The Use of Sonication for the Efficient Delivery of Plasmid DNA into Cells

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Received December 9, 1996; accepted March 6, 1997

Purpose. Ultrasonic methods have considerable potential for the introduction of macromolecules into cells. In this paper we demonstrate that, under controlled conditions, application of 20 kHz ultrasound to a suspension of yeast cells facilitates the delivery of plasmid DNA into these cells.

Methods. Aliquots of growing yeast cells (Saccharomyces cerevisiae, strain AH2) were suspended in buffer and exposed to 20 kHz ultrasound from a laboratory (probe-type) sonicator in the presence of microgram quantities of plasmid DNA. Efficiency of DNA delivery was scored as the number of cells transformed.

Results. Cell transformation was optimal at 30 seconds sonication using an output of 2.0 watts and resulted in a 20 fold enhancement over control values. At extended sonication times, fewer cells showed evidence of transformation because of reduced cell viability. The increased DNA uptake and the decreased cell viability were both attributable to acoustic cavitation events during sonication. The extent of acoustic cavitation was measured and it was found that there was an increase in cavitation events with increased sonication time. Cell viability was shown to be directly related to the number of cavitation events. The effects of sonication on plasmid DNA were investigated and indicated that the structural integrity of plasmid DNA was unaffected by the sonication conditions employed.

Conclusions. Under controlled conditions, ultrasound is an effective means of delivering plasmid DNA into cells. The subsequent expression of DNA molecules in cells depends upon a balance between transient cell damage and cell death.

KEY WORDS: DNA delivery; cells; sonication; cavitation; transformation.

INTRODUCTION

Several methods have been developed for the delivery of DNA into cells. These include:

(i) chemically-facilitated methods, e.g., calcium phosphate and DEAE-dextran;
(ii) vector-mediated methods, e.g., liposomes and retroviruses;
(iii) mechanical methods, e.g., Biolistics™ and electroporation.

Each method has inherent limitations, such as low efficiency; complex protocols and/or cost, but in particular, many cells are only responsive to one or a few specialised methods. In this respect, mechanical methods are often more versatile, as they are based on disruption of the cell membrane and are less dependent on cell type.

The investigation described here examines the use of ultrasound as a novel mechanical method for the delivery of DNA into cells. The earliest report of sonication-mediated DNA delivery was the introduction of a plasmid containing the thymidine kinase gene into fibroblasts using pulsed ultrasound from a probe-type sonicator (1). The efficiency of transformation in these early experiments was low. For example, at a DNA concentration of 50 µg/ml only an average of 23 transformants per 106 cells were obtained. In these early studies conditions were not optimised with respect to the cell line used, rather, conditions were adapted from previous experiments which demonstrated ultrasound enhanced uptake of exogenous molecules (2). Subsequent studies demonstrated the delivery of viral particles into plant cells (3) and a sonication-mediated method was developed for the introduction of plasmid DNA into plant cells (4). It was found that a single pulse of ultrasound (0.5–1.0 second) from a 20 kHz probe-type sonicator could enhance the uptake of DNA into plant protoplasts. Experimental conditions were also partially optimised and transformation efficiencies in the region of 1% were achieved. However, as in previous reports (1), large amounts of DNA were required. Further reports of sonication-mediated DNA delivery include delivery to intact plant cells (5), leaf segments (6), and plant embryos (7,8). Cavitation was suggested as the mechanism responsible although no evidence was presented. Unfortunately, comparison of results from the various studies is difficult since little attention is paid to the influence of ultrasound variables (e.g. frequency, intensity and duration of exposure) on the extent of DNA uptake, or to the effects of ultrasound on the cells or macromolecules. However, these reports do illustrate that ultrasound may offer an attractive means of DNA delivery into cells and that cavitation is the most likely explanation for DNA transfer.

The advantages of sonication-mediated DNA delivery are that it is rapid, relatively inexpensive, and by virtue of being a mechanical method, should be applicable to a wide range of cell types and tissues. The method has applications in the areas of biotechnology, medicine, and basic cellular research. In biotechnology for example, the range of cell types utilised nowadays is much broader and more complex than previously. Thus, there is a need for a highly efficient and versatile DNA delivery technique which can be used with a variety of cell types, and which will eliminate the need to develop specific transformation protocols. In this respect ultrasound may prove very useful for the delivery of DNA to animal cells both in vitro and in vivo.

