, which may not be feasible in clinical practice.

Cell permeabilization mediated by low frequency ultrasound is generally associated with cavitation. Cavitation is the disturbance of lipid packing by the explosion of air bubbles in the bilayer [44]. The minimum pressure amplitude that satisfies the cavitation threshold increases with the pressure wave frequency, f_{PW}. To occurs cavitation, the Mechanical Index of the pressure wave, defined by $MI=p_{max}/\sqrt{(f_{PW)}}$, where p_{max} is expressed in MPa (1 MPa = 10 bar) and the pressure wave center frequency f_{PW} expressed in MHz, should be lower than 0.5 [31]. Thus, this mechanism should not occur with our waves (in the MHz range) because the Mechanical Index associated with them is below the cavitation threshold (MI < 0.2 in all cases), in contrast to low-frequency sonophoresis (with MI > 0.5) or shock waves produced by the high-energy lasers (MI > 10) [27].

The efficiency in the permeabilization of the cell membrane, and thus in the transfection, seems to be dependent on the pressure applied and in the PA wave rise time which explains why higher pressures and waves with wider distribution of frequencies (and therefore lower wavelength acoustic waves, more commensurate with the size of the cells) yielded higher efficiencies. Thus, cell permeabilization may be due to mechanical effects associated with steep pressure gradients (> 1 bar / ns and > 0.1 bar / μm). Such effects may directly perturb the biomembranes or mediated cytoskeleton rearrangement [45].

Terakawa *et al* [15] suggests that the mechanism will probably depend on the size of the molecule that crosses the membrane. If the molecule has a low molecular weight, the permeation mechanism must go through denaturation and expansion of membrane proteins. However, for high molecular weight molecules such as plasmids, shear forces that momentary disrupt of the plasma membrane seem to at the basis of the transfection mechanism.

CONCLUSION

In conclusion we demonstrate that the application of PA waves with pulses of duration in the nanosecond and picosecond range, *in vitro*, can permeabilize the cell membrane and thus, delivering to the cells a DNA plasmid. This non-viral transfection method demonstrated to be safe, with cell viabilities higher than 94%. The best results obtained so far are a percentage of transfected cells of at least 1%. This is probably an underestimate because only part of the cell present in the medium were subject to the PA waves. The best estimate if all the cells were exposed to photoacoustic waves is about 5%.

Gene transfection using PA waves is safe and attractive method for targeted gene therapy but still needs further studies. Optimization of some conditions like experimental setup, plasmid concentration, temperature, piezophotonic materials and other variables proved to be essential to achieved best efficiencies and thus increase the PA waves potential as a tool for gene therapy.

The appropriate design of a piezophotonic materials to efficiently respond to picoseconds lasers (i.e., a dye that strong absorbs in a thin film with good thermoelastic properties, $\gamma > 1$ and very short lifetimes), will lead to PA waves with broadband of GHz frequencies and thus to pressure gradients commensurate with the size of an individual cell. Such piezophotonic devices, would revolutionize gene therapy.

It should be will be possible to introduce this method in clinical practice through the use of optical fibres installed in catheters, making this technique one of the most specific and flexible in gene therapy.

A possible therapeutic aim may be, for example, the introduction of a gene that produces a coagulation factor in liver cells of patients with haemophilia [46]. Conventional therapy used nowadays involves the regular injection of protein (coagulation factor) in the patients, and is one of the more expensive therapies in the world, with an annual cost per person (in the USA) above 300.000$.

The safe, effective, affordable and scalable non-viral transfection method studied in this project may provide nem opportunities to manage haemophilia.

