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The application of ultrasound as a rapid method to provide DNA fragments suitable for detection by DNA biosensors

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Abstract

Contamination of food and water supplies by microorganisms such as *Escherichia coli*, the need for point-of-care bedside analysis of biological samples, and concerns about terrorist attacks using biological organisms, have made the development of fast, reliable, and sensitive analytical methodologies for use in monitoring of pathogens very important. With a variety of biosensors being developed for extremely sensitive and rapid nucleic acid diagnostics, it has become even more important to shift focus towards creation of methods to decrease the amount of time and effort necessary for sample preparation. The application of ultrasound has the potential to create DNA fragments from genomic material with lengths that are suitable for determination using biosensors and microarrays. For example, application of 85 W power at a frequency of 20 kHz can produce a preponderance of fragments of 100–400 base pairs (bp) within several seconds, and sample processing can lead to over 75% conversion from genomic material to fragments in times of 20–30 s. A proportion of these fragments are in a single-stranded state and are suitable for hydridization with immobilized single-stranded DNA probe oligonucleotides using a fiber optic biosensor. Control of factors such as salt concentration, exposure time, ultrasound power, and the initial temperature of the solution, can affect the length and form (single- or double-stranded) of DNA fragments that are generated by ultrasound, and average fragment length can be adjusted by selection of these operating parameters.

Keywords: Ultrasound; Cavitation; DNA; Biosensors; Fragmentation; Fiber optic

1. Introduction

The development of fast, reliable, and sensitive analytical methodologies for use in monitoring of environmental pathogens is of great interest. Recent events associated with contamination of municipal water supplies by *Escherichia coli* (*E. coli*) (O'Connor, 2002), with food products by *Listeria* (Donnelly, 2001), and concerns about terrorist attack of the food and water supplies, have generated substantial interest in expediting the development of rapid dedicated test kits and monitoring devices. This interest is also evident in clinical situations such as point-of-care bedside analysis (Kluge et al., 2003). Examples of rapid testing platforms include those that use immunochemistry, and alternatively, those that use nucleic acid detection.

DNA biosensors can ideally offer rapid, dedicated, and reversible detection of bacteria, viruses, and genetic screening for diseases. Commonly, single-stranded DNA (ssDNA) probe molecules are immobilized on the surface of a device and a signal is generated upon the hybridization of target nucleic acid sequences to these probes. This requires that the target genomic DNA is available in single-stranded form, and that, preferentially, it is available as segment lengths that are sufficiently short to allow for fast hybridization kinetics and limited steric hinderance, while sufficiently long enough to ensure specificity by virtue of the energetics associated with hybridization of the nucleic acid target sequence. With the development of biosensors that are capable of fast speeds and low detection levels (for example, 20–40 s for DNA at concentrations of approximately 1 pg/mL, for targets of 25–300 mer length (Watterson et al., 2001)), the problem of developing an appropriate rapid sample preparation method to convert genomic DNA into single-stranded material of short sequence lengths becomes of paramount importance. Optimally, one sample preparation method would provide short fragments from genomic DNA that could be detected by a variety of different biosensors, such as acoustic (Cavic and Thompson, 2002; Su et al., 1994), surface

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plasmon resonance (SPR) (Georgiadis et al., 2000; Peterson et al., 2002), electrochemical (Azek et al., 2000; Yang and Thorp, 2001; Millan et al., 1994; Wang, 2002), and optical systems (Piunno et al., 1995; Jordan et al., 1997; Graham et al., 1992).

There are several methods that can be used to generate target material for DNA biosensors. Some of the more common approaches include cleavage by restriction enzymes, and polymerase chain reaction (PCR) (Saiki et al., 1985). Restriction enzymes are useful because they can be chosen to cleave at specific nucleic acid sites, and can be efficient in producing fragments of known sequences and lengths. However, the drawback to using restriction enzymes is that it requires additions of reagents to the system, and careful control to ensure conditions are optimal for enzyme activity. PCR is an analytical technique that allows for the amplification of specific DNA sequences. The target to be amplified acts as a template, and can be copied during successive cycles of heating and cooling. The advantage of this approach is an exponential increase in the amount of target (amplicon) that is formed during the progression of cycles. Shorter amplicons can hybridize with immobilized ssDNA probes, and this is the basis for a number of microarray protocols. Real-time PCR (Walker, 2002) not only amplifies a target sequence but also quantifies the amount of material that was initially available, eliminating the need for biosensors. The drawback of PCR methods is that they require the presence of the desired sequence, primers that define the region to be copied, DNA polymerase, the presence of deoxynucleoside triphosphates, and a lack of any other similar sequence that might be copied. PCR methods have their place in experiments where samples are very limited in the number of target copies of a sequence, but are not ideal when there are sufficient copies for detection. Therefore, it is important to consider physical methods such as syringe shearing and the application of ultrasound as rapid and simple means to fragment DNA. Syringe shearing can be effective in producing fragments from genomic DNA, but the manual approach is not readily controlled in terms of reproducibility. Ultrasound is more versatile and more readily controlled in terms of energy delivery and selection of environmental conditions.