Biotechnology has enabled several new categories of molecules to be used as therapeutic agents. For example, protein based drugs e.g. insulin and erythropoietin, and DNA based treatments (gene therapy (9), DNA vaccines (10) and antisense oligonucleotides) are now being routinely investigated. The inherent problem with all such macromolecular bioactive drugs is that they are susceptible to degradation in the gastrointestinal tract, exhibit poor gastrointestinal uptake, and are cleared rapidly from the blood stream. Therefore, they need to be delivered to the blood stream at a sustained rate. At present, in clinical use, the mode of delivery is generally limited to injection. However, there have been reports of ultrasound-mediated transdermal protein delivery (11), therefore ultrasound may also prove to be a viable alternative for the transdermal delivery of therapeutic macromolecules. Furthermore, entry into the blood...
stream may not be the only problem with delivery of macromolecular bioactive drugs. Therapeutic use is often limited, especially for gene therapy, where there are additional problems of targeting and achieving uptake at the active site. Since the degradation of polymer matrices may be enhanced using therapeutically acceptable levels of ultrasound (12), there is potential for a polymeric device, incorporating DNA or protein drug, where release and uptake at the active site can be facilitated by sonication.

In order for ultrasound to be successful in any of these areas, it is of prime importance that the effects of sonication on cells and DNA, during the procedure, are fully understood. The investigation presented here is a preliminary study into the effects of ultrasound upon cells and DNA during sonication-mediated DNA delivery and attempts to establish the general mechanisms by which DNA transfer occurs.

MATERIALS AND METHODS

Materials

Chemicals used were of AnalaR grade or above, and obtained from BDH Chemicals (Poole, England), or Sigma Chemical Company (Poole, England). Agarose was supplied by Gibco BRL (Paisley, Scotland). All restriction endonucleases and buffers were supplied by Boehringer Mannheim (Lewes, England). Lambda DNA, Hin dIII-digested, DNA fragment size markers were obtained from Promega (Southampton, England). The components for the culture media were supplied by Difco Laboratories (West Molesey, England) and Oxoid Limited (Basingstoke, England). The pGad1If plasmid was constructed and supplied by J. Rosamond, Manchester University.

Calibration of the Sonication Apparatus

Ultrasound was applied using a probe-type sonicator (VC60, Sonics and Materials Inc., Connecticut, USA) with an operating frequency of 20kHz. The machine was used in continuous mode at 2.0 watts with a 3mm diameter probe placed 3mm below the surface of the sample to be sonicated. Thermal effects were minimised by placing the sonication sample in a vessel maintained at 4°C by a circulating water bath.

The amount of cavitation within a sample was quantified by an assay of the amount of molecular iodine formed from a potassium iodide solution. The iodine is formed by free radicals that are produced upon collapse cavitation and is therefore directly related to the amount of cavitation that occurs in the system. The method used was based on that described by Kondo and Yoshii (13).

Sonication-Mediated Transformation

Yeast cells were selected as a model system for the preliminary development of a sonication-mediated DNA delivery protocol as yeast is relatively easy to culture and yields results more rapidly than similar experiments carried out on mammalian cells. Gene transfer to the haploid AH22 (leucine requiring) strain of Saccharomyces cerevisiae was performed using the plasmid pGad1If. The plasmid contains the Saccharomyces cerevisiae LEU2 reporter gene. DNA delivery was carried out using a modification of a previously described heat-shock protocol (14). A single colony of AH22 was used to inoculate 4ml of YPD broth and grown overnight at 30°C in a shaking incubator (200 rpm). The overnight culture was diluted into 100ml of YPD to an OD<sub>600</sub> = 0.05 and grown at 30°C to an OD<sub>600</sub> = 0.2. The cells were harvested by centrifugation at 2000g for 5 minutes at 4°C, washed by resuspension in 5ml of sterile distilled water, then harvested again. The cells were resuspended in 0.5ml of sterile TE-LiAc (10mM Tris-Cl, 1mM EDTA, 100mM lithium acetate, pH 7.5). Aliquots of plasmid DNA (1µg) plus 50µg of carrier DNA (salmon sperm DNA) were added to Eppendorf tubes. The salmon sperm DNA was denatured immediately prior to use, by boiling 5µl of a 10mg/ml stock for 5 minutes, then placing on ice for 5 minutes. A 50µl aliquot of cells and 440µl of sterile PEG-TE-LiAc solution (TE-LiAc + 40% w/v polyethylene glycol 4000) were also added to each Eppendorf tube giving a final volume of 500µl. The suspensions were sonicated for various time periods. Following sonication, the cells were microfuged for 20 seconds then resuspended in 500µl TE. Cells were plated onto a medium which selected for the presence of the LEU2 reporter gene (yeast omission medium with all essential amino acids except leucine). Serial dilutions were also made from each tube and the cells plated onto a full medium (yeast omission media with all essential amino acids including leucine) to give a viable count. The plates were inverted and incubated at 30°C for 2–4 days before scoring the number of colonies.

