

Removal of PCR inhibitors using dielectrophoresis as a selective filter in a microsystem

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Diagnostic PCR has been used to analyse a wide range of biological materials. Conventional PCR consists of several steps such as sample preparation, template purification, and PCR amplification. PCR is often inhibited by contamination of DNA templates. To increase the sensitivity of the PCR, the removal of PCR inhibitors in sample preparation steps is essential and several methods have been published. The methods are either chemical or based on filtering. Conventional ways of filtering include mechanical filters or washing *e.g.* by centrifugation. Another way of filtering is the use of electric fields. It has been shown that a cell will experience a force when an inhomogeneous electric field is applied. The effect is called dielectrophoresis (DEP). The resulting force depends on the difference between the internal properties of the cell and the surrounding fluid. DEP has been applied to manipulate cells in many microstructures. In this study, we used DEP as a selective filter for holding cells in a microsystem while the PCR inhibitors were flushed out of the system. Haemoglobin and heparin – natural components of blood – were selected as PCR inhibitors, since the inhibitory effects of these components to PCR have been well documented. The usefulness of DEP in a microsystem to withhold baker's yeast (*Saccharomyces cerevisiae*) cells while the PCR inhibitors haemoglobin and heparin are removed will be presented and factors that influence the effect of DEP in the microsystem will be discussed. This is the first time dielectrophoresis has been used as a selective filter for removing PCR inhibitors in a microsystem.

Introduction

The polymerase chain reaction (PCR) has been widely used to detect microorganisms in clinical diagnostics and the food industry. However, when conducting PCR on complex biological materials (blood, milk, faeces *etc.*), residual materials from the samples or from pretreatment steps can inhibit and significantly reduce the efficiency of the PCR process.^{1,2} The mechanism of the inhibition can *e.g.* be a competition between inhibitor and DNA template or chelation of the ions used in the process.³ It has been well documented that the PCR inhibition can be solved by selecting a suitable method for pretreating the sample, by selection of a suitable method to isolate DNA templates, or by selecting a DNA polymerase that is less affected by the inhibitor.⁴ Sample pretreatment is an essential step to limit the PCR inhibition effects. Pretreatment is either chemical or mechanical.⁵ Filtering or washing by centrifugation can be used to wash away the inhibitors as long as the inhibitors do not bind to the cells or are not inside the cells, but both methods are laborious and are not suitable for microsystems. In addition, filters holding the cells can get clogged or collect air, and the cells tend to stick onto the surface. Dielectrophoresis (DEP)^{6,7} has previously been used for manipulation and sorting of cells in microsystems.^{8–10} In this paper we describe a new approach to the use of DEP as a selective filter in a DEP-chip to remove PCR inhibitors. By using DEP it is possible to attach the cells to electrodes, while other materials with different dielectric characteristics (in this case inhibitors) are passing unhindered through the system. Interestingly, when using DEP as a selective filter in the DEP-chip, the DEP force can be turned off and the cells released as a ready sample for the PCR.

Principle of dielectrophoresis (DEP)

When an electric field is applied upon particles such as biological cells, a dipole is induced.^{11,12} If the electric field is non-uniform, a part of the dipole will experience a stronger field, thereby creating an overall force on the cell. This force is not dependent on the polarity of the external field, but on internal properties of the cell compared to its surroundings. The time average of the DEP force on a spherical cell can be written as

$$\langle F_{\text{dep}} \rangle = 2\pi\epsilon_r R^3 \text{Re}[\underline{K}(\omega)]\nabla E^2 \quad (1)$$

Where ϵ_r is the permittivity of the fluid, R is the cell radius, E the electric field strength and $\underline{K}(\omega)$ is the complex Clausius-Mossotti factor, which is the strength of the effective polarization of the cell. With the electrode geometry in our system, the direction of the force is defined purely by the Clausius-Mossotti factor, which is

$$\underline{K}(\omega) = \frac{\epsilon_c - \epsilon_f}{\epsilon_c + 2\epsilon_f} \quad (2)$$

Here ϵ_c and ϵ_f is the complex permittivity of the cell and fluid, respectively. The complex permittivity is defined as $\underline{\epsilon} = \epsilon - i\sigma/\omega$, where $i = \sqrt{-1}$ and $\omega = 2\pi f$ is the radial frequency of the imposed electrical field. σ is the conductivity of the current media (σ_c for a cell and σ_f for fluid) and ϵ is the permittivity.