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Photoacoustic Waves for Gene Therapy

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SUPPLEMENTARY INFORMATION

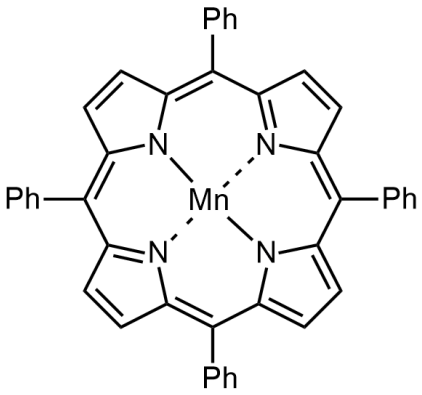


Figure 1. Molecular structure of Mn-TPP (Mn^{III} 5,10,15,20-tetraphenylporphyrinate).

Preparation of culture medium

For 1 L of DMEM medium, 13.4 g of DMEM, 2.4 g of HEPES and 2.0 g of sodium bicarbonate were added to 890 mL of milli-Q water, 100 mL of FBS (fetal bovine serum), and 10 mL of PS (antibiotics). The mixture was stirred until a homogeneous solution was obtained. Solution was filtered, inside the laminar hood, through a filter with a porosity of 0.2 μm.

Preparation of cells to Flux Cytometry

Aspirate off culture medium and wash cells with 0.5 mL PBS and after that aspirate off PBS. Add 0.5 mL 1x trypsin to the cells and let sit for 5-6 minutes in the incubator. With the micropipette aspirate and blast off several times the trypsin to help in the detachment. Remove all cells and trypsin and centrifuge for 8 minutes at 1350 rpm. Discard the supernatant and resuspend in 0.5 mL of PBS.

Measurement of absorbance

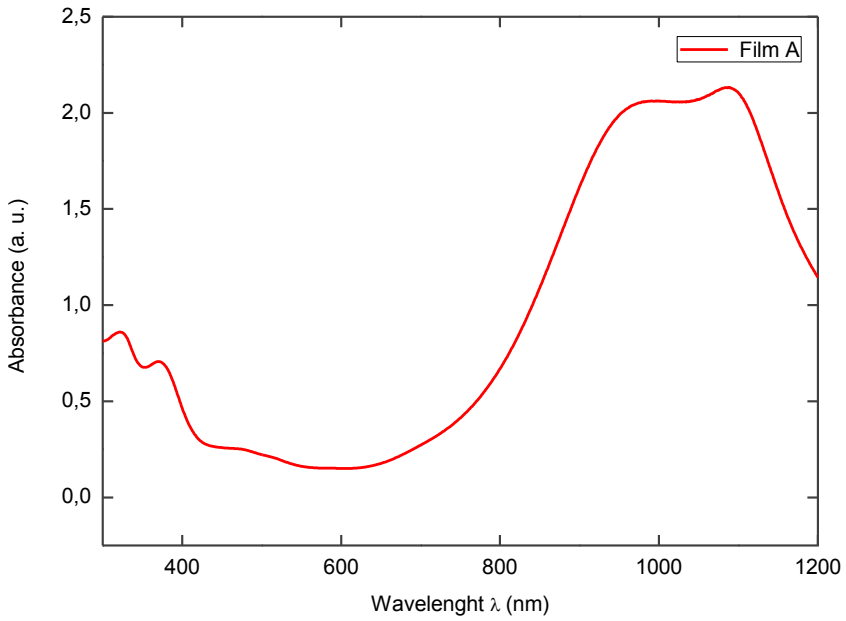


Figure 2. Absorbance spectra of Film A (Epolight 1178).

