Influence of polymer adjuvants on the ultrasound-mediated transfection of cells in culture

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Running head: Adjuvants for microbubble-mediated sonoporation

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Abstract

The purpose of this study was to further understand the mechanisms involved in ultrasound–mediated delivery of DNA (sonoporation); in particular, to understand how a plasmid should be formulated with an ultrasound contrast agent (UCA). Different polymer adjuvant-UCA combinations were formulated, and their impact on in vitro DNA transfection, was determined, under various experimental conditions. When present in the medium surrounding a cell suspension, and in the presence of a plasmid encoding for the green fluorescent protein (GFP), expression following sonoporation was increased by more than 1.5-fold compared to that achieved in control experiments (without the adjuvants). The effects of the adjuvants were not influenced by the nature of the UCA, nor that of the transfected cells; in contrast, the adjuvant concentrations, their physicochemical properties, and the manner in which they were used, did have an impact on transfection. Close association of the adjuvants to the UCA inhibited their action, suggesting that these substances must have access to the cell membrane to be effective. Indeed, Pluronic® F127 appeared to improve the efficacy of transfection (%GFP-positive cells and cell viability), via fluidization of the cell membrane, perhaps facilitating thereby the formation of transient pores and their re-sealing. The mechanism of action of PEG, on the other hand, remains unclear.

Introduction

Ultrasound–mediated delivery of DNA (sonoporation) is a relatively new and promising method for gene therapy (1-5). Compared to viral and non-viral transfection techniques, sonoporation introduces the genetic material into cells or tissues with high spatial and temporal specificity and control, and with minimal toxicity. However, the approach is yet to realize its full potential for a number of reasons: first, the mechanisms involved in sonoporation are not completely understood; second, the technology is complex; and third, the manner in which a plasmid should be formulated with an ultrasound contrast agent (UCA), i.e., gas-filled microbubbles or microcapsules, has not been investigated in terms of physico-chemical composition, in any detail whatsoever. These issues clearly need to be addressed and, to a certain extent, resolved, before a safe, effective and practical treatment can be evolved. This work contributes to the further elucidation of the mechanism(s) of sonoporation, and focuses on the impact of the UCA formulation (6-8). In the present study, it was hypothesized that some biocompatible polymers used as pharmaceutical adjuvants in drug formulation such as polyethylene glycols (PEG) and amphiphilic molecules such as Pluronic®, Tween® and Triton X-100 could be useful for improving the efficacy of sonoporation since these polymers have been known for their capacity to increase the fluidity, permeability of cell membrane (9, 10); to preserve and repair against membrane damage (11-13); to facilitate drug and plasmid transport and to enhance gene transfer (14-17). Thus, the effect of polymer adjuvants (Pluronic F127, F68 and L61, PEG 4000 and PEG 35000) to the UCA on DNA transfection has been assessed under a range of experimental conditions (different cell types transfected, different adjuvants and UCA, and different incubation methods). The additional or synergistic effect of the adjuvant to UCA was evaluated with the efficacy of transfection (%cells positive to reporter protein GFP; and cell viability post-sonoporation). The effect of polymeric adjuvants (PEG4000 and Pluronic® F127) on cell membrane fluidity was further assessed by measuring fluorescence anisotropy and compared with the results of the transfection.

Materials and Methods

Cell culture

Rat mammary carcinoma cells (MAT B III, CRL-1666, ATCC-LGC) were incubated at 37°C, in 225 cm^2 tissue culture flasks in a solution of MacCoy's 5A medium containing Glutamax (Life Technologie, Basel, Switzerland), and supplemented with 10% v/v foetal calf serum (FCS) and 1% v/v antibiotics (initial concentration: 10,000 IU/ml Penicillin, 10,000 µg/ml Streptomycin, 25 µg/ml Fungizone, Bioconcept, Alschwil, Switzerland) under a 5% CO_2 atmosphere.
Human embryonic kidney cells (HEK 293-H, 11631-017, GIBCO) were incubated at 37°C, 5% CO_2 atmosphere in 75 cm^2 tissue culture flasks in a DMEM medium supplemented with 10% FCS and 1% non-essential amino acids, under a 5% CO_2 atmosphere. Prior to ultrasound exposure, 293-H cells were carefully detached from culture flasks using 0.05% trypsin solution containing 0.53mmol of EDTA. All cells were re-suspended in the culture medium without FCS for ultrasound exposure.