To illustrate one important aspect of the system, whether cells are attracted or repelled from the electrodes, it is useful to rewrite $\underline{K}(\omega)$ in terms of the characteristic relaxation time constants¹¹

$$\underline{K}(\omega) = \frac{\sigma_c - \sigma_f}{\sigma_c + 2\sigma_f} \cdot \frac{i\omega\tau_o + 1}{i\omega\tau_{mv} + 1} \quad (3)$$

Where $\tau_o = (\epsilon_c - \epsilon_f)/(\sigma_c - \sigma_f)$ and $\tau_{mv} = (\epsilon_c + 2\epsilon_f)/(\sigma_c + 2\sigma_f)$. The combining of eqns. (1) and (3) reveals that when the conductivity of the fluid is higher than the effective conductivity of a cell, the cell will experience a negative DEP force and be repelled from the electrode (negative DEP). When σ_f is lower than σ_c the cell will be attracted to the electrodes (positive DEP). To ensure that the cells always experience a positive DEP force σ_f must be lower than σ_c . For the experiments described in this study sterile water with lower conductivity is always used for washing. The cells attached to the electrodes in the beginning of a washing process are therefore not released during the washing and knowing the exact value of σ_c is not important.

Materials and methods

Fabrication of the microsystem

The microsystem was a DEP-chip fabricated on a silicon substrate. A 2 μm LPCVD (Low Pressure Chemical Vapour Deposition) nitride layer was deposited on the silicon substrate to insulate the electrodes from the substrate. On top of that a 180 μm LPCVD polysilicon layer was deposited. An 80 nm layer of titanium was deposited using E-beam evaporation and structured using a lift-off process. To remove the natural oxide a 30 s HF (hydrofluoric acid) dip was applied just before titanium deposition. The silicide was formed using rapid thermal annealing for 1 min at above 700 $^{\circ}\text{C}$ in an argon atmosphere. The electrode design was an interdigitated-finger structure⁶ with a characteristic dimension of 10 μm and a length of 2 mm (Fig. 1). The walls of the channel were made of SU-8¹³ and the channel was height \times width = 70 \times 400 μm . The total channel length in the microstructure was 11 mm.

Yeast cells

The baker's yeast (*Saccharomyces cerevisiae*) (Danisco A/S, Denmark) cell was chosen as a model in the experiment. The yeast cells were grown overnight at 37 $^{\circ}\text{C}$ in Yeast extract Nitrogen Base (YNB) medium supplemented with 40% glucose and 40 $\mu\text{g l}^{-1}$ histidine (Bie & Berntsen A/S, Denmark). Cells were collected by centrifugation, and the medium was removed. The cell pellet was washed three times in 1 ml water (ultra-clean water, MERCK eurolab). 100 μl of the cell suspension (10^7 cells per ml) were collected, and used as positive PCR controls (without PCR inhibitor).

Inhibitors

Heparin (Sigma, St. Louis, Missouri, USA) and bovine haemoglobin (Sigma, St. Louis, Missouri, USA) were selected

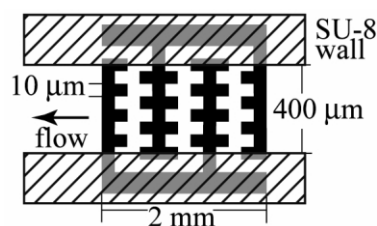


Fig. 1 Schematic outline of the DEP microsystem used in this study. The electrode structure is of the interdigitated-finger geometry.⁶

as inhibitors for testing the ability to remove inhibitors from the cells using the DEP microstructure, since both heparin and haemoglobin are well known as PCR inhibitors. 100 μl of a solution with the inhibitors in 10 \times the final concentration were added to 900 μl of the cell samples. This gave a final concentration of 1.3 mg ml⁻¹ for heparin (giving a conductivity of $\sigma_f = 23 \text{ mS m}^{-1}$) and 10 mg ml⁻¹ for haemoglobin (giving $\sigma_f = 52 \text{ mS m}^{-1}$). 30 μl of this mixture – containing both cells and the chosen inhibitor – was used as PCR controls. The final concentration of inhibitors was made several orders of magnitude higher than the concentration that gives a PCR inhibition effect.³ The mixture of yeast cells with inhibitors were prepared and kept at room temperature for 1 h before use.