Plasmid Integrity Following Sonication

Supercoiled plasmid DNA was purified by agarose gel electrophoresis and subsequently isolated from a gel slice using the GeneClean<sup>™</sup> DNA purification kit (BIO 101, La Jolla, USA). Aliquots of this supercoiled DNA, at the same concentration as that used in the transformation protocol, were placed into Eppendorf tubes. The volumes were made up to 500µl with freshly prepared PEG-TE-LiAc solution. Samples were sonicated for various time periods. DNA was recovered using the GeneClean<sup>™</sup> kit. Plasmid DNA integrity was assayed using agarose gel electrophoresis. 0.8% GibcoBRL ultrapure agarose in TAE buffer (0.4M Tris pH 8.0, NaOAc 0.2M, EDTA 10mM) was used. Gels were run for three hours at 5V/cm⁻¹ and subsequently stained with ethidium bromide (0.5mgL⁻¹).

Plasmid Integrity Following Sonication-Mediated Transformation

Plasmid DNA was isolated from yeast colonies which screened positive for the LEU2 reporter gene (15). The isolated DNA was used to transform Escherichia coli HB101 (ATCC 33694) by the calcium chloride method (16). Plasmid DNA was isolated from E.coli using the alkaline lysis method (17). Plasmid integrity (size and restriction pattern) were assayed using agarose gel electrophoresis.

RESULTS AND DISCUSSION

Calibration of the Sonicator with Respect to Cavitation

The use of potassium iodide to monitor cavitation has been widely employed (18–20). The sonication system described in the present study was calibrated using such a method. It can
be seen from Fig. 1 that there is an increase in the formation of iodine molecules with increasing sonication times. There is a rapid increase in the first 30 seconds. From 30 to 100 seconds the rate of iodine production decreases. Beyond 100 seconds very little additional iodine is produced. The assay measures a colour change initiated by free radical species released into the bulk liquid as a consequence of collapse cavitation and gives a direct measurement of the occurrence of transient cavitation events. Therefore, the results indicate that the greatest amount of cavitation occurs within the first 30 seconds in this system. Between 30 and 100 seconds there are fewer new cavitation events and even less beyond 100 seconds. However, there is a general increase in cavitation events with increased sonication time.

DNA Delivery via Sonication

Figs. 2 and 3 show the results of the investigation into sonication-mediated DNA delivery. Fig. 2 shows that increased sonication time results in higher transformation numbers until an optimum sonication time of approximately 30 seconds is reached. Beyond this optimum time, transformation numbers decrease rapidly up to 100 seconds then remain virtually constant between 100–250 seconds sonication. Fig. 3 shows the effect of sonication time on the viability of the cells (number of colony forming units) and indicate a general decrease in viability with increasing sonication time. However, the first part of the curve is shouldered, indicating only a small loss in viability during the first 15 seconds of sonication. At 30 seconds sonication, approximately 70% of the cells remain viable. Further sonication continues to give an exponential decrease in viability to 100 seconds sonication where only approximately 20% of the cells remain viable. After 100 seconds sonication the curve tails off and the viable count remains reasonably constant with approximately 10% of cells remaining.

Fig. 1. Iodine liberated from 0.1M KI solution versus sonication time at 2.0 watts. Each point is the average of 10 experiments (±SD).

Fig. 2. The number of transformants obtained during the study of sonication-mediated transformation at 2.0 watts. Each point on the graph represents the mean (±SE) of between 8 and 27 experiments. Each experiment represents the number of transformants obtained when transforming AH22 with 1 μg of pGadIf and was the average of triplicate plate counts.