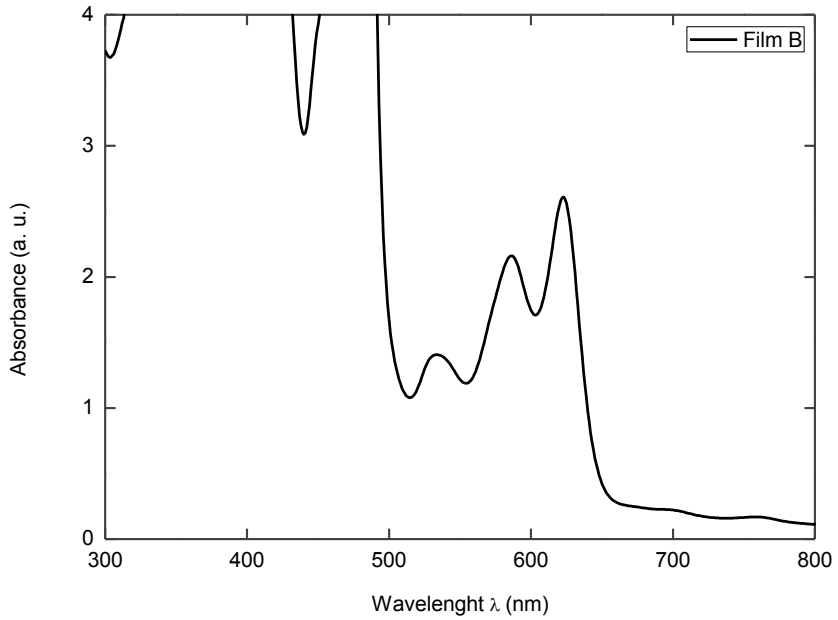


Figure 3. Absorbance spectra of Film B (Mn-TPP).

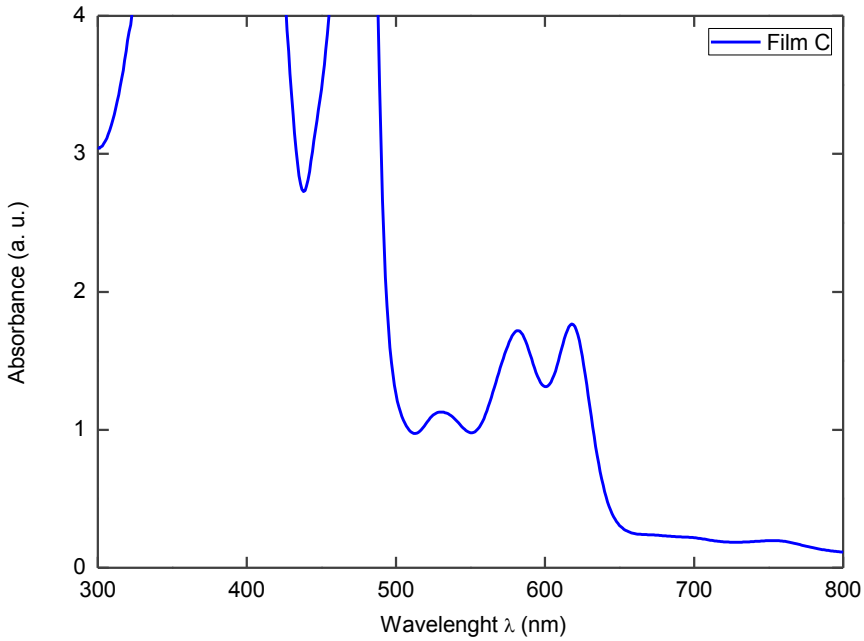


Figure 4. Absorbance spectra of Film C (Mn-TPP).

Gene transfection – Fluorescence Microscopy Photos

The diffusion and exchanges of substances from the extracellular environment to the intracellular one is limited by the molecular size. Large entities, such as Green Fluorescent Protein plasmids accounting for thousands of Dalton in molecular weight and are not capable of transversing the plasma membrane (Figure 5).

Figure 5. Representative photo of negative controls in cell line COS-7.

To determine de relevance of some variables in the transfection efficiency were performed several experiments beyond the previously reported. The results of these experiments allowed to identify some possible optimizations. In transfection assays using "indirect method" was tested applying PA waves with pulse duration of 30 ps for 5 minutes (Figure 6) and have proved less efficiency than the application for 10 minutes. This result indicates that higher application times allow higher levels of transfection.