Preparing the DEP-chip for experiments

The DEP-chip was cleaned thoroughly before use. Firstly, the DEP-chip was flushed through with sterile water, then with ethanol followed by sodium dodecyl sulfate (SDS), 10%, and finally with sterile water to remove any residues of SDS. The system was then ready for use.

Primers and PCR conditions

Two primers, namely Ribo.Prot.S3-forward and Ribo.Prot.S3-reverse were used in a PCR to amplify the yeast ribosomal protein S3 – a housekeeping gene. The Ribo.Prot.S3-forward primer was a 21-mer primer with the sequence 5'-AAT CCT CAG GCA AAT GTA AAA-3'. The Ribo.Prot.S3-reverse primer was a 20-mer primer with the sequence 5'-CTT AGG CAA ATC AAA AGC AT-3' (TAG Copenhagen A/S, Denmark). PCR amplicons of 199 bp of the yeast ribosomal protein S3 gene were generated in a PCR with these primers.

All PCR mixtures (20 μl) contained 1 \times AmpliTaq Gold PCR Master Mix (Applied Biosystems, CA, USA) and 0.156 μM of primers. The PCR was performed in a thermocycler (PTC-200; MJ Research, Inc., MA, USA) for 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 59 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 1 min after a hot start at 94 $^{\circ}\text{C}$ for 10 min. The last step was an extension at 72 $^{\circ}\text{C}$ for 10 min. 10 μl of yeast cells suspension was used as templates. Yeast cells in pure water were used in PCR positive controls, water in negative controls, and yeast-inhibitors (heparin or haemoglobin) with yeast cells in inhibition controls.

DNA analysis

All PCR products were analysed on the microchip based Agilent 2100 Bioanalyzer (Agilent Technologies, CA, US) using the DNA 500 kit, which can analyse DNA fragments up to a length of 500 bp.

Results

Using the DEP-chip as selective filter to remove PCR inhibitor

An overview of the experimental set up using the DEP-chip to wash away the inhibitor from the yeast cells is presented in Figs. 2 and 3. For initialisation of the chip, the DEP-chip was filled with water from a syringe used for washing. Details of the 3-step of manipulation of the DEP-chip are illustrated in Fig. 4. In step 1, the withdrawing syringe was started, thereby sucking the sample into the channel. A voltage of 20 V_{pp} at 1 MHz was applied to the electrodes and the yeast were collected on the electrodes by positive DEP. In Fig. 5 a series of images illustrates the build up of cells on the electrodes. After 1–2 min

(depending on the density of cells), the electrodes were saturated with yeast cells, the suction was stopped, and the sample container vial was removed from the structure (Fig. 4, step 1). In step 2, pure water was flushed through the channel (Fig. 4, step 2). The flow rate of pure water was 0.2 ml h⁻¹ for the experiments in Table 2 and 1 ml h⁻¹ for the experiments in Fig. 6. After a selected time (0 to 30 min), step 3 was started. The voltage applied to the electrodes was turned off, the cells were released, and the pump was set to 1 ml h⁻¹ to flush out the cells (Fig. 4, Step 3). Sometimes a short pressure pulse was applied (by flicking the inlet tube) to release the cells. One drop (approximately 20 µl) of the released cells was collected from the output for PCR analysis. Between each run in an experiment with the same inhibitor, the DEP-chip was flushed with clean water by setting the syringe pump with a speed of 3 ml h⁻¹ for a few minutes.