Ultrasound exposure

In a 3 ml polystyrene round-bottom tube, serving as ultrasound exposure chamber, 500 µl of cell suspension (1 x 10^6 cells/ml) were mixed with 5 µg of plasmid gWiz™-GFP (Aldevron, Fargo, ND, USA) encoding for the green fluorescent protein (GFP) and an ultrasound contrast agent (UCA) consisting of 15 x 10^6 microcapsules (i.e., a particle-to-cell ratio of 30 capsules/cell). The UCAs used were air-filled microcapsules made of tripalmitine (BG1593 and BG1766, Bracco Research SA, Geneva, Switzerland). The tube was held in a mechanically rotating system and immersed in a water bath thermostated at 37°C. The distance between the tube and the ultrasound transducer was 7.6 cm (optimized for a maximum ultrasound exposure). MAT B III cells were insonified at a peak negative pressure of 570 KPa for 10 seconds, using a focused transducer (Panametrics, Waltham, MA, USA) operating at a transmitted frequency of 2.25 MHz, a pulse repetition frequency of 100 Hz and a duty cycle of 20%. Sonoporation was performed in the absence or presence of a polymeric adjuvant, either a polyethylene glycol (PEG) or a Pluronic®; the surfactants were added to the cell-UCA suspensions at various concentrations prior to ultrasound exposure (except in one instance with Pluronic® 127 which had been incorporated directly into the UCA shell during its preparation). (Pluronic® F127 and F68 were purchased from Sigma Aldrich (Buchs, Switzerland) and Plutonic® L61, PEG 4000

and PEG 35000 were purchased from Fluka (Buchs, Switzerland). After sonoporation, the suspension was supplemented with 2 ml of cell medium containing 10% of FCS and placed in a 12-well plate. The cells were incubated at 37°C under a 5% CO_2 atmosphere for 24 hours prior to the analysis.

Assay of reporter gene

GFP-positive cells were analyzed using flow cytometry (FACS Calibur, Becton Dickinson Biosciences, Allschwill, Switzerland). After trypsinization, the cell suspension was placed in a 5 ml polystyrene round-bottom tube and washed twice in phosphate buffered saline (PBS). After the last wash, the pellet was re-suspended in 250-300 µl of PBS. Just before analysis, 20 µl of a 40 µg/ml propidium iodide solution (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was added to the sample to assess the cell viability. The results of the flow cytometry analyses were expressed as the percentage of cells positive to GFP with respect to the total number of cells treated by ultrasound, and in terms of fluorescence intensity, using the software CellQuest Pro (Becton Dickinson Biosciences, Allschwill, Switzerland). The percentage of positive cells was calculated for the entire sample, including dead cells. The fluorescence intensity was expressed in arbitrary units (AU) relative to an internal standard.

Cell membrane fluidity

Cell membrane fluidity was measured by fluorescence anisotropy of 1,6-diphenyl-1, 3, 5-hexanetriene (DPH) (Molecular Probes, Eugene, OR, USA) (18). Briefly, 1 ml of a 2 µM solution of DPH was introduced into the cell suspension. The mixture was stirred for one hour in the dark at room temperature. Exactly one hour prior to anisotropy measurement, a known volume of PEG or Pluronic® F127 solution was added to cell suspension (the final concentration of which was 0.5 x 10^6 cell/ml). Fluorescence intensities were measured using a Fluoromax-1 spectrophotometer (SPEX Industries, Stanmore, UK), equipped with a polarizer set. The fluorescence anisotropy r was calculated from the equation: $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} is the intensity of the components parallel to the original polarization, I_{VH} is the intensity of the components perpendicular to the polarization and G is an instrument factor. The excitation wavelength for DPH was 358 nm and the fluorescence intensity was calculated by integrating the emission between 420 and 440 nm.

Statistical analysis

All the results (%GFP-positive cells, %viable cells, intensity of GFP-positive cells and measurement of fluorescence anisotropy) are reported and displayed as mean ± standard deviation. Tests of significance were performed using a one-way analysis of variance (Minitab version 13.20, MINITAB Inc., Paris, France), with $p<0.05$ considered to be statistically significant.

Results

Different contrast agents, such as Definity® (Bristol-Myers Squibb Canada, Inc., Saint-Laurent, Quebec) and Byk963 (Byk Gulden Lomberg Pharmaceuticals, Clinical Pharmacology, Konstanz, Germany), are formulated with adjuvant: the former contains a pegylated phospholipid (DPPE-PEG 5000), while the latter has Pluronic® F68 introduced during the microbubble preparation process (19). BR14 (Bracco Research SA, Geneva, Switzerland), which has already been tested in sonoporation experiments, is also prepared in a solution of PEG 4000. As these adjuvants are commonly used in pharmaceutical applications, for example to stabilize formulations, their potential to enhance ultrasound-mediated gene delivery and/or improve cell viability post-sonoporation, was considered a useful question to pursue.

Effect of "free" and "grafted" polymers on transfection

Air-filled BG 1766 microcapsules were used as control, and the presence of polyethylene glycol (PEG) 4000, Pluronic® F68 or Pluronic® F127 was subsequently evaluated. Figure 1 shows that all three polymers, when unassociated with the UCA ("free") in the cell suspension, enhanced transfection efficiency as measured by the % of GFP-positive cells (increase from 20% to 30%). However, when Pluronic® F127 was incorporated ("grafted") into the UCA during its preparation, transfection (% GFP-positive cells) was not different from the control value. With respect to the fluorescence intensity observed, no differences were found between the formulations.