Fig. 3. Viability curve showing the number of colony forming units obtained during the study of sonication-mediated transformation at 2.0 watts. Each point on the graph represents the mean (±SE) of between 8 and 27 experiments. Each experiment represents the number of colony forming units obtained when transforming AH22 with 1 μg of pGadIf and is an average of triplicate plate counts.
The events in the transformation curve (Fig. 2) can be related to the events in the viability curve (Fig. 3). For example, high transformation numbers are observed at shorter sonication times where there is a shoulder in the viability curve indicating a small loss in viability. From 30–100 seconds there is a rapid decrease in transformation number and there is also an exponential decrease in cell viability. The cells used in this study were a heterogeneous population at all stages of budding. It has been suggested that dividing cells are more susceptible to the effects of sonication (21, 22) therefore they may be killed off during this phase, which could explain the rapid decrease in transformation number between 30–100 seconds sonication.

After 100 seconds sonication there is a tail in the viability curve which could explain the constant tail in the transformation curve after 100 seconds sonication. It has been suggested that these cells may be a resistant sub-set of cells in the population, which are smaller in size than the rest of the population, and therefore more likely to move in the streaming forces during stable cavitation without mechanical damage (22, 23). However, the calibration curve (Fig. 1) indicates that very few new cavitation events occur at longer sonication times, which may result in the damaging mechanisms of sonication becoming exhausted, and would therefore explain the profile.

It is likely that the number of transformants obtained in a sonication-mediated transformation protocol is dependent upon a balance between transient cell damage and eventual cell death (Figs. 2 and 3). A number of reports in the literature provide evidence that cavitation is the mechanism responsible for cell damage during sonication (24–27). Presumably, cavitation transiently damages the cell membrane allowing enhanced uptake of plasmid DNA and leading to the observed increase in transformation efficiencies. However, this same cavitation mechanism can also lead to decreased cell viability. This hypothesis is consistent with the results obtained in the present study.

Fig. 1 shows the amount of iodine liberated from a potassium iodide solution, as a direct consequence of cavitation events, as a function of sonication time and it is interesting to note that the time course of the cavitation curve also reflects similar events in both the viability curve (Fig. 3) and the transformation curve (Fig. 2). Therefore, there appears to be a direct correlation between cavitation events, viability, and the number of transformants. To test this hypothesis, viability data from the sonication-mediated transformation experiments were plotted against the amount of cavitation (Fig. 4). A single exponential relationship can be seen from 30–250 seconds sonication which indicates that viability is directly related to the cavitation events which occur between 30–250 seconds sonication in this system. As no tail exists when viability is plotted against cavitation events (Fig. 4), it follows that the tail in the transformation and viability curves (Figs. 2 and 3) is unlikely to be a result of a resistant sub-population of cells. Therefore, degassing of the system at longer sonication times is probably the most likely explanation of this effect.

At sonication times less than 30 seconds it was noted that there was a large shoulder in the viability curve (Fig. 3). A significant shoulder in a viability curve generally indicates sub-lethal damage that can be repaired, which would also account for high viabilities at shorter sonication times and hence high transformation numbers. However, yeast generally exist in budding groups rather than as single cells. Therefore, it was possible that breakdown of groups of cells to single cells could account for the apparent increase in transformation numbers at short sonication times. Microscopic observations of the cells during the procedure showed that the yeast cells existed in budding groups which were rapidly broken down to single cells when exposed to ultrasound. This explained why the viability curve could be extrapolated back to a theoretical value of 120% and why complications arise when scoring colony forming units as opposed to single cells. However, the shoulder observed when the viability data is plotted against amount of cavitation (Fig. 4) is much larger than when it is plotted against sonication time (Fig. 3). An increase in cell number of approximately 20–30% is consistent with the breakdown of clumps to single cells but could not possibly account for all of the shoulder seen in Fig. 4. Therefore it is likely that both increased cell numbers and repair of sub-lethal damage contribute to the much larger shoulder observed in figure 4 and this in turn is what leads to the high transformation numbers observed at short sonication times.

In conclusion, the results suggest that cavitation is the mechanism which determines cell viability and hence transformation numbers in a sonication-mediated transformation protocol. The number of transformants obtained will depend upon sufficient transient damage to enable entry of DNA to the cell, but not enough damage to significantly decrease cell viability.