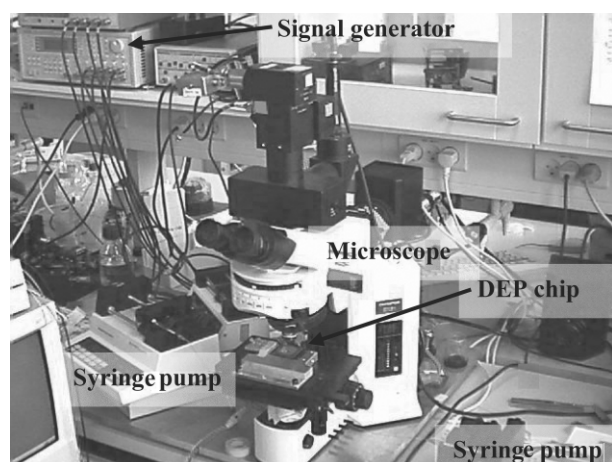


Fig. 2 Experimental set up using the DEP-chip to wash away PCR inhibitors from the yeast cells.

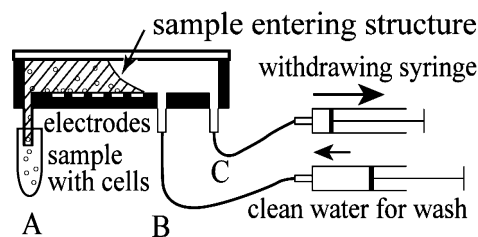


Fig. 3 Schematic overview of the set up, showing the first step in which the sample is sucked into the structure.

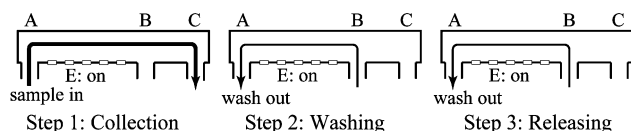


Fig. 4 Functional overview of the experiment set up. Step 1, the pump withdraws and the sample is sucked over the electrodes. Step 2, the inhibitor is washed away while the cells are attached to the electrodes. Step 3, the voltage applied to the electrodes is turned off. The cells are released and collected outside.

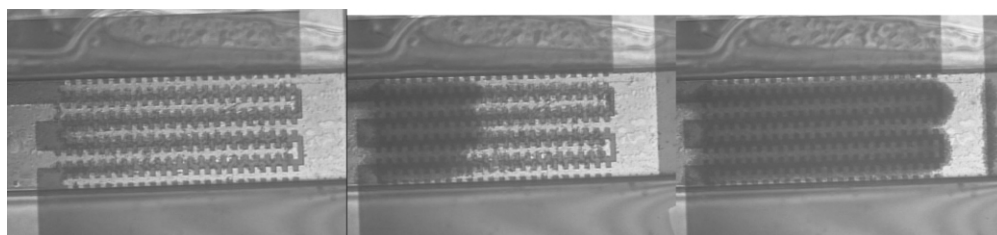


Fig. 5 A series of 3 images showing the build up of cells as they collect on the electrodes under the influence of positive DEP.

The influence of different factors on the efficiency of the DEP-chip

DEP force. When attracted to the electrodes, the cells were lying side by side, forming a thick layer that might be several times thicker than the cell diameter. The electric field strength decreases with the distance from the electrodes, and cells at the top of the layer were therefore exposed to a lower DEP force and more sensitive to the flow. Applying a low flow rate and a high field-strength will maximize the amount of collected cells.

Conductivity. To determine the highest effective conductivity that is applicable for the DEP-chip, a dilution row of KCl in water was made and mixed with the yeast cells and the mixture was pumped through the DEP-chip. We determined that with the flow set to 0.2 ml h⁻¹ and a field of 20 V_{pp} at 1 MHz, yeast cells could be collected when conductivity reached up to 140 mS m⁻¹, but at very low efficiency. A useable conductivity of the sample applicable in our DEP-chip using the yeast cell was determined to be 70 mS m⁻¹.