Ultrasound is an oscillating pressure wave with a frequency of approximately 20 kHz and higher (Wilson and Buffa, 1997). Since ultrasound is mechanical in nature, it requires a medium for propagation. In aqueous solutions, the application of ultrasound causes a phenomenon known as cavitation. Cavitation occurs when the gases dissolved in liquid are induced to form cavities or microbubbles. The radius of these bubbles can range from 100 to 250 μm (Elsner and Lindblad, 1988). There are two types of cavitation that can be said to influence biomolecules in solution in a direct or indirect manner: stable cavitation (or gas body activation) and transient (or vaporous or inertial) cavitation (Fry, 1978; Leighton, 1994; Miller et al., 1996; Riesz and Kondo, 1992). Stable cavitation occurs in solutions when the application of low intensities of ultrasound (ca. 1 W/cm^2) generates a current of microbubbles (Elsner and Lindblad, 1988). During the negative half-pressure cycle of the ultrasound wave, the microbubbles increase in size; during the positive half-pressure cycle, the bubbles decrease in size. Transient cavitation occurs during the application of higher intensities of ultrasound (greater than 1 W/cm^2) (Elsner and Lindblad, 1988). In this case, the microbubbles may begin to oscillate in size but at some point during sonication, they reach a critical size and collapse. The extreme heat generated at the point of this collapse, several thousand Kelvin, is enough to cause the formation of free radicals from water (Todd, 1970; Henglein, 1987; Suslick et al., 1986; Makino et al., 1983). The collapses that occur due to transient cavitation are considered high energy events and can be quite destructive. In fact, they can be responsible for erosion, cell disruption, sonoluminescence, and shearing of biological molecules (Leighton, 1994).

When cellular materials are exposed to ultrasound, there are two mechanisms that are primarily responsible for the observed effects on biological molecules. The first is direct mechanical damage via bulk heating. The second is by indirect interaction and is chemical action in which free radicals attack molecules. This oscillation in microbubble size causes shearing stress as microstreaming occurs at the bubble surface and bubbles interact with each other, solution, and vessel walls. Mechanical stress is responsible for causing cell lysis and is suspected to be responsible for most of the DNA degradation in solution (Pritchard et al., 1966; Peacocke and Pritchard, 1968). Besides cavitation, direct mechanical or thermal degradation is the other process by which ultrasound ruptures DNA. This type of fragmentation can be found in the absence of cavitation and is caused by the acceleration of molecules, causing increased interaction between solvent and DNA therefore resulting in a thermally derived degradation (Elsner and Lindblad, 1988). It is generally accepted that the fragmentation of DNA by mechanical stress in solutions exposed to ultrasound is via successive halving while that of free radical attack is more random (Freifelder and Davison, 1962).

Ideally, the sample preparation procedure to produce fragments for analysis will be very fast and have a minimal number of steps. Experiments reported by our laboratory have confirmed that a signal can be obtained from a fluorescence-based fiber optic biosensor using 25 mer immobilized single-stranded probes, intercalating dye for detection of hybridization, and samples of sonicated purified genomic *E. coli* DNA (Almadidy et al., 2002). This work was done using an initial concentration of 1 pg/mL of DNA, and the fluorescence signal reached equilibrium in about 20 s. The results suggested that there was availability of some single-stranded target of a size that was appropriate for hybridization and detection by the biosensor.

The present report provides insight about the nature of the samples that were able to produce signals using the fiber optic biosensor. The work evaluates the primary factors that can be used to control the size of fragments formed upon

sonication of genomic material, and considers whether this material is in a form that is suitable for detection by use of biosensors that rely on hybridization. The fragmentation of purified genomic material from *E. coli* is compared with results using solutions containing live whole cells to determine whether extraction of genomic material is necessary at all. Cumulatively, the results suggest that it is possible to design a rapid and efficient sample preparation system to create a distribution of target fragments of a desired length and form.

2. Experimental

2.1. Solutions and reagents

The genomic DNA used in most of the experiments was salmon sperm nuclei type II-S purchased from Sigma–Aldrich (Oakville, ON). Unless otherwise indicated, the DNA solutions were made and diluted using TE buffer consisting of 10 mM Tris–HCl from GIBCO BRL LifeTechnologies Inc. (Gaithersburg, MD) and 1 mM EDTA (Sigma–Aldrich) and the pH was adjusted to approximately 8. The sodium chloride that was used to alter the salt concentration of the samples was from EM Science (Darmstadt, Germany). Ultra Pure Electrophoresis Grade agarose was used for agarose gels (GIBCO BRL LifeTechnologies Inc). The agarose gels were diluted using TBE buffer. A 10× TBE buffer was made using 49 g of Trizma® Base or tris(hydroxymethyl)aminomethane (Sigma–Aldrich), 25.05 g boric acid of from BDH Inc. (Toronto, ON), and 1.68 g ethylenediaminetetraacetic acid (EDTA) (Sigma–Aldrich) in 450 mL of cartridge purified water (MilliQ) obtained from a Millipore water purification system from Millipore Corporation (Bedford, MA). This was diluted to 1× as a running buffer for agarose gel electrophoresis and as a solvent to make the agarose gels. SYBR Gold nucleic acid stain was from Molecular Probes (Eugene, Oregon). The DNA standard used in the gel electrophoresis was the AmpliSize™ Molecular Ladder 50–2000 bp from BioRad (Mississauga, ON). Loading buffer was prepared using 55% (v/v) of glycerol (BDH) and 45% (v/v) of 10× TBE buffer.

2.2. Instrumentation

Centrifugation was done using a Beckman Microfuge™ 12 (Beckman Coulter Canada Inc, Mississauga, ON). Microcentrifuge tubes were from VWR (Misissauga, ON), and were autoclaved for sterilization. A Libra S22 UV/visible Spectrophotometer from Biochrom Ltd (Cambridge, England) and quartz cells (104-QS, 10 mm, K80) from Hellma GMBH & Co (Mullheim, Germany) were used for hyperchromicity experiments. A Vibra Cell™ high intensity ultrasonic processor with a microtip attachment (having a 5 mm tip diameter) from Sonics & Materials Inc (Danbury, CT), was used for the application of ultrasound. Electrophoresis was done using a Bio-Rad Sub-Cell GT system (Bio-Rad, Mississauga, ON). A BioRad Gel Doc 1000 was used to visualize the gels and photographs were taken using the associated Molecular Analyst software. For some experiments, the pictures of the gels were analyzed using ImageQuant® by Molecular Dynamics® Inc. (Sunnyvale, CA).