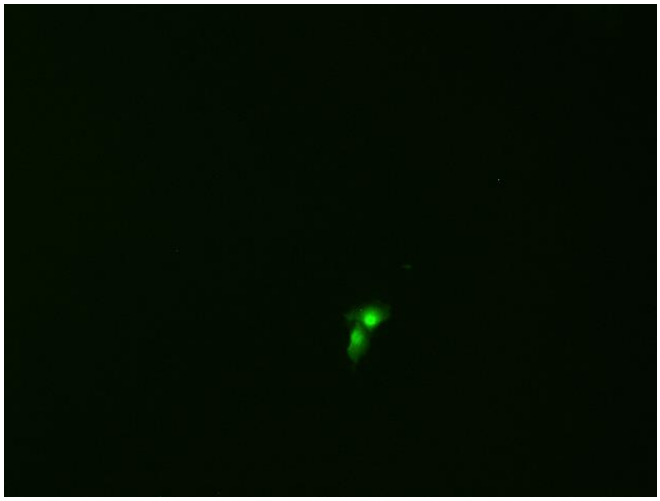


Figure 6. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Indirect Method" for 5 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

Still for the "indirect method" was tested the application of PA waves for 10 minutes using the other laser, with pulse duration of 8 ns, and has been verified a evident reduction in transfection levels (Figure 7). This confirms the importance of PA waves with higher pressures and with the presence of higher frequencies when generated by the laser of a shorter pulse duration.

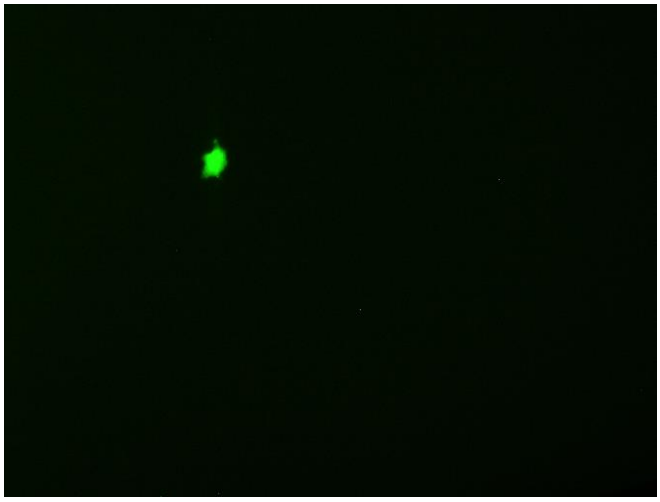


Figure 7. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Indirect Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 8 ns of pulse duration.

The tests performed for “Indirect Method” were also reproduced for the “Contact Method” and under similar conditions always showed better results. The application time of PA waves return to show that higher periods of application of pressure waves increase de levels of transfection as is indicated in Figures 8, 9, 10 and 11 for periods of application of 3, 5, 10 and 15 minutes, respectively, with laser pulse duration of 30 ps at fluence of 100 mJ/cm^2. Is evident a significant increase in the number of transfected cells for larger periods of application notwithstanding that between 10 minutes and 15 minutes the increase appeared to be less pronounced.

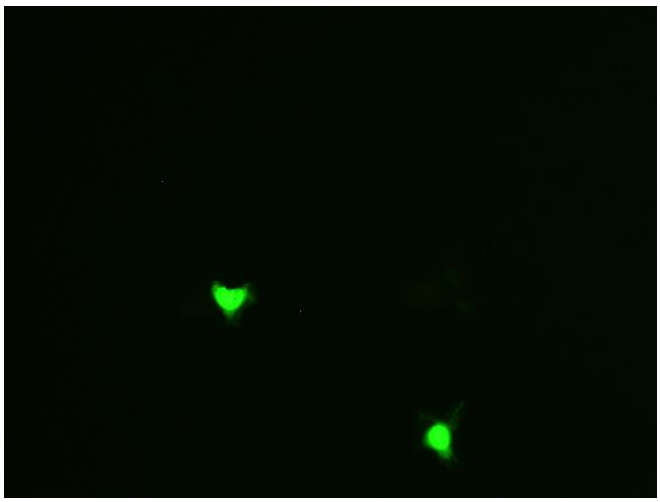


Figure 8. Representative image of gene transfection with gWizGFP [100 µg/mL] 24 hours after de application of PA waves with “Contact Method” for 3 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