Flow rate. The flow rates of the DEP-chip were varied from $f = 0.1$ ml h⁻¹ to 1.0 ml h⁻¹. A syringe pump manually controlled the flow rate. With a cross-section of the structure, $A = 400 \mu\text{m} \times 70 \mu\text{m}$, the flow rate was calculated to have an average flow velocity ($v = f/A$) around 1 mm s⁻¹ to 10 mm s⁻¹. We also found that applying a higher velocity reduced the amount of collected cells.

Determination of PCR inhibitor effect of haemoglobin and heparin. The effect of haemoglobin and heparin on the PCR amplification of the yeast ribosomal protein S3 gene was investigated. Yeast cells and haemoglobin or heparin were mixed and 10 µl of this sample was used as template (Table 1). These initial experiments showed that the PCR reactions were completely inhibited by a haemoglobin concentration in the sample of 1 mg ml⁻¹ and by a heparin concentration of 13 µg ml⁻¹.

Effects of the DEP-chip to remove the PCR inhibitor effect of heparin and haemoglobin. The results of PCR amplifications of the yeast ribosomal protein S3 gene using the yeast cells washed in the DEP-chip as template is presented in Table 2 and in Fig. 6, respectively. The yeast cells in water (10⁷ cells per ml) were mixed with haemoglobin (10 mg ml⁻¹), or heparin (1.3 mg ml⁻¹), respectively, and the cell/inhibitor mixtures were applied to the DEP-chip for washing as described above. The flow rate of pure washing water was 0.2 ml for the experiments in Table 2 and 1 ml h⁻¹ for the experiments in Fig. 6. The cells were collected after washing 0, 2.5, 5, 7.5, 10 or 30 min, respectively (Table 2) or after washing 0, 2, 4, 6, or 8 min, respectively (Fig. 6). The cells were used directly as PCR templates. At a flow rate of 0.2 ml h⁻¹ (Table 2) a 199 bp PCR amplicon of the yeast ribosomal protein S3 gene was observed in all the PCR in which the yeast cells collected after 10 or 30 min washing were used as templates. With haemoglobin, positive PCR results were obtained for samples collected after 5

min of washing while with heparin, positive PCR were only observed with samples collected after 10 min of washing. We also found that with the flow rate of 0.2 ml h^{-1} , a sufficient washing time was approximately 10 min. A series of 48 experiments were performed at a 5-times higher flow rate (1 ml h^{-1}). The percentage of positive PCR reaction increased gradually from 33% or 40% (after 2 min washing) to 100% or 89% (after 8 min washing) for haemoglobin and heparin, respectively. For the positive PCR reactions, the concentrations of PCR product were $0.91 \pm 0.51 \text{ ng } \mu\text{l}^{-1}$ and there was no significant relation between the concentration of PCR product and the time for washing.

Discussion

In this study, the baker's yeast (*S. cerevisiae*) cell was used as a model organism for investigating the capability of dielec-

trophoresis in a microsystem to remove inhibitors from the cells. Yeast was chosen for several reasons. It is unicellular, safe to handle, fast growing in cheap culture medium, easily manipulated and with its size on average $5\text{--}10 \mu\text{m}$ it is visible under normal light microscopy.¹⁴ Furthermore, with a very strong membrane the yeast cells can stand a wide variation in salt concentrations.

Heparin and haemoglobin were selected as inhibitor models in the study since both are natural components of the blood. A number of publications have shown that haemoglobin has a great inhibition effect on PCR and similar effects have been reported with heparin.^{3,15,16} Haemoglobin is known as a multi-chained protein that is the oxygen-carrying protein of red blood cells. Haemoglobin is made up of four polypeptides or globin chains; two identical α -chains and two identical β -chains. The globin chains of the hemoglobin interact and are connected with each other by the haeme group which contains an iron ion (Fe^{2+}) in the centre. The haeme group with the iron ion has been shown to be involved in inactivating several DNA polymerases in PCR reactions.¹⁷ In an initial experiment we determined that a concentration of 1 mg ml^{-1} haemoglobin or of 0.013 mg ml^{-1} heparin had a great inhibitive effect on our PCR amplifications. A concentration of 10 mg ml^{-1} haemoglobin in water and 1.3 mg ml^{-1} of heparin in water were therefore selected as suitable inhibitor concentrations for all the tests throughout the study. These concentrations were 1 and 2 orders of magnitude, respectively, higher than the concentration giving the PCR inhibition effect.