2.3. Hyperchromicity studies to consider conversion to single-stranded DNA fragments

A stock solution of DNA was prepared at a concentration of 100 μg/mL of MilliQ water. All of the samples were diluted to 50 μg/mL using TE buffer. Samples were mixed well and their absorbances from 200 to 320 nm were measured. Peak locations and absorbances were determined using the proprietary software. The blank used for these experiments contained a 1:1 ratio of TE to water. Three different conditions were used including non-sonicated DNA, sonicated DNA, and thermally denatured DNA. Sonicated DNA was kept on ice and exposed to ultrasound in the continuous mode for 2 min at an output power of 5 (ca. 85 W) followed by the wavelength scan. In order to thermally denature DNA, the solution was placed in a 50 mL centrifuge tube and then submersed in boiling water for at least 1 h. A wavelength scan was taken immediately following this. A comparison was made to wavelength scans for samples that were cooled on ice for 5 min prior to the scan and there was no statistically significant difference in the results at a 95% confidence level (results not shown). Therefore, a cooling step was eliminated. The results shown are the average of three trials for each treatment condition.

2.4. Agarose gel electrophoresis

DNA fragment separation was done by gel electrophoresis using 1% agarose gels to which the appropriate concentration of SYBR Gold nucleic acid stain had been added (1:10,000 dilution). It was then poured into the gel casting tray and left to cool and polymerize. The gel was placed into the electrophoresis apparatus and 1× TBE running buffer was used. All samples were vortexed to ensure they were mixed well and then portions were mixed with loading buffer in a 90:10 ratio. Unless otherwise indicated, 20 μL of this solution were loaded into the gel. The molecular ruler was mixed with loading buffer in a 1:1 ratio before adding to the wells, and one standard was always run on each side of the gel. A current of 100 mA was applied for 1 h.

2.5. Analysis and optimization of factors influencing fragmentation

The Yates pattern analysis is mathematical matrix that allows variables be screened to determine if they are statistically significant and if they are independent or dependent. Four variables were investigated using the Yates pattern analysis. The initial temperature of the DNA solution and ionic

strength were selected as factors. Preliminary experiments to determine whether there were any effects with regard to changes caused by other factors such duration of ultrasound exposure (or sonication), and the power (by changing the output control setting) were done to see if these factors should be examined further.

The effect of changing output control power and the duration of ultrasound exposure were studied using salmon sperm DNA at 50 μg/mL. For the power studies, settings of 1–5 (the microtip limit) were used and the duration of sonication was 1 min. These output control settings correspond to a range of power between approximately 8 and 85 W. The effects of sonication time were studied by exposing samples to ultrasound at an output control setting of 5 (ca. 85 W) and the times were varied from 10 s to 5 min. All studies were done in continuous mode energy delivery. These variables were evaluated using the Yates pattern analysis.

A 1:1 ratio of stock solution to TE buffer was placed into sterilized microcentrifuge tubes with a total volume of 800 μL. In this analysis, the maximum salt concentration (+) of the final sample was 1 M, the minimum (−) was 10^{-2} M, and the intermediate (0) was 10^{-1} M. These required the addition of NaCl solutions made in TE buffer in concentrations of 2, 2×10^{-2}, and 2×10^{-1} M, respectively to the DNA stock solution. Samples were processed using the appropriate power control setting (+ = 5, − = 3, 0 = 4), amount of time for sonication (+ = 2 min, − = 10 s, 0 = 1 min), and the initial temperature of the solution (+ = 90 °C, − = 0 °C, 0 = 45 °C). Table 1 indicates the variable settings used for each trial. The temperatures required for these experiments were set by placing the appropriate samples contained in microcentrifuge tubes into hot or warm water baths or on ice until the desired temperature had been reached. The temperatures were ca. 5 °C for samples on ice and between 85 and 90 °C for those submersed in boiling water.

Table 1
Variables identified by the Yates pattern

Trial number	Variables			
	[NaCl] (M)	Ouput control setting	Duration of sonication	Initial temperature (°C)
1	10^{-2}	3	10 s	0
2	1	3	10 s	0
3	10^{-2}	5	10 s	0
4	1	5	10 s	0
5	10^{-2}	3	2 min	0
6	1	3	2 min	0
7	10^{-2}	5	2 min	0
8	1	5	2 min	0
9	10^{-2}	3	10 s	90
10	1	3	10 s	90
11	10^{-2}	5	10 s	90
12	1	5	10 s	90
13	10^{-2}	3	2 min	90
14	1	3	2 min	90
15	10^{-2}	5	2 min	90
16	1	5	2 min	90
Intermediate	10^{-1}	4	1 min	45

These values selected for experiments were associated with the maximum and minimum values chosen for the Yates pattern matrix design.

Two trials of each sample were done to ensure that the observed effects were real and that the adjustment of the variables could be correlated with the measurable effects. Samples were processed in a random order to ensure that results were not biased. Every ninth sample processed used intermediate values associated with the variables. Four intermediate trials were done to help ensure, again, that true effects were being observed, there was a degree of reproducibility, and that there was no outside influence on the fragmentation. Following ultrasound exposure, samples were placed directly on ice. The salt concentration of each sample was adjusted to ca. 0.5 M prior to mixing with the loading buffer to maintain a consistent ionic strength for gel electrophoresis and visualization. The standard DNA ladder was also adjusted to the appropriate salt concentration to maintain consistency and also had loading buffer added (1:1:1 ratio of standard:loading buffer:salt solution). Gel electrophoresis was done according to the procedure in Section 2.4.