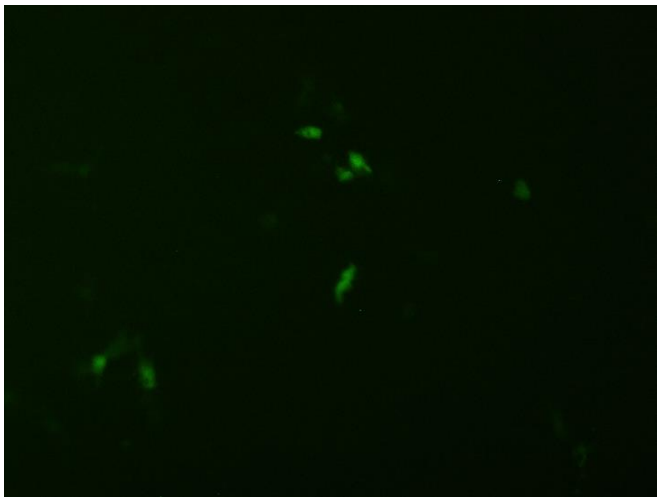


Figure 9. Representative image of gene transfection with gWizGFP [100 µg/mL] 24 hours after de application of PA waves with “Contact Method” for 5 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

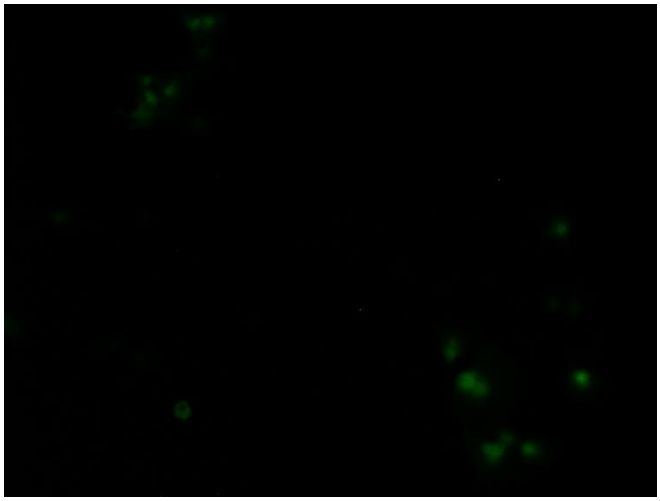


Figure 10. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

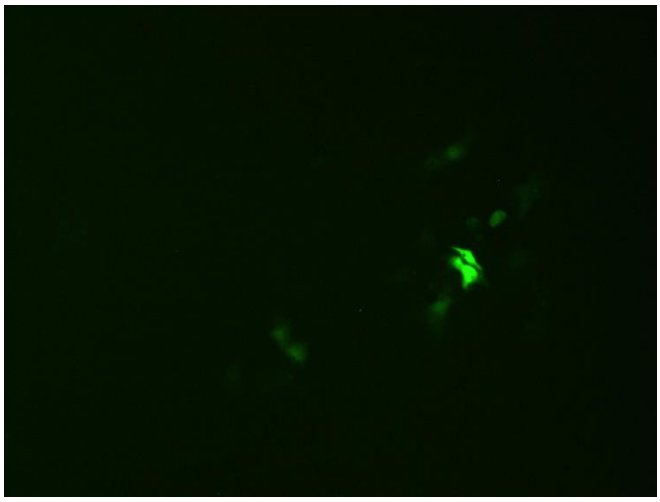


Figure 11. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Contact Method" for 15 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

In the “contact method” was also studied the influence of the laser fluence used and thus the maximum pressure of the PA waves generated. The results are shown in Figures 12, 13 and 14 for fluences of 33 mJ/cm^2, 66 mJ/cm^2 and 100 mJ/cm^2 respectively during 10 minutes of exposition to PA waves. The results confirmed again the importance of the maximum pressure of the stress waves produced in cell membrane permeabilization.