Several factors define the amount of cells that can be collected by the DEP-chip such as the designed geometrical electrode, the conductivity of the sample, the flow-rate, and the field-strength. The conductivity of the sample is an essential factor that influences the effectivity of the DEP-chip. Adding ions to water will increase the conductivity of the solution, and almost any inhibitor will therefore increase the conductivity of the mixture in comparison to clean water. As one can see from eqn. (3) there is a cross-over point, where positive DEP ceases to exist and the electrodes will not attract the cells. The force by which cells are attracted to the electrodes is reduced as σ_f gets closer to σ_c . The transition from useable to non-useable conductivity to apply for collecting cells is not abrupt, but graduated, as the layer of cells attached to the electrodes becomes thinner as σ_f increases. A high cell collection efficiency of the DEP-chip of more than 90% was observed

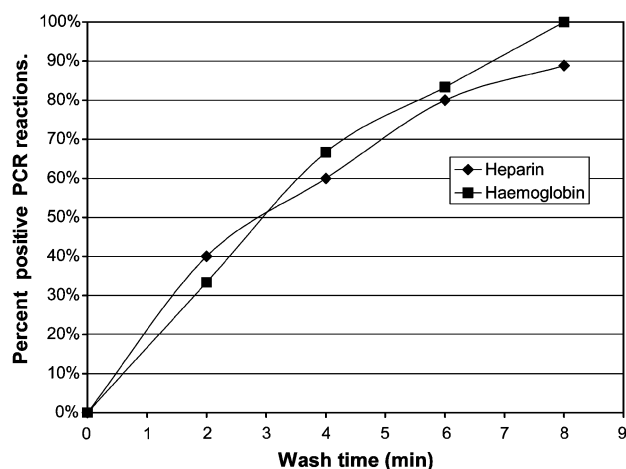


Fig. 6 Removal of haemoglobin and heparin inhibition effects on PCR. The baker's yeast cells were mixed with PCR inhibitors – haemoglobin or heparin, respectively. The cell mixture was applied to the DEP-chip to wash away the inhibitors with a flow rate of 1 ml h^{-1} (see text). The cells were collected at different time points and PCR was performed with the yeast ribosomal protein S3 primers (see Materials and Methods for more details). The graph shows the percentage of positive PCR reactions at each wash time.

Table 1 The inhibition effects of haemoglobin and heparin on PCR

| | 10 mg ml^{-1} | 1 mg ml^{-1} | $100 \mu\text{g ml}^{-1}$ | $10 \mu\text{g ml}^{-1}$ | $1 \mu\text{g ml}^{-1}$ | 100 ng ml^{-1} | pos | neg |
|------------------------|--------------------------|---------------------------|---------------------------|---------------------------|--------------------------|--------------------------|-----|-----|
| Haemoglobin PCR result | – | – | + | + | + | + | + | – |
| Heparin PCR result | 1.3 mg ml^{-1} | $130 \mu\text{g ml}^{-1}$ | $13 \mu\text{g ml}^{-1}$ | $1.3 \mu\text{g ml}^{-1}$ | 130 ng ml^{-1} | 13 ng ml^{-1} | pos | neg |
| | – | – | – | + | + | + | + | – |

+ PCR positive, – no PCR product, pos/neg: positive and negative controls.

Table 2 Removal of haemoglobin and heparin inhibition effects on PCR. The baker's yeast cells were mixed with PCR inhibitors – haemoglobin or heparin, respectively. The cell mixture was applied to the DEP-chip to wash away the inhibitors with a flow rate of 0.2 ml h^{-1} (see text). The cells were collected at different time points and PCR was performed with the yeast ribosomal protein S3 primers (see Materials and Methods for more details).