Images of the gels were analyzed using ImageQuant®. Lines were drawn down the centre of each lane, the width of the lanes were defined as 5 pixels on either side, and intensity profiles were created. This was done for each sample lane including one in which no sample was run. The empty lane was used for background subtraction. Since there were two standard lanes on each gel, two standard intensity profiles were created. The peaks representing each standard in the DNA ladder were visible and it was possible to record the pixel number at which the maximum intensity for each peak occurred. This data was averaged for each gel and a new standard graph representing pixel number and base pairs was created. Standard graphs were made by choosing the pixel number at which the maximum intensity occurred for each standard peak on the intensity profile graph and re-plotting pixel number versus the number of base pairs (Fig. 1).

In order to determine and optimize the roles of the factors participating in the fragmentation of DNA by ultrasound, it was necessary to choose parameters to measure from the agarose gel eletropherograms. One response parameter that was chosen for analysis was the pixel number, and corresponding fragment length, at which the maximum intensity occurred in the fluorescence intensity profile. This was used as an indicator of the fragment that was most predominant after the ultrasound treatment. However, this did not yield any information about the shape of the intensity profile or the predominance of other fragments of different lengths. As a means to indicate the overall number of fragments formed based on intensity measurements, the area under the normalized intensity profiles was calculated as the second parameter for analysis.

These parameters were organized into tables and the average and standard variance of the duplicates for each trial

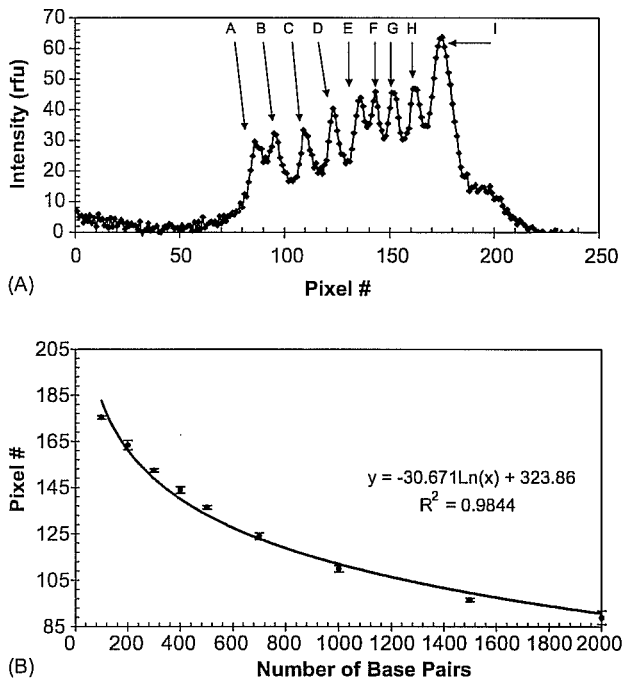

Fig. 1. Standard curve to convert pixel number to the number of base pairs. (A) An example of fluorescent intensity data collected with respect to the pixel number, which correlates to migration distances for the DNA molecular ruler. The pixel number positions of the peaks are representative of a specific length in base pairs (bp) of DNA where A = 2000 bp, B = 1500 bp, C = 1000 bp, D = 700 bp, E = 500 bp, F = 400 bp, G = 300 bp, H = 200 bp, and I = 100 bp. (B) Conversion of the data from (A) into a standard curve. All standard curves had an R^2 value of at least 0.97.

was calculated. A series of summations of the parameters were computed according to the Yates pattern analysis technique and the minimum significant factor effect [MIN] was calculated (Massart et al., 1978). Any factors considered significant, along with the associated scalar values, were combined into the parameter equations. Two sets of equations were generated, one representing the number of base pairs at which the maximum fluorescent intensity occurred and the other for the area under the curve in the fragment region. The factors involved, as well as the relationships between factors, were compared at the 90 and 95% confidence levels.

2.6. Effect of high ionic strength on fragmentation

Salmon sperm DNA (50 μg/mL) in a 1 M NaCl solution was exposed to ultrasound while on ice for varying amounts of time up to 5 min at an output setting of 5 (ca. 85 W). All samples, including standards, were adjusted to a salt concentration of 0.5 M prior to separation and visualization using agarose gel electrophoresis as previously described. The fragmentation patterns were visualized using agarose gel electrophoresis according to Section 2.4 and compared using ImageQuant®.

2.7. Comparison of fragmentation from purified genomic DNA and whole cell material

E. coli (Klone 6) cells were used as the whole cell material for this experiment. They were grown in Luria-Bertani (LB) media at 23 °C. 1.5 mL increments of the cellular solutions were placed in previously autoclaved microcentrifuge tubes, and centrifuged for 3 min at 5000 rpm. The supernatant was removed and the cells were resuspended in 1 mL of TE buffer. The cell density of the suspension to be sonicated was ca. 2.5×10^8 cells (determined by light scatter at 600 nm). Suspensions were sonicated on ice for 3 or 5 min, under pulsed and continuous settings, at an output setting of 5 (ca. 85 W). Any pulsed studies were done using a 50% duty cycle. Non-sonicated cells in suspensions were used as controls. Ultrasound was applied using the same settings but over a wider range of times to solutions of salmon sperm DNA (50 μg/mL). DNA fragments were separated using gel electrophoresis and visualized as previously outlined with the exception that the mixture of loading buffer to sample had a ratio of 90:20.