Concentration and nature of adjuvant

Sonoporation experiments were performed using different adjuvants at different concentrations. Thus, PEGs 4000 and 35000 and Pluronic® F68, F127 and L61 were evaluated for their effect on plasmid transfection at two concentrations: 0.05 and 0.1% w/v. The results (Figure 2) showed that both PEGs significantly enhanced gene transfection, especially at the lower concentration; over 33% of transfected cells were positive to GFP in the presence of PEG 4000 and PEG 35000 compared to 26.2 ± 0.3% for the control without PEG. Increasing the adjuvant concentration to 0.1% w/v had no beneficial effect with PEG 35000 (Figure 2A). The Pluronic® behaviour was rather complex: Pluronic® L61 decreased transfection, while Pluronic® F68 and Pluronic® F127 slightly improved gene transfer. In terms of the viability of sonoporated cells, the higher concentration (0.1%) of the PEG adjuvants was generally more beneficial; on the other hand, cell

viability remained unaffected for the Pluronic®, except for Pluronic® L61 where higher cell death was observed (Figure 2B), in agreement with previous results (20), 2006 1 /id}.

To examine more closely the impact of polymer concentration, a more detailed transfection experiment was performed on two cell types, MAT B III and 293-H cells, using Pluronic® L61 at levels ranging from 0.0002% to 0.002% w/v. The cells and the Pluronic® concentration range were chosen based upon previous results (18;21)} which demonstrated that PL 61 increased membrane fluidity at very low concentrations (0.0002-0.002% w/v, or 1-10 μM). For both cell types, transfection rates ranged from 15% to 25%. In the case of MAT B III cells, no differences from control were observed at any concentration of Pluronic® (Figure 3A). For 293-H cells, 0.001 and 0.002% w/v polymer produced a small but significant increase in the % of GFP-positive cells ($p = 0.005$ and 0.004 respectively). Viability was relatively low (40-60%) for both cell types over the entire range of polymer concentrations examined (Figure 3B). Similarly, fluorescence intensity was not affected by the concentration of polymer; however, it was noted that the fluorescence detected was 3-times higher in 293-H cells than in MAT B III (Figure 3C).

Timing of adjuvant administration

To explore whether polymer adjuvants elicit their effects via an action on the plasmid (e.g., DNA stabilization) or on the cell membrane (e.g., fluidization), the manner in which PEG 4000 and Pluronic® F127 were added to a MAT B III cell suspension before ultrasound exposure, was varied: (i) the adjuvant, UCA, and then the DNA were separately added to the cell suspension and the mixture was immediately insonified (denoted as "no incubation"); (ii) adjuvant was first incubated with the DNA solution for 20 minutes, then mixed with the cell suspension containing the UCA and insonified (denoted as "incubation with DNA"); (iii) the adjuvant was first incubated with the cell suspension for 20 minutes, then mixed with DNA and UCA, and finally insonified (denoted as "incubation with cells").

The control experiment utilized the BG1766 UCA together with 10 μg/ml of DNA but without adjuvant. Figure 4 shows that the transfection efficiencies with "no incubation" and with "incubation with DNA" for both PEG 4000 and PF 127 were similar and significantly better than the control (16% ± 1.4 compared to 22% ± 1.2 respectively). Transfection efficiency was significantly improved when the adjuvant molecules were first incubated with the cells to be transfected, and an increase of about 5% of GFP-positive cells was obtained. However, no significant differences were observed between the various preparations with respect to fluorescence intensity (data not shown).

In all these experiments, adjuvant was introduced before insonification of the cells. Subsequently, the Pluronic® was added to the cells after sonoporation; three conditions were compared: (a) without adjuvant (control); (b) after 1 hour incubation of PF 127 with the cells before sonoporation; and (c) addition of polymer to the cells after ultrasound exposure. Figure 5 indicates that, when polymer was added immediately after ultrasound exposure, transfection rate was decreased 2-fold ($p = 0.000$) and cell viability, relative to the other conditions, was reduced from approximately 70 to 50%. In contrast, when cells were pre-incubated with Pluronic® F127, GFP expression rate reached 28% vs 20% for the control conditions.

Study of membrane fluidity of cells in the presence of adjuvants

The preceding findings prompt us to evaluate whether the improved transfection in the presence of PEG 4000 and PF 127 was directly related to membrane fluidization. Transfection efficiency, cell viability and membrane fluidity were therefore evaluated as a function of the polymer concentration (0 to 0.1%w/v). Cell membrane fluidity was measured by fluorescence polarization of 1,6-diphenyl-1, 3, 5-hexanetriene (DPH)(18).

Figure 6A shows that transfection efficiency (% GFP-positive cells) in the presence of PF 127 was well-correlated with measurements of (1/anisotropy). As the fluorescence anisotropy reflects the rigidity of the membrane, its reciprocal is logically an expression of membrane fluidity. Transfection rate and membrane fluidity increased in parallel with increasing polymer concentration before reaching a plateau at 0.05% w/v. The overlap in behaviour between transfection and membrane disordering suggests that the mechanism involves bilayer fluidization. It should be noted, however, that no correlation was observed between anisotropy and fluorescence intensity (data not shown). Also, while the highest polymer concentration (0.1% w/v) did not further improve gene transfection nor increase membrane fluidity (Figure 6A), cell viability increased essentially linearly with adjuvant concentration (Figure 6B).