| Washing time (min) | Haemoglobin (10 mg ml^{-1}) | Haemoglobin (10 mg ml^{-1}) | Heparin (1.3 mg ml^{-1}) | Heparin (1.3 mg ml^{-1}) |
|--------------------|---|---|--------------------------------------|--------------------------------------|
| 0 | – | – | – | – |
| 2.5 | – | – | – | – |
| 5 | + | + | – | – |
| 7.5 | nt. | + | nt. | – |
| 10 | + | + | + | + |
| 30 | + | nt. | + | nt. |
| Positive control | + | + | + | + |
| Negative control | – | – | – | – |
| Inhibitor control | – | – | – | – |

+ Indicates a strong peak on the electropherogram, – indicates no peak, nt. indicates not tested.

when the conductivity of the fluid was lower than 50 mS m⁻¹. Experimental data revealed that conductivity of 70 mS m⁻¹ is applicable in our DEP-chip using the yeast cells. We also found that, applying a sample with lower conductivity will ensure a larger DEP force and higher flow velocities can be applied for the DEP-chip. When the electrodes were saturated, no further cells were collected and every new cell entering the DEP-chip would be wasted. We determined that with a yeast culture of 10⁷ cells per ml and an electrode surface of 0.8 mm², 1 min was sufficient time to saturate the electrodes.

A number of advantages of the DEP-chip used as a selective filter are: (1) the cells needed for analysis are attached on the electrodes, and the application of purified water, with lower conductivity, for washing, ensures a positive DEP (the cells are attracted to the electrodes); (2) the cells can easily be released after washing by switching off the electric field; (3) the released cells are ready for analysis in the next step; and (4) the chip is ready for another sample analysis. The DEP-chip also has a number of disadvantages. A main concern is the limit of suitable conductivity of the fluid carrying the cells. In contrast to stabilization of pH, which can be achieved by a pH-stabilizing buffer, the only way to reduce the conductivity of a solution is to dilute with a solution having a lower conductivity. In our system with yeast cells we used pure water for dilution and washing. However, not all cells can tolerate solutions with such low osmotic pressure. In such cases a sugar solution with physiological osmolarity (e.g. 9% sucrose) can be used.

A potential problem in DEP experiments is cell lysis or sample heating that may occur when applying the electric field. However, we have seen no sign of cell lysis or sample heating even in an ac field of 20 Vpp. Another potential problem in DEP experiments is adhesion of cells to the electrodes. For the yeast cells used in this study the cells could simply be released by switching off the field and flushing out. Sometimes a short pressure pulse was applied (by flicking the inlet tube) to release the cells. For other types of cells adhesion may be a severe problem. In such cases the electrodes can be coated to prevent cell adhesion.¹⁸

With the DEP-chip, it is possible to sort different types of cells on the basis of differences in their dielectric properties. Such a method requires a fluid with known conductivity, and the specific DEP spectrum of each type of cell. The DEP spectrum of a certain cell type is in most cases unknown and, when so, laborious to obtain. However, knowing these, it is possible to adjust the frequency and conductivity needed, making the DEP-chip capable of discriminating between different cell types e.g. cancer and normal cells.¹⁹

In this study we used pure water for washing sample and the fluid conductivity was therefore lowered during the wash. It is, however, possible to use a fluid with a specific conductivity in the wash to keep σ_r constant when discriminating based on cell type.

The DEP-chip described in this study was designed with only the electrodes used for collecting and holding the cells. Therefore, it is necessary to release the cells from the electrodes and collect them outside for the PCR analysis. Research on a newly designed integrated chip, in which a PCR amplification chamber is integrated with the DEP part on the chip, is progressing. The newly designed integrated DEP/PCR-chip makes it possible to move the pretreatment of cells on chip, this will reduce the loss of rare cells as well as minimizing the amount of external handling before a diagnostic PCR analysis.

In conclusion, in the present study we showed that yeast could be selectively withheld while PCR inhibitors are removed by using dielectrophoresis in a microsystem. The required volumes (sample and wash) are small, and of the order of 30 μ l. The dielectrophoretic filter can be used to collect any cell type, without altering the set up much. In contrast to conventional methods the sample preparation method presented here is suited

for integration with microstructures for PCR reaction and DNA analysis. This work is an important contribution towards sample preparation, PCR reaction and DNA analysis in a micro total analysis system for molecular diagnostics.

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