3. Results and discussion

In order to reliably use DNA fragments for detection of pathogens, it is important to understand the quality of the fragments as they pertain to use in DNA biosensors. For example, it is important to have an understanding of the sizes of fragments generated by the application of ultrasound, whether these sizes can be controlled, and whether they are in single- or double-stranded form. Using gel permeation chromatography and gel electrophoresis, Fukudome et al. (1986) found that calf thymus DNA that was sonicated using 20 kHz ultrasound under different conditions was broken down into molecular weights ranging from 7×10^4 to 70×10^4. This corresponded to approximately 200–1000 bp, with a decrease in distribution as the 1000 bp mark was approached. Elsner and Lindblad (1988) indicated that during sonication, a lower limit of 100–500 bp was reached depending on the intensity, time of exposure, and the gases present in solution.

3.1. Exploring the formation of single-stranded DNA fragments

In order for hybridization to occur between probe nucleic acids at a sensor surface, target DNA must be in single-stranded form. It is possible to follow transitions between the single- and double-stranded states using spectroscopic or calorimetric methods (Tinoco, 1996). Spectrophotometry can also be used as a semi-quantitative means to find the concentration of DNA in solution since a wavelength of 260 nm is near the absorption maximum for all of the nucleotide units. When DNA at a constant concentration undergoes an increase in temperature that

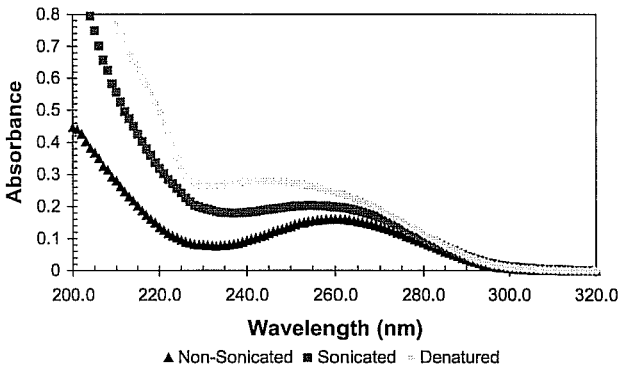

Fig. 2. Normalized absorption spectra for non-sonicated, sonicated, and thermally denatured DNA.

converts double-stranded DNA to a single-stranded form, the absorbance at 260 nm (A_{260}) increases and is known as the hyperchromicity effect (Puglisi and Tinoco, 1989).

A comparison of wavelength scans over the range of 200–320 nm showed changes in absorbance and maximum wavelength (λ_{max}) for non-sonicated, sonicated, and thermally denatured DNA (Fig. 2). Table 2 shows the values for A_{260} and λ_{max} for the different treatments. The hyperchromicity effect was observed when comparing the spectra of thermally denatured and non-sonicated DNA, and is seen as a significant increase in absorbance at 260 nm by more than 50%, and a peak shift from 260.4 ± 0.4 nm to shorter wavelengths. For both absorbance values and maximum wavelength, DNA that was exposed to ultrasound for 2 min falls almost directly in between the values for double- and single-stranded DNA. The change in light absorbing properties of sonicated DNA suggests that there has either been a change in the nucleotide bases or the form of the DNA (single- or double-stranded). Since DNA fragmentation occurs as backbone scission mainly between C–O bonds (Richards and Boyer, 1965) and limited amounts of thymidine undergo modification (Elsner and Lindblad, 1988), it is likely the form of DNA that has changed and is no longer all double-stranded. In some cases, post-sonication temperatures were found to climb as high 80 °C depending on the starting temperature of the solution. Under these circumstances, some of the DNA fragments could have been converted to a single-stranded form due to melt rather than due to sonication. The melting temperature of DNA from salmon testes, which is about 2000 bp in length, is 87.5 °C in 0.15 M sodium chloride with 0.015 M sodium citrate

Table 2
Comparison of A_{260} and λ_{max} for non-sonicated, sonicated, and thermally denatured DNA

Treatment	A_{260} (absorbance units)	λ_{max} (nm)
Non-sonicated	0.160 ± 0.004	260.4 ± 0.4
Sonicated	0.198 ± 0.01	254.6 ± 0.1
Thermally denatured	0.244 ± 0.01	<250

Results are the average of three trials.

(Sigma–Aldrich, 1999). Some fragments produced by ultrasound can be reduced in size to below 100 bp in length, and would have lower melt temperatures than the genomic DNA.

Since there is ambiguity with regards to the form of the target material, fragments are referred to by their number of base pairs as opposed to bases from this point forward.

3.2. Identification of primary factors influencing the fragmentation of DNA

It was clear from the preliminary experiments that both the duration of exposure to ultrasound and the power affect the formation of fragments. Fig. 3 shows the intensity profiles for representative samples exposed to ultrasound for varying amounts of time. The peaks close to pixel number 40 are the result of non-fragmented genomic DNA whereas any intensity at higher pixel numbers comes from fragments. Fragments of about 100 bp (pixel number near 240) were evident, and the signals for such small fragments increased in intensity as the duration of sonication increased. The size of fragments seemed to remain approximately the same even at longer exposure times, but the total number of fragments increased. The data suggested that by increasing exposure time, ultrasound had the ability to quantitatively fragment genomic DNA while still maintaining fragments of approximately the same size.