Plasmid Integrity Following Sonication

A concern with a sonication-mediated DNA delivery system is whether the integrity of the DNA would be compromised during the procedure. In order to investigate this, DNA samples were prepared and sonicated as described above. During isolation, the plasmid DNA was purified using the Geneclean™ procedure and yielded a preparation containing three different plasmid forms (supercoiled, linear and open circular). From Fig. 5 it is evident that all the forms of plasmid DNA are unaffected by the sonication procedure. In addition, this is a very sensitive assay as one single strand break would abolish a supercoil and convert it to the open circular form. Therefore,
in conclusion, plasmid DNA can be sonicated for 200 seconds in a similar manner to the sonication transformation protocol without measurable degradation.

Plasmid Integrity After Sonication-Mediated Transformation

There have been concerns that physical methods of transformation, such as electroporation, affect the integrity of the plasmid DNA entering the cell. This may not be detected immediately if the selection marker is unaffected. The investigation detailed above illustrates that no strand breakage occurs to plasmid DNA as a result of sonication. However, this does not preclude the presence of other more subtle forms of DNA damage. For example, damage to the DNA bases or sugars would not be detected by gel electrophoresis but might render the DNA liable to repair/recombination within the cell. Therefore the quality of the DNA once it has transformed a cell was also examined. If sonication is to prove useful as a transformation method, it is necessary to ensure that size and sequence of DNA are also unaffected once it has entered the cell. The aim of this study was to assess the quality of the plasmid DNA in cells transformed by sonication. This was achieved by checking the overall size and partial restriction map of the plasmids isolated from the transformed cells.

It is time-consuming to obtain pure plasmid DNA directly from yeast cells, therefore a crude preparation was made and transformed into E.coli to enable rapid purification. Yeast colonies transformed with pGad1f which screened positive for the LEU2 reporter gene were selected and small scale preparations of the pGad1f plasmid were made from the transformed yeast cells and transformed into the E.coli strain HB101. Samples of the pGad1f plasmid (not isolated from yeast cells) were also transformed into HB101 as a control. Bacterial colonies which screened positive for the ampicillin resistance gene were selected and small scale preparations of the pGad1f plasmid were made. Preparations were made from bacterial colonies which were transformed with the pGad1f plasmid isolated from the yeast clones, and also from the control pGad1f plasmid. A fraction of each preparation was cut using restriction enzymes.

Cut and uncut samples were analysed by agarose gel electrophoresis.

Samples transformed by sonication, heat shock, and background transformants (no sonication or heat shock) all had 22 yeast clones selected and prepared by this procedure. The samples shown in Figs. 6 and 7 are representative of 22 yeast clones from each method. Control DNA samples were prepared from Escherichia coli HB101 transformed with the pGad1f plasmid by heat shock.

Figure 6 illustrates there is no difference in the plasmid DNA prepared from the yeast clones transformed by sonication, heat shock, or in the absence of sonication or heat shock (lanes 3–11). It also illustrates that plasmid DNA isolated from yeast clones is identical to the control (lanes 12–14). All samples appear the same as the original pGad1f plasmid DNA sample (lane 2).

Figure 7 illustrates restriction digests of the samples with Eco R1 and Hin dIII. The pGad1f plasmid is 13.3 kilobases. Eco R1 should yield three fragments of 5.8, 5.3 and 2.2 kilobases. Hin dIII should yield two fragments of 13.0 and 0.3 kilobases, although it is difficult to observe the 0.3 kilobase fragment on a 0.8% AEF gel. Lanes 2–4 show control samples of pGad1f. The Eco R1 digest (lane 3) showed the expected fragments i.e. 5.8, 5.2 and 2.2 but only the 13 kilobase fragment of the Hin dIII digest was observed (lane 4). Lanes 5–7 show control samples of original plasmid. The Eco R1 digest is only partial but it is possible to see the very faint 5.8 and the 5.3 fragments plus partially digested fragments. The 2.2 kilobase fragment was too faint to be seen. This is not unexpected as longer lengths of DNA will bind more ethidium bromide and will be seen more clearly. The 13 kilobase fragment of the Hin dIII fragment is clear (lane 7).