Figure 7A demonstrates that PEG 4000 at 0.02% w/v increased significantly transfection rate, but that further augmentation of the polymer concentration had no effect. Fluorescence anisotropy was not affected; on the other hand, as for Pluronic® F127, and no plateau was observed for cell viability at high amounts of PEG 4000. In contrast to Pluronic® F127, cell viability progressively increased with PEG 4000 concentration (Figure 7B).

Discussion

PEG has been extensively examined in biochemical and pharmaceutical applications, e.g., for membrane fusion (22), as a particle/membrane surface-modulating agent (23) and for membrane solubilization (24). Pluronic® (or Poloxamers) are known to enhance the permeation of hydrophobic molecules, such as doxorubicin, across cell membranes, to increase membrane fluidity (21), and to facilitate gene transfer (25). Equally, the action of both PEGs and Pluronic® depend (*inter alia*) upon their hydrophilic-lipophilic balance and their concentration (21).

While our work was in progress (26), Chen and co-workers showed that ultrasound alone – *i.e.* without contrast agent - and Pluronic® work synergistically in enhancing gene delivery. However, they did not unravel the mechanism of action of the Pluronic molecules. Indeed, we showed positive effects of adjuvants, such as PEG 4000 and Pluronic® F68 and F127, on transfection efficiency, particularly when the polymers were added "free" to a cell suspension (Figure 1); no improvement in transfection was observed when Pluronic® F127 was grafted onto the UCA microcapsule shell. This initial result suggested that the adjuvants acted either at the cell membrane or at the level of DNA; the polymers' impact on transfection appeared to be independent of contrast agent formulation.

PEGs and Pluronic® may affect gene transfection at different levels. For example, they may interact with plasmid to form a complex which facilitates gene transfer by stabilizing DNA and reducing its degradation by cytosolic DNases. Alternatively, the polymers may fluidize cell membranes thereby enhancing the extent and duration of ultrasound-induced poration and DNA permeation into the cytoplasm; in the same way, membrane re-sealing may be improved resulting in higher cell viability. The exact mechanism of action almost certainly depends upon many different parameters, such as the polymer hydrophilic-lipophilic balance (HLB) and concentration. For the three Pluronic®, their HLB values are quite different: respectively, 3, 22 and 29 for Pluronic® L61, F127, and F68 (27;28). Likewise, it is known that high PEG concentrations (>20% w/v) are necessary to induce membrane fusion, whereas much lower levels (0.1-1% w/v) are sufficient to change membrane fluidity (29).

The transfection of NIH/3T3 cells using PEI-DNA complexes was improved by addition of Pluronic® (30). Six different Pluronic® were tested and compared (F68, F127, P105, P94, L122 and L61). Those with higher HLB, at higher concentration (1 or 3% versus 0.1% w/v), were more effective. It was postulated that the polymers protected the DNA complex by sterically hindering the access of serum components. Similarly, Pluronic® L61 and F127 significantly increased (by an order of magnitude) transfection relative to the naked plasmid in skeletal

muscle (25). However, this polymer mixture had no effect on the efficiency of *in vitro* sonoporation in the work presented here (data not shown). The mechanism of gene transfer in skeletal muscle remains unclear, although both polymer-DNA complexation and increased cell membrane fluidity have been suggested.

The latter mechanism has been demonstrated for Pluronic® L61 and P85; the degree of effect depended upon cell type (normal versus tumor) (18;21). Normal cells appeared to predominantly adsorb Pluronic® polymers on their surface, for example. Moreover, Pluronic® L61 increased the microviscosity of normal cell (splenocyte) membranes but decreased that of myeloma cells. The binding efficiency of the Pluronic® polymers also depended upon their HLB values; for example, binding of the hydrophobic L61 (HLB = 3) was 1.5-3 times higher than that of the more hydrophilic P85 (HLB = 16). The results in Figure 2B suggest that the relatively hydrophilic Pluronic® F68 and F127, may improve cell membrane re-sealing (unlike L61). Cell viability with L61 was the lowest among all the adjuvants tested: at concentrations of 0.05% and 0.01% w/v, only about 63% and 52%, respectively, of cells were viable. This apparently high toxicity of hydrophobic Pluronic® has been reported *in vivo*. Morphological and biochemical assays following injection into muscle tissue indicated that the more hydrophobic the polymer, the more severe the lesions induced (31).

At lower concentrations (0.0002-0.002% w/v), Pluronic® L61 again resulted in cell viabilities of ~50-60% (Figure 3B), but eventually improved transfection rate to more than 25% (Figure 3A). However, the amount of DNA internalized was not affected in the same way (Figure 3C), which makes it difficult to draw mechanistic conclusions. Also intriguing is the observation that fluorescence intensities in 293-H cells were 3-fold higher than in MAT B III cells; this would imply easier transfection and nuclear uptake in the former despite their longer population doubling time (>20 hours compared to 12 hours for MAT B III cells).