Any changes in the power of ultrasound strongly affect fragmentation patterns (Bankier, 1993). In the study by Fukudome et al. (1986), it was found that the molecular weights of DNA fragments after ultrasound exposure were remarkably decreased when ultrasonic power was increased from 25 to 105 W. A steady size distribution of fragmentation was reached between powers of 100 and 200 W. This was also observed when looking at the effects of acoustic power on other polymers, where it can be seen that increased power increased the number of breaks in the polymer and decreased the molar mass of the fragments (Schmidt-Naak et al., 2002). Our data indicated that for output power of 25 W or less, the amount of fragmentation was limited and there were a very large range of fragments formed with the average length centered at about 500 bp. Increasing the power to 40 W or higher resulted in the formation of much shorter fragment lengths and a narrowing of the distribution of fragment sizes. It is important to note that fluorescence intensity profiles are due to fluorescent intercalator that binds to DNA, and that more dye can intercalate into longer fragments than is possible for shorter ones. Therefore, the fluorescence intensity data is of limited value, but location and distribution of fluorescence can be used to interpret fragmentation behavior.

3.3. Analysis and optimization of factors that influence fragmentation

Optimization and predictive models enable the creation of empirical equations that relate a response parameter to

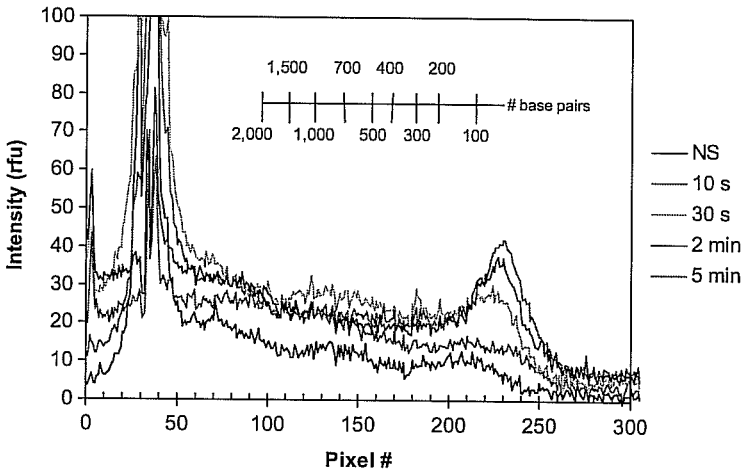

Fig. 3. Effect of ultrasound exposure times on fragmentation patterns. The intensity profiles showed that as the extent of exposure to ultrasound increased, so did the formation of more small fragments (peak intensities near pixel 240), although the range of fragment sizes stayed approximately the same.

variables. These models allow the significance of variables to be identified, as well as any interaction between variables to be assessed. Models create a means by which the best combination of variables can be chosen for a process. Three presumptions are made for this type of experimental design, the response parameter function is smooth and continuous, interaction between variables exist and cannot be ignored, and any variable dependence must have statistical significance. Based on these principles, the Yates pattern analysis is an appropriate full factorial two level experimental design. The use of this mathematical approach provided an opportunity to work towards developing an optimized procedure for fragmenting genomic DNA to be used in DNA biosensors. Fig. 4 is representative of the typical normalized intensity profiles observed for one of the trials in the Yates pattern analysis. The high fluorescence just after pixel number 50 is the result of unfragmented DNA that did not permeate into the agarose gel. The intensities in the pixel range associated with fragmented DNA (i.e. all pixel numbers higher than 70) increased under any of the ultrasound exposure conditions when compared to non-sonicated DNA. The corrected (normalized) profiles were used to provide quantitative information that could be interpreted using the Yates pattern analysis. The two response parameters measured were the pixel number (and corresponding number of base pairs) at which the most intense fragment peak occurred and the area under the intensity profile in the fragment region. The data collected and subsequently used for chemometric analysis is organized in Table 3.

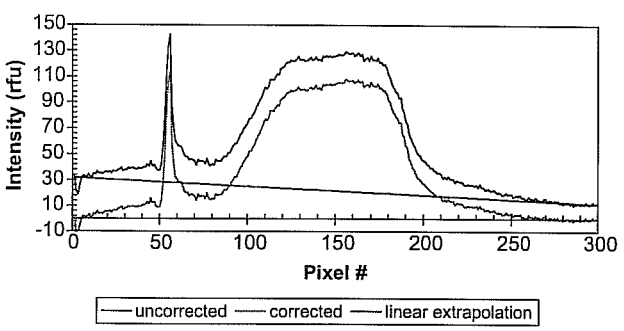

Fig. 4. Intensity profile for trial 2A. This sample was sonicated using the conditions of 1 M NaCl, output 3, duration of 10 s, and with a starting temperature of 0 °C. The graph shows the original data (relative fluorescence units, rfu) as well as the normalized (corrected) data after the linear ramping background signal was subtracted.

Using the values determined from the intensity profiles the following equation was generated at the 90% confidence level with a minimum significant factor affect [MIN] of 216. Only three variables were found to participate in determining the fragment at which the maximum fluorescent intensity occurred.

$$\text{Average \#BP} = 344 + 174X_3 - 141X_1X_3X_4 \quad (1)$$

where X_1 represents salt concentration, X_2 is output control (power), X_3 is time in seconds, and X_4 is the initial temperature. There are real values for each of these variables that can be calculated using the following equations:

$$X_1 = \frac{S - 0.1}{0.495} \quad (2)$$

$$X_2 = \text{OC} - 4 \quad (3)$$

$$X_3 = \frac{t - 60}{55} \quad (4)$$

$$X_4 = \frac{T - 45}{45} \quad (5)$$

where S is the NaCl concentration in molarity, OC is the output control setting, t is the amount of time used for ultrasound exposure, and T is the initial temperature of the solution. This equation remained the same when the confidence level was increased to 95% where the [MIN] value for comparison was 281.