Lanes 8–10 show samples from yeast clones transformed with 50 seconds sonication. The Eco R1 digest (lane 9) again shows the 5.3 and the 5.8 kilobase fragments plus partially digested fragments of 13.3, 11.1, 8.0 and 7.5 kilobases. Again the 2.2 kilobase fragment is too faint to be seen. The 13 kilobase fragment of the Hin dIII digest is clear (lane 10). Lanes 11–13 show samples from yeast clones transformed by heat shock. The fragments resulting from the Eco R1 (lane 12) and Hin dIII (lane 13) digests are as expected except again the 2.2 fragment was too faint to be observed on the Eco R1 digest. All the uncut samples appeared identical and at a position on the gel which was expected.

In conclusion, plasmid DNA isolated from samples transformed with sonication, heat shock and in the absence of sonication or heat shock appeared identical. Therefore the plasmid DNA is unlikely to have been adversely affected by the sonication step in the sonication-mediated transformation protocol.

CONCLUSIONS

The results of this study have demonstrated the use of ultrasound for the introduction of DNA into cells. Using yeast as a model system, it has been established that transfer of DNA is optimal at an output of 2.0 watts using short sonication times (30 seconds). Plasmid DNA appears to be unaffected by the sonication conditions. It was evident that the number of transformants obtained in a sonication-mediated protocol was dependent upon cell viability, and that the mechanism which determined cell viability also determined transformation num-
ber. The present study also strongly suggests that the mechanism involved in the delivery of DNA into cells is cavitation, as previously proposed (4–8). However, cavitation is not a single entity but a broad spectrum of related phenomena. Cellular damage that is attributed to cavitation damage may occur, (i) by tensile forces on the cells during microstreaming as a result of stable cavitation, (ii) by the liquid jets or shock waves following unstable cavitation and (iii) by the free radicals that may be produced during the final stages of bubble growth and collapse. The study presented here did not distinguish between the processes. The contribution of each cavitation phenomenon warrants further investigation.

The use of ultrasound as a method for the introduction of therapeutic macromolecules into cells was considered in the introduction. To evaluate this possibility, the work presented here would need to be repeated with mammalian cells and animal studies to assess the therapeutic potential of such a system. It is interesting to note that a recent report by Lauer et al. demonstrated that shock waves from a lithotripter enhanced the uptake of plasmid DNA into mammalian cells and suggested that cavitation was the mechanism responsible (28). In a later study, Delius et al. (29), based on the results of Lauer et al., suggested that lithotripsy could be used for gene therapy. Shock waves from a lithotripter can be non-invasively focused (through the skin) on to the target organ. Application of shock waves in combination with either local transcutaneous DNA application or delivery via the circulation may enable efficient DNA transfer to target cells.

Current delivery systems for gene therapy all have inherent problems. For example, viral vectors are very efficient delivery systems but have a number of pathogenic/immunogenic problems associated with them e.g. even non-replicative viral forms can recombine in vivo if a wild-type virus subsequently infects the treated cells. Similarly, long term exposure to non-native proteins can lead to local inflammatory and allergic reactions. Liposomes and polylysine-DNA conjugates do not have the toxicity problems that are associated with viral vectors but are not as efficient, since delivery via receptor-mediated endocytosis often leads to destruction of the endosomal contents. Development of an ultrasonic gene delivery system may offer efficient gene transfer without toxicity problems.

Various strategies have evolved in gene therapy depending upon the disease state e.g. the ex vivo approach, where a gene construct is transfected into cells which are re-injected into the host, gene replacement, where a defective or missing gene is introduced into the host cell and gene therapeutics, where the transfected gene effectively generates a "protein drug" with a specific pharmacological effect. Another approach is to introduce a potent inhibitor of a gene which is expressed to the detriment of the host. This involves the binding of short oligonucleotide sequences to mRNA to block RNA translation (anti-
sense approach), or to DNA (antigene approach), or to target molecules which bind to proteins involved in the regulation and expression of genes, and inhibit their activity (aptamer approach) (30). Virtually all these approaches to gene therapy depend upon the efficient delivery of large numbers of DNA molecules or chemically modified (nuclease-resistant) oligonucleotides to the cell. A clinically acceptable mechanical method of delivery based on ultrasound may offer a better means of delivering oligonucleotides into cells than presently available methods. An investigation of an oligonucleotide system would be an interesting extension of the area of ultrasonic delivery of macromolecules.

ACKNOWLEDGMENTS

This work was supported by an award from the BBSRC to J.-A.W. and grants from the Nuffield Foundation and Society for General Microbiology to A.D.

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