Pluronic® enhanced gene transfer and cell viability even when added shortly before sonoporation (Figures 2 and 4). Adsorption of the polymers at the plasma membrane may be very rapid, or the polymers might simply be effective at very low surface concentrations. A 20-minute incubation of Pluronic® F127 with cells, in the absence of DNA, seemed to further improve transfection (Figure 4). This implies that the adjuvant is not acting directly on DNA itself, but that there is a polymer effect on the cell membranes. Pre-incubation of the adjuvant with the cells before sonoporation would allow more surfactant molecules to adsorb onto and/or integrate into the cell membrane. Figure 5 supports this contention in that transfection was enhanced when the Pluronic® was present during sonoporation, and was even more successful with pre-incubation.

The increase in cell viability with increasing Pluronic® F127 concentration (Figure 6) suggests that this adjuvant improves cell repair post-sonoporation. This is consistent with the observation that Poloxamer 188 (which is similar to Pluronic® F68: HLB = 29 (28) improved tissue (and cell membrane) recovery in a dose-dependent way following electroporation (32). Facilitated re-sealing of membranes porated by electrical, thermal, or other forces, has been deduced as the mechanism of action of this (and other) non-ionic, polymer surfactants (33). It was noted, however, that transfection rate reached a plateau at 0.05% w/v PF 127. This may be due to the polymer reaching its critical micelle concentration (34;35), at which no further "free" surfactant molecules are available for interaction with the cell membrane (34). Alternatively, DNA might form a complex with the Pluronic® micelles (36;37), and decrease its availability for transfection. The mechanism of action of PEG 4000 in sonoporation is more difficult to understand. PEG can fuse lipid bilayers, enhance drug permeation into cells, and repair membranes which have been perturbed (21). PEG has also been used to improve gene delivery by lipofection (39). The polymer may promote membrane fusion by dehydrating lipid headgroups (40). PEGs can also alter membrane fluidity at concentrations ranging from 0.1-1% to 10% w/v (39;41). When gene delivery with lipofection (Lipofectamine 2000, Invitrogen, Carlsbag, CA, USA), in the presence of 0.05% w/v of PEG 4000, was tested in the present work, no enhancement in transfection rate was observed (data not shown). Yet low concentrations of PEG 8000 have induced liposome aggregation, and increased the association between cell membranes and lipid/DNA complexes (42), leading to better plasmid uptake. Other studies have reported similar results (39;43); for example, PEG 8000 at concentrations between 1 and 8% (v/v) increased (by up to 10-fold) the transfection of 9L cells by cationic liposomes complexed with plasmid pUT650 (43).

PEG and Pluronic® polymers are quite different; the former is hydrophilic, the latter amphiphilic. The mechanisms of action as adjuvants in DNA delivery may be quite distinct, therefore. The fluorescence anisotropy data indicate that Pluronic® PF 127 increases membrane fluidity while PEG 4000 does not (Figures 6 and 7). It may be that the concentration of PEG used was simply insufficient to provoke any effect; after all, much higher levels are needed to induce membrane fusion. Indeed no change in membrane anisotropy was noted across the whole range of PEG concentration tested whereas even slight amount of Pluronic 127 decreased anisotropy. This demonstrates that PEG and Pluronic may exert their protective action through separate mechanisms. It would be of high interest to combine both reagents to unveil potential synergetic effects.

Further speculation on a putative mechanism of action for PEG 4000 in sonoporation is inappropriate in the absence of further experiments designed to test specific hypotheses. Given

the quite modest effects observed to-date, the value of a detailed and systematic investigation must be weighed against the pursuit of alternative strategies that provide much greater enhancement of gene delivery.

Conclusions

Polymeric adjuvants present in UCA can improve *in vitro* transfection and cell viability even at moderate concentrations (0.05-0.1% w/v). PEG 4000, and Pluronic® F68, F127, enhanced GFP-expression more than 1.5-fold compared to the contrast agent alone. The adjuvant effect was indifferent to the UCA and cell type used, but strongly depended upon the nature of the polymer, the manner in which it was presented to the cells, and its concentration. The adjuvants were not effective when grafted to the UCA, suggesting that their adsorption onto, or integration into, the cell membrane was necessary for efficacy. Pluronic® F127 appears to fluidize the cell membrane, facilitating transient pore formation and re-sealing. The mechanism of action of PEG, on the other hand, remains unclear.

Abbreviations

UCA	Ultrasound contrast agent
FCS	Foetal Calf Serum
GFP	Green Fluorescent Protein
DPH	1,6-diphenyl-1, 3, 5-hexanetriene
HLB	Hydrophilic-Lipophilic Balance

Legends to Figures

Figure 1: Effect of adjuvant (PEG 4000, Pluronic® F127, Pluronic® F68 at 0.05%w/v) on transfection efficiency (bars) and fluorescence intensity (▲), expressed in arbitrary units (AU), using BG1766 microcapsules. Sonoporation was performed at 2.25 MHz (P- = 570 KPa) in the presence of 30 capsules/cell (MAT B III). Control is a standard UCA (BG1766) without adjuvant. Statistical significance: * = $p < 0.05$ versus "free".

Figure 2: Effect of the nature and concentration of adjuvant on (A) percentage of GFP-positive cells and (B) cell viability. Two adjuvant concentrations were tested: 0.05% w/v (♦) and 0.1% w/v (□). Sonoporation was performed at 2.25 MHz (P- = 570 KPa) in the presence of 30 capsules BG1593/cell (MAT B III).