Table 3
Area and fragment with the highest intensity data used in the Yates pattern analysis

Trial #	Location of fragment region with highest intensity (bp)				Relative area of the fragment region of the intensity profile			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
1	654	661			1808	1715		
2	282	407			9263	8807		
3	574	566			1302	6325		
4	146	566			6745	10523		
5	99	79			2187	2776		
6	103	685			2393	4396		
7	91	91			2582	938		
8	537	102			6323	2252		
9	89	91			4088	2232		
10	1280	587			6537	8870		
11	760	73			3473	5240		
12	873	746			7274	5515		
13	87	98			2286	4812		
14	132	103			4999	4136		
15	99	99			4010	4024		
16	107	125			4719	5297		
Intermediate	89	101	73	79	3078	2016	5253	2619

That data shown here corresponds to the duplicate trials done for each sample and the quadruplicate trials for the intermediate.

By far the greatest independent contributing variables were ionic strength or salt concentration (X_1) and the ultrasound exposure time (X_3). The amount of time samples were exposed to ultrasound was clearly the most important factor since it was the only one that remained as an independent variable when the confidence limits were increased to 95%. When the salt concentration was increased, so was the average size of the fragments. When the duration of sonication was increased, the average fragment size decreased. None of the factors played a completely independent role.

It is important to consider that the fragment sizes were not being directly observed, but rather the fluorescent intensity of the intercalating dye was detected. SYBR Gold was chosen as the fluorescent stain for these experiments because it is more sensitive to single-stranded DNA than other dyes such as ethidium bromide (Tuma et al., 1999), and hyperchromicity data indicated that there was a mixture of both single and double-stranded DNA formed. SYBR Gold has twice the relative fluorescence in the presence of double stranded DNA when compared to single-stranded DNA (Tuma et al., 1999). Therefore, by looking at the fragment size at which the maximum fluorescent intensity occurred, there may be more involved than the abundance of fragments in that region since the form of the DNA (single- or double-stranded) can impact intensity as well. Because the fragment size having the highest fluorescent intensity was not indicative of the number of fragments formed, it was necessary to consider another parameter for measurement.

Measuring the area under the curve associated with any fragmentation was chosen as a factor that would be reflective of the overall intensity measured and was anticipated to correspond to the quantity of fragments produced by the ultrasound exposure. The equation generated with respect to the area under the curve was for a confidence level of 90%.

$$\text{Area} = 4620 + 1508X_1 - 987X_3 - 827X_1X_3 \quad (6)$$

where X_1 represents salt concentration, and X_3 is time in seconds and their real values are defined in Eqs. (2)–(5). When the confidence level was increased even higher to 95%, the area equation was dependant solely on the salt concentration, and this parameter was the largest positive contributor to area by far. It was apparent that samples that had a starting salt concentration of 1 M were much more intense in the gel photographs. However, it is important to recall that all of the samples were adjusted to the same ionic strength (ca. 0.5 M NaCl) before separation on the gel to eliminate any differences in migration or fluorescent intensity that would be caused by the presence of salt. Rather than interpreting this contribution to area to mean that more fragments of DNA are formed when solutions of high ionic strength are exposed to ultrasound, it is possible that the double-stranded DNA structure was stabilized in the presence of 1 M NaCl and may in fact be more resistant to cavitation effects.

3.4. Effect of high ionic strength on DNA fragmentation by ultrasound

A study considering the fragmentation patterns of DNA in solution of high ionic strength (1 M NaCl TE buffer) suggested that the range of fragment sizes could be altered. Fig. 5 shows fluorescence intensity profiles for non-sonicated DNA and DNA solutions that were sonicated for 1, 2 and 5 min. Fig. 5A compares the intensity profiles for non-sonicated samples prepared with no additional salt and in 1 M NaCl solution. There is a peak in the 1 M NaCl non-sonicated sample near 220 pixels (or 125 bp) and the overall intensity profile is higher than that of the control containing no salt. Keeping in mind that these samples were

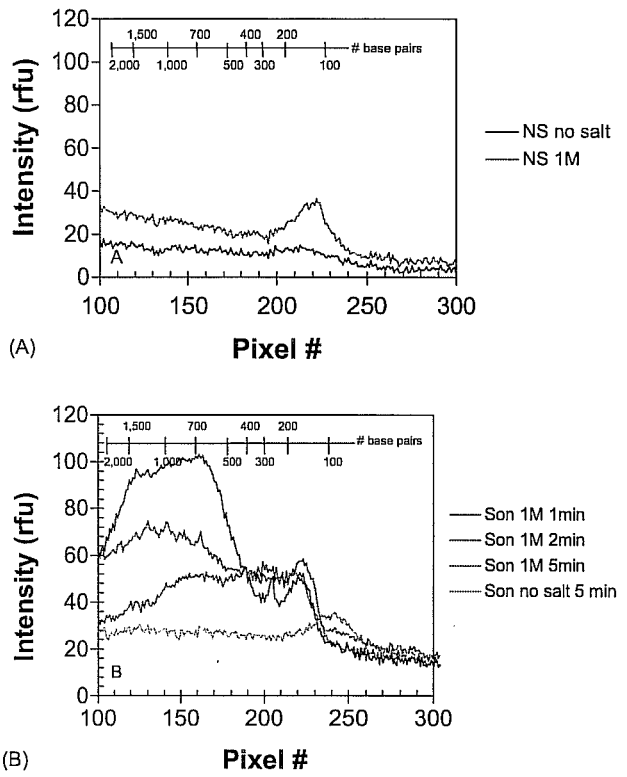

Fig. 5. Effect of increasing ultrasound exposure times in high salt solutions. (A) comparison of intensity profiles for non-sonicated samples prepared with no additional salt and in 1 M NaCl solutions. (B) Comparison of intensity for samples sonicated for times ranging from 1 to 5 min.

adjusted to the same ionic strength before gel electrophoresis, this suggested that salt increased the stability of the double-stranded structure or associated with the fragments to cause increased fluorescence. Experiments that investigated fluorescence from DNA in the presence of dye using NaCl concentrations from 0 to 1 M demonstrated that the intensity of fluorescence remained constant in this range of ionic strength. Therefore, the intensity changes seen in the analysis of gels was associated with the alteration of stability of double-stranded DNA by the salt concentration. This is not surprising since it is well known that raising the ionic strength of a DNA solution by adding additional salt causes an increase in the melting temperature of DNA (Puglisi and Tinoco, 1989). However, the results also suggest that not only is the double-stranded structure resistant to separation at ionic strengths, the DNA backbone itself resists fragmentation to smaller sizes.