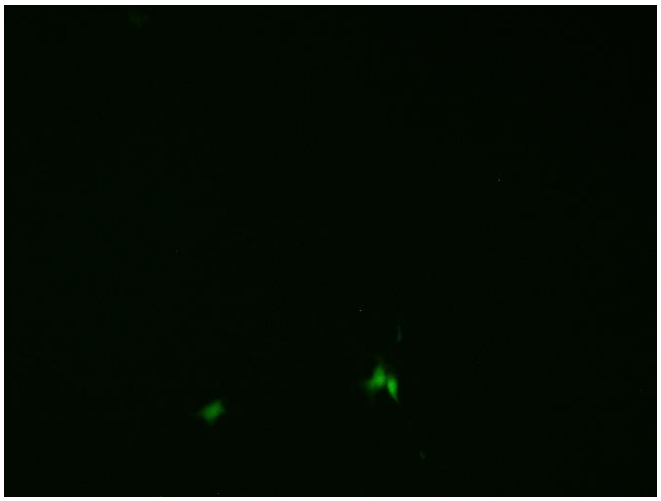


Figure 12. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with “Contact Method” for 10 minutes in COS-7 cell line with 33 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

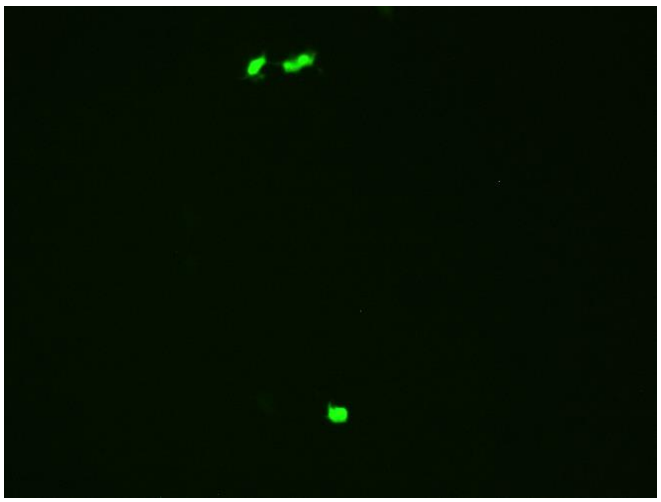


Figure 13. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with “Contact Method” for 10 minutes in COS-7 cell line with 66 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

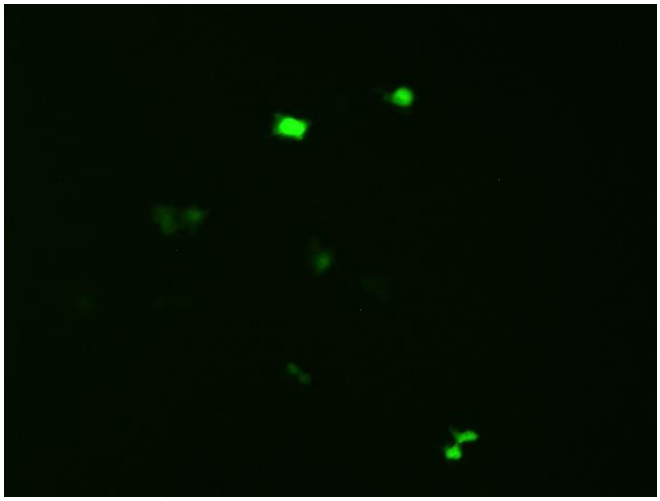


Figure 14. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

Higher plasmid concentrations also increase the number of transfected cells as is visible in Figures 15, 16 and 17 for 100 μg/mL, 250 μg/mL and 500 μg/mL, respectively, for PA waves application of 10 minutes in COS-7 cell line with 100 mJ/cm^2 by "contact method".