Figure 3: Sonoporation of MAT B III (—▲—) and 293-H (- -□- -) cells in the presence of BG1766 (2.25 MHz at P- = 570 KPa, 30 capsules/cell). Effect of Pluronic® L61 concentration on (A) percentage of GFP-positive cells, (B) cell viability, and (C) fluorescence intensity expressed in arbitrary units (AU).

Figure 4: Effect of incubation of PEG 4000 (0.05% w/v) and Pluronic® F127 (PF 127 – 0.05% w/v) with DNA or with MAT B III cells on transfection efficiency at 2.25 MHz (P- = 570 KPa; 30 capsules BG1766/cell). Control contained no adjuvant. Statistical significance: * = $p < 0.05$ versus all others; ** = $p < 0.05$ versus control, no incubation, and incubation with DNA.

Figure 5: Transfection experiment in the presence of 0.05% w/v Pluronic® F127, under three different incubation protocols: in the absence of Pluronic® F127, with the addition of Pluronic® F127 1 hour before ultrasound exposure, and with the addition of Pluronic® F127 after sonoporation (2.25 MHz, P- = 570 KPa, 30 capsules BG1766/cell). GFP-positive cells (bars) and cell viability (♦) were assessed. Control contained no Pluronic® F127.

Figure 6: Effect of Pluronic® F127 (PF 127) on membrane fluidity, expressed as 1/anisotropy (- -□- -) and (A) cell transfection (- -▲- -) or (B) cell viability (- -♦- -). MAT B III cells were sonoporated at 2.25 MHz, P- = 570 KPa in the presence of 30 capsules BG1766/cell.

Figure 7: Effect of PEG 4000 on membrane fluidity, expressed as 1/anisotropy (- -□- -) and (A) cell transfection (- -▲- -) or (B) cell viability (- -♦- -). MAT B III cells were sonoporated at 2.25 MHz, P- = 570 KPa in the presence of 30 capsules BG1766/cell.

Figures

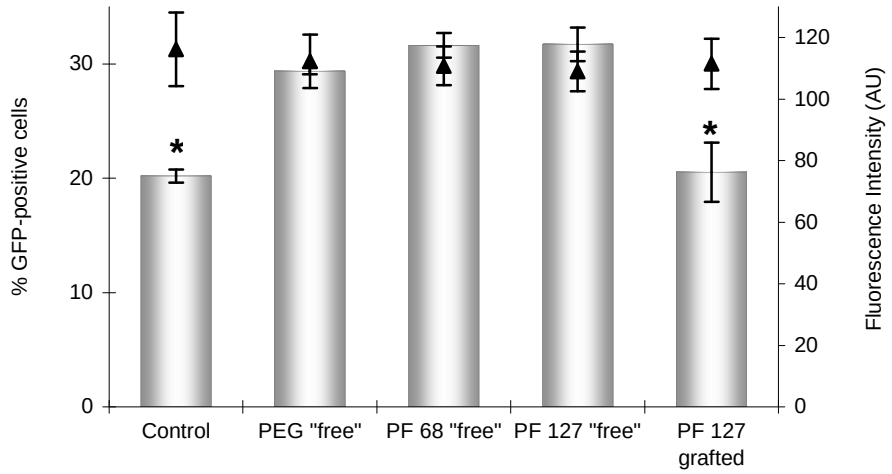

Figure 1

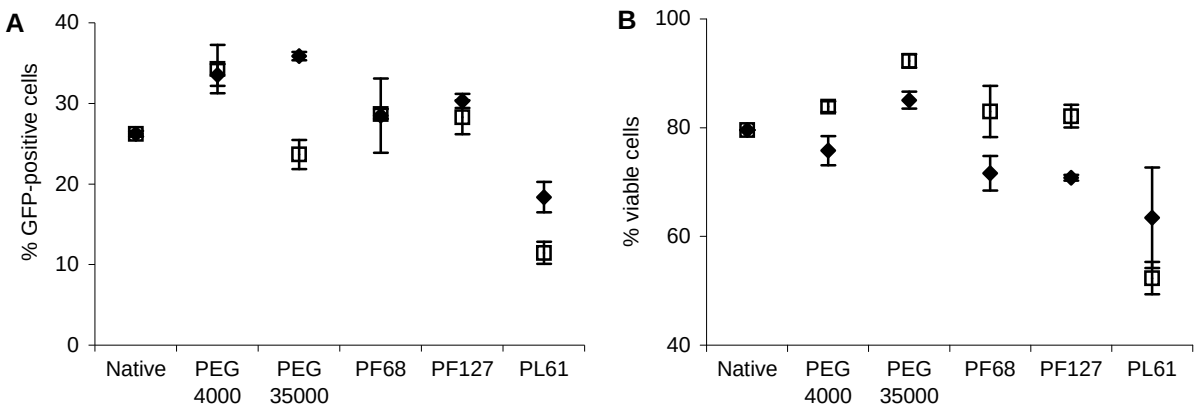
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Figure 2