Fig. 5B compares the intensity profiles for samples sonicated for times ranging from 1 to 5 min. As sonication time increased, there was a decrease in the range of fragment sizes with a shift towards smaller fragment sizes (larger pixel numbers). For solutions processed at a high ionic strength with application of ultrasound for 1 min, there was a large amount of fragmentation associated with the migration range for 450–1400 bp with some fragmentation centered in the 150 bp area. As the exposure time increased, the intensity for fragments in 450–1400 bp region decreased while the fluorescent intensity in the 150 bp region increased only marginally. Given that one region decreased significantly in intensity while the other increased by only a small amount, it can be suggested that the size of fragments decreased. It also seems possible that some of the strands potentially changed to a single-stranded form while at the same time disrupting the arrangement of any salt associated with the DNA, causing a decreased fluorescent intensity in that region. As the duration of sonication was increased, the distribution of fragment sizes shifted closer to the 150 bp region. A hybridization assay would normally be used to discover the amount of available single-stranded target material. However, given that there may be entire fragments in either a double- or single-stranded form or fragments that are composed of both double- and single-stranded regions, it would be incredibly difficult to accurately interpret any results from a hybridization experiment.

The changing fragmentation patterns in 1 M NaCl DNA solutions seen for increased times of sonication suggested that the effects of high ionic strength on fragmentation patterns could be overcome. The larger fragments produced under conditions of high ionic strength have the potential to be used in DNA biosensors. However, given that the optimal target strands should be 100 bases or less (Chan et al., 1995), it would seem that care should be taken to keep salt concentrations as low as possible if sonication is to be used to fragment DNA. Any use of salt or other ions in buffers used for DNA biosensors should be added to the sample after it has been fragmented by ultrasound if possible.

3.5. Comparison of ultrasound sheared purified genomic DNA and whole cell material

The use of ultrasound to fragment genomic DNA is an energy transfer method, which means that other components exist in the media that may absorb the ultrasound or react with the free radicals. This can become very important when considering applications on whole cell cultures since several other materials, like protein, will be present and are known to absorb ultrasound (Fry, 1978). The purified genomic material used in the studies was salmon sperm DNA (ca. 2000 base pairs) (Tanaka and Okahata, 1996). The effect of ultrasound on *E. coli* cell cultures (ca. 4 million base pairs) (Cooper, 1997) and a comparison with the products formed from sonication of purified genomic material from salmon sperm was done.

The results seen in Fig. 6A indicate that the DNA fragment size with the highest fluorescence for sonicated purified genomic DNA was centered near 300–500 bp, but that fragments ranged in size from 50 to 1500 bp regardless of the mode of sonication that was used (continuous or pulsed, or interjected periods of cooling). As can be seen in Fig. 6B, the most predominant fragment size resulting from the sonication of whole cell material was once again in the range

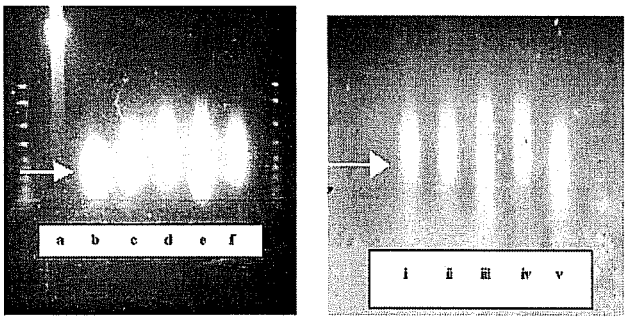

Fig. 6. Comparison of predominant fragment size and distribution of sonicated purified genomic material (A) and sonicated whole cell material (B). The arrows mark the 300–500 bp fragment regions. The reference mass ladders cover the range from 50 to 2000 bp. The conditions of sonication for the above trials were: (a) non-sonicated, (b) 5 min pulsed, (c) 5 min constant, (d) 3 min constant, (e) 3 min pulsed, (f) 3 min continuous with periods of cooling, (i) 3 min pulsed, (ii) 3 min continuous, (iii) 1 min pulsed, (iv) 1 min continuous, and (v) 30 s continuous.

of 300–500 bp. The duration of sonication for these studies ranged from 30 s to 3 min, although some of the purified genomic material studies ranged up to 5 min. No migration of DNA was observed for non-sonicated cells. Preliminary experiments using a fiber optic biosensor (Almadidy et al., 2002) to investigate samples of whole cell suspensions that had been sonicated indicated that there was no significant difference in fluorescence signal development in comparison to use of purified genomic DNA for similar quantities of total genomic DNA.

4. Conclusions

Ultrasound is a rapid method to fragment DNA for use in biosensors applications. It can be applied directly to cellular samples or purified genomic material with very similar results. Salt concentration, exposure time, power, and temperature can be manipulated to control the length and potentially the form of fragment desired (single- or double-stranded). With careful control of sonication and hybridization conditions, this sample processing method has the potential to move nucleic acid biosensor technology towards "real-time" analysis.

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