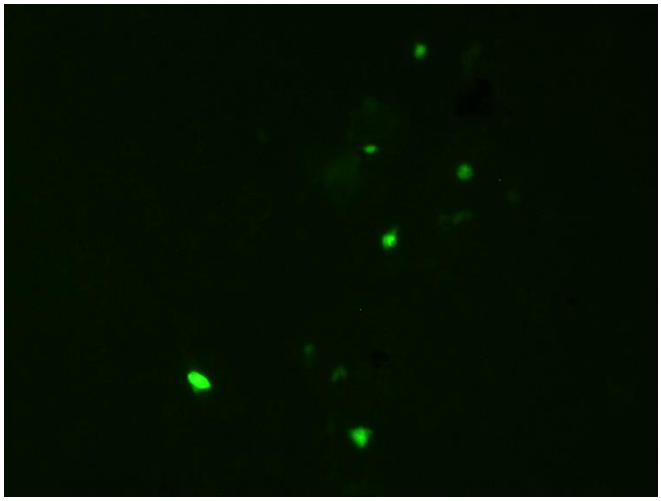


Figure 15. Representative image of gene transfection with gWizGFP [100 μg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

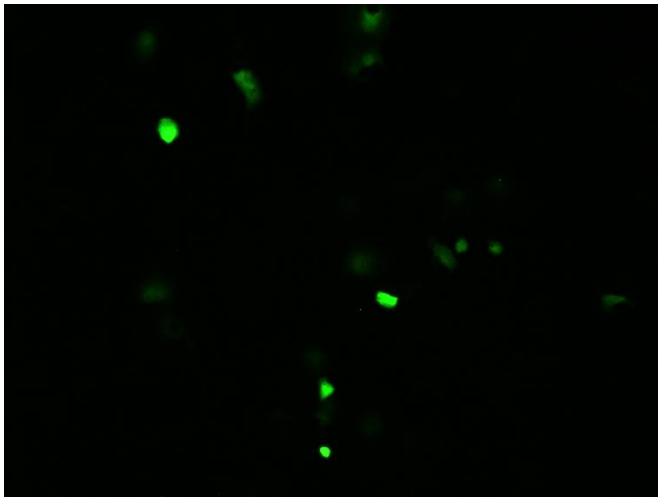


Figure 16. Representative image of gene transfection with gWizGFP [250 µg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

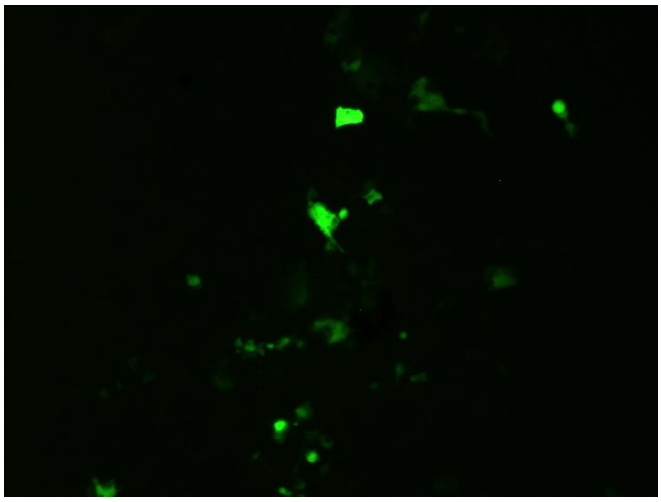


Figure 17. Representative image of gene transfection with gWizGFP [500 µg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 30 ps of pulse duration.