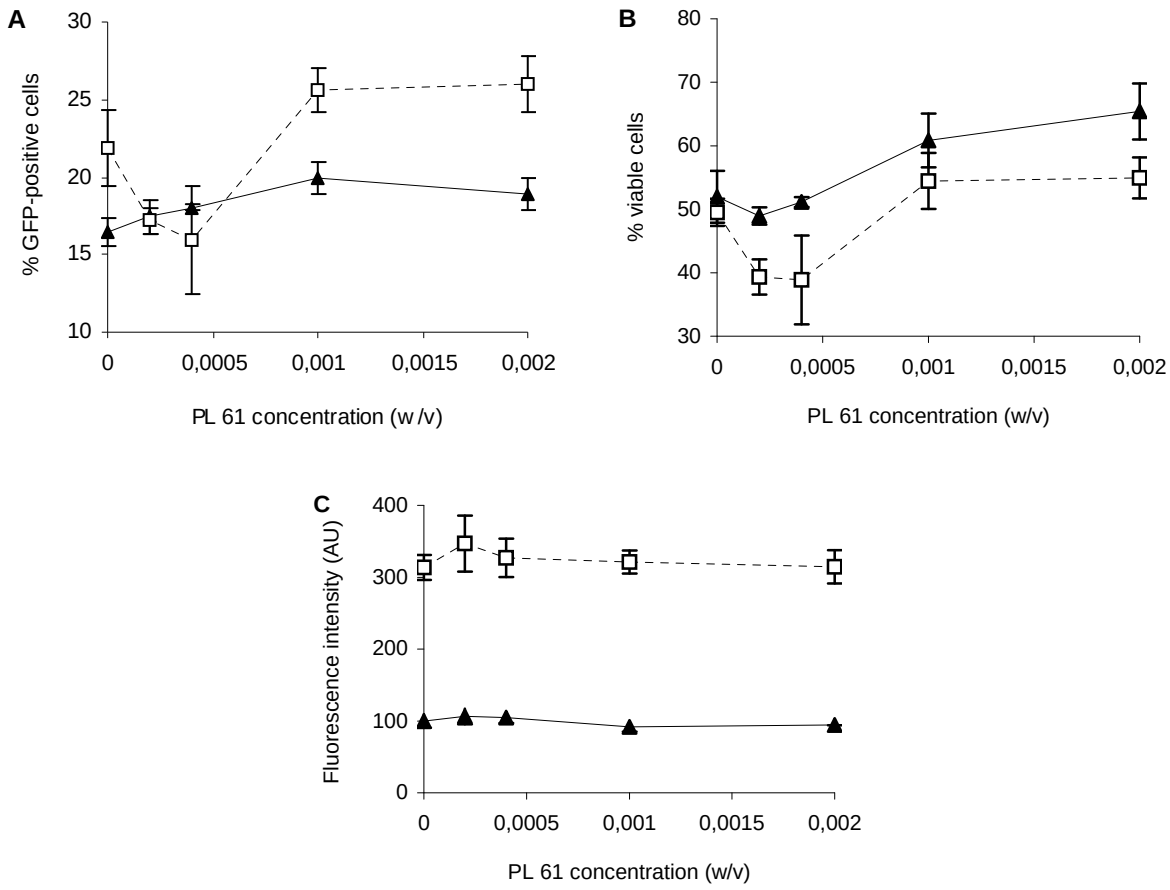
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Figure 3

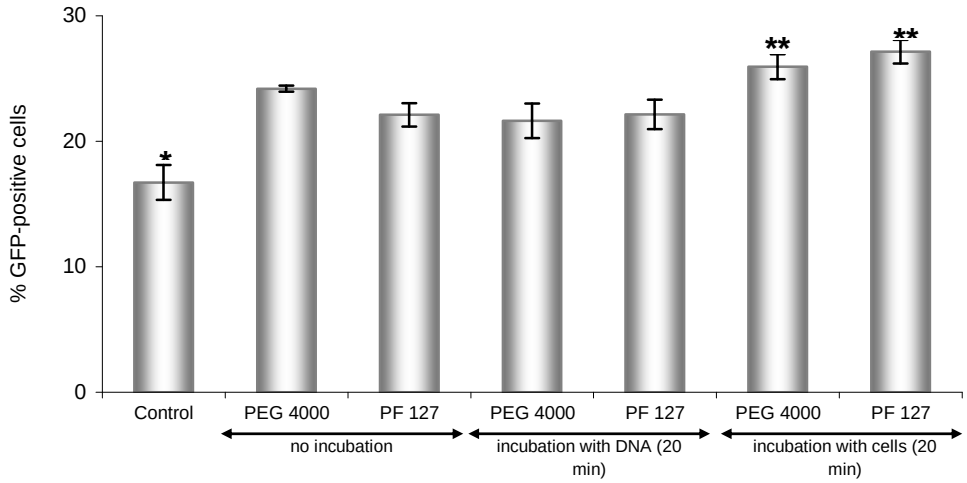
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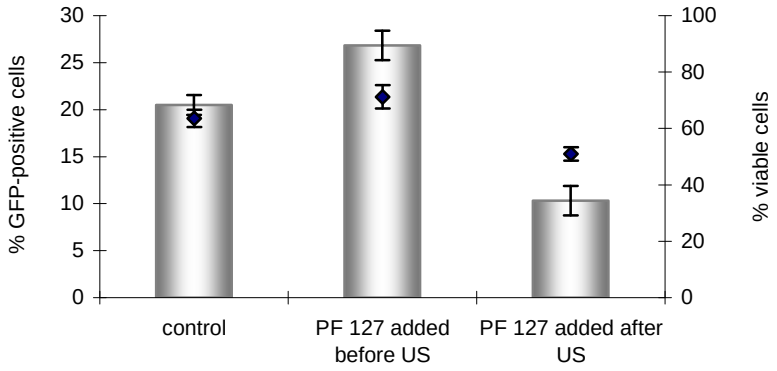
Figure 4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PF 127 added before US
PF 127 added after US

Figure 5

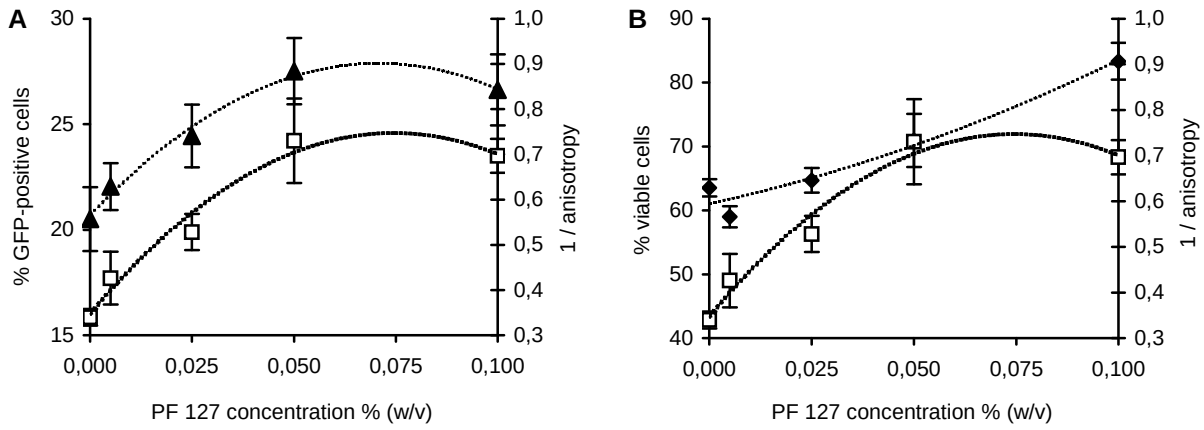
A
30
25
20
15
% GFP-positive cells
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
1 / anisotropy
0,000
0,025
0,050
0,075
0,100
PF 127 concentration % (w/v)
B
90
80
70
60
50
40
% viable cells
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
1 / anisotropy
0,000
0,025
0,050
0,075
0,100
PF 127 concentration % (w/v)

Figure 6

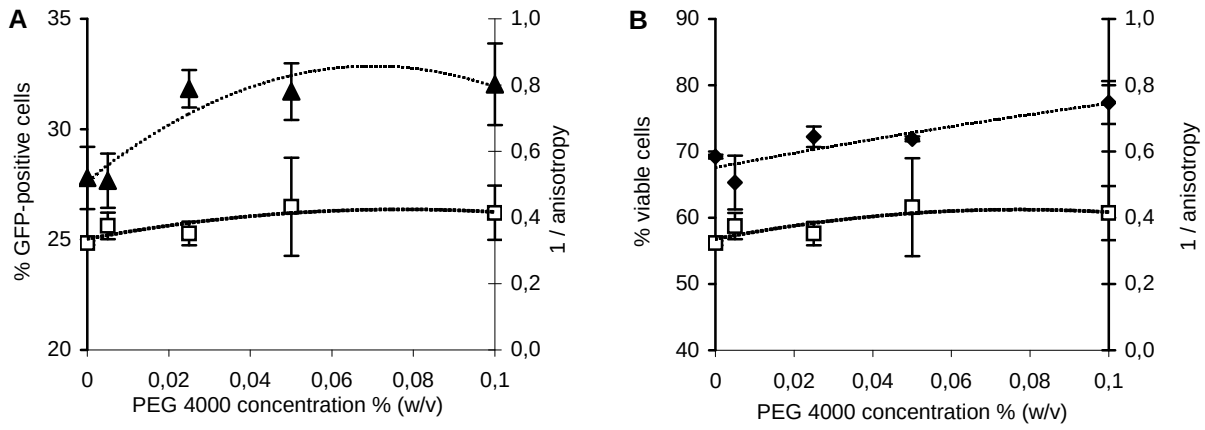
A
35
30
25
20
% GFP-positive cells
1,0
0,8
0,6
0,4
0,2
0,0
1 / anisotropy
0
0,02
0,04
0,06
0,08
0,1
PEG 4000 concentration % (w/v)
B
90
80
70
60
50
40
% viable cells
1,0
0,8
0,6
0,4
0,2
0,0
1 / anisotropy
0
0,02
0,04
0,06
0,08
0,1
PEG 4000 concentration % (w/v)

Figure 7

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