It was also tested the 8 ns pulse duration laser for this experimental design and it demonstrate again a significant decrease of percentage of transfection at a fluence of 100 mJ/cm^2 and with application time of 10 minutes (Figure 18)

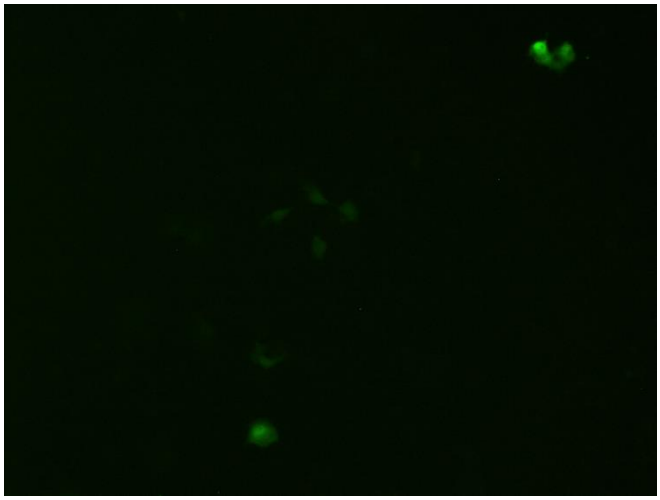
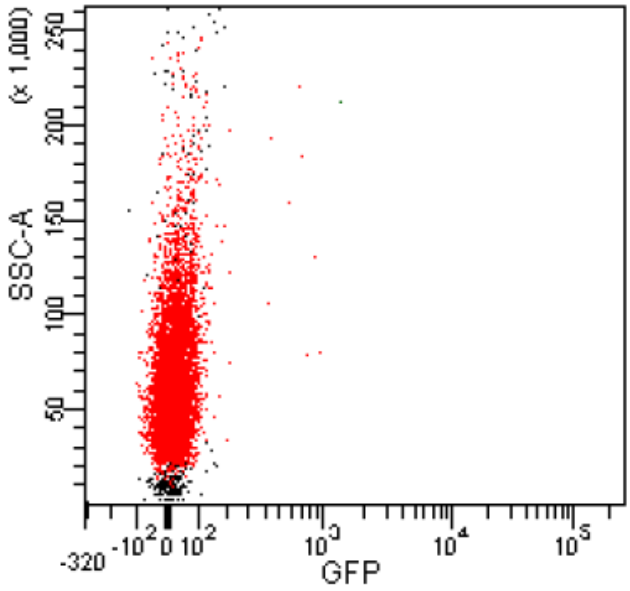


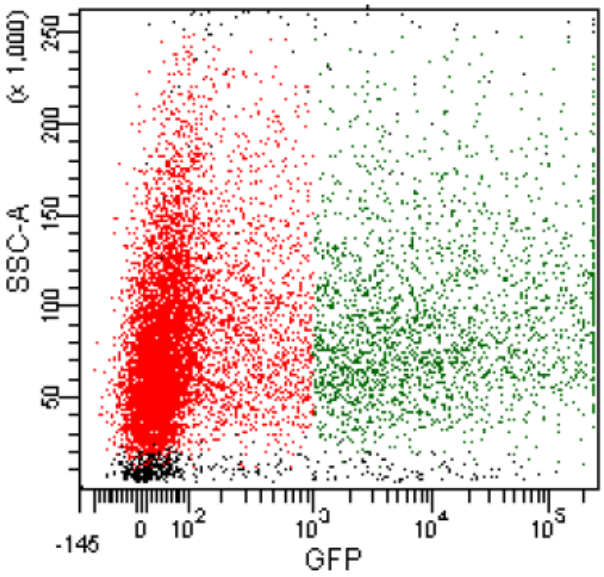
Figure 18. Representative image of gene transfection with gWizGFP [100 µg/mL] 24 hours after de application of PA waves with "Contact Method" for 10 minutes in COS-7 cell line with 100 mJ/cm^2 of laser fluence with 8 ns of pulse duration.

Flow Cytometry Results

The negative control showed no transfected cells (Graph 1) confirming the results already observed under fluorescence microscope. The transfection using lipofectamine reached 23% of transfected cells (Graph 2).



Graph 2. Negative Control (only in presence of plasmid DNA)



Graph 1. GFP expression by the cells (green dots) in relation to their side-scattered light (SSC) 24 hours after transfection with Lipofectamine using plasmid gWizGFP.