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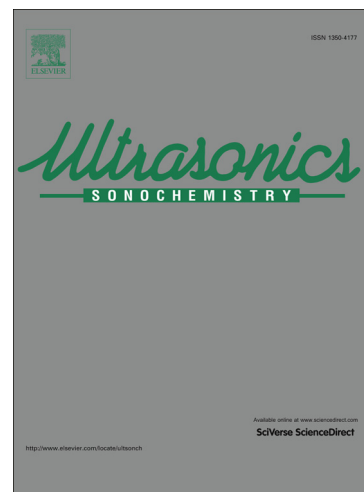
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Title

Effect of Sonication Frequency on the Disruption of Algae

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Abstract

In this study, the efficiency of ultrasonic disruption of *Chaetoceros gracilis*, *Chaetoceros calcitrans*, and *Nannochloropsis* sp. was investigated by applying ultrasonic waves of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz to algal suspensions. The results showed that reduction in the number of algae was frequency dependent and that the highest efficiency was achieved at 2.2, 3.3, and 4.3 MHz for *C. gracilis*, *C. calcitrans*, and *Nannochloropsis* sp., respectively. A review of the literature suggested that cavitation, rather than direct effects of ultrasonication, are required for ultrasonic algae disruption, and that chemical effects are likely not the main mechanism for algal cell disruption. The mechanical resonance frequencies estimated by a shell model, taking into account elastic properties, demonstrated that suitable disruption frequencies for each alga were associated with the cell's mechanical properties. Taken together, we consider here that physical effects of ultrasonication were responsible for algae disruption.

Highlights

The disruption of algae is frequency dependent and algae specific.

The resonance frequencies of algae are calculated using elastic modulus measures.

Cavitation bubbles are necessary for the algae disruption process.

Chemical effects are not the main mechanism for algal cell disruption.

Suitable disruption frequencies are associated with the cell's mechanical properties.

Keywords: algae; disruption; physical effects; high-frequency ultrasonic

1. Introduction

Algal cells provide a readily available source of lipids for the biofuel industry [1–3] and a variety of different lipid extraction methods have been proposed [4]. One of the more recent developments in extraction technology has been the use of ultrasound, and at present, there have been reports of inactivation via ultrasound for many microorganisms [5–7]. Inactivation of microorganisms by ultrasound was first reported in the 1920s [8], and the specific mechanism began to be reported in the 1960s [9]. The inactivation mechanism differs with different parameters, such as temperature, ultrasonic frequency, and acoustic power, and between microorganisms; the inactivation mechanisms for ultrasonic inactivation of *Escherichia coli* [10], *Listeria monocytogenes* [11] and *Alicyclobacillus acidiphilus* [12] have been reported. Ultrasound has been found to affect *Microcystis aeruginosa* at several tens to several hundreds of kilohertz [13–16]. However, in another study, both ultrasonic frequency and power were reported to be necessary for ultrasonic inactivation in *Chlamydomonas concordia* and *Dunaliella salina* [17]. Yet despite these reports, there remain many unanswered questions about the mechanisms of algal disruption by ultrasound.

Ultrasonic waves exert several different effects, grouped broadly into two types. The first type comprises direct effects without cavitation at very low power, such as radiation force and acoustic streaming [18]. Bioeffects caused by acoustic streaming have been confirmed to occur in cell cytoplasm [19]. The second type comprises cavitation effects, which consist of both chemical and physical effects, for example, free radical reactions, shock waves, shear stress, and microjet [20]. Although chemical effects have been reported as a major cause of inactivation [21], inactivation mechanisms due to shock waves [22] and shear stress of microstreaming [23] have also been suggested. Against this background, we investigate the effects of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz ultrasound on suspensions of *Chaetoceros gracilis*, *C. calcitrans*, and *Nannochloropsis* sp. to clarify the mechanism by which ultrasound causes disruption in algae.

2. Materials and methods

2.1. Algae

C. gracilis, *C. calcitrans*, and *Nannochloropsis* sp. were selected for this study. *C. gracilis* and *C. calcitrans,* which are diatoms, contain chlorophyll a and c, and *Nannochloropsis* sp. belonging to Eustigmatophyceae, contains only chlorophyll a. These algae are widely available; *C. gracilis* and *C. calcitrans* were purchased from Yanmar Co., Ltd. and *Nannochloropsis* sp. was purchased from ISC Co., Ltd. All algae were cultured and concentrated under appropriate conditions recommended by the manufacture, and were transported at a controlled temperature. Initial concentrations of *C. gracilis* and *C. calcitrans* were 1.1×10^8 cells/mL, and that of *Nannochloropsis* sp. was 1.1×10^{10} cells/mL. All experiments were performed within 48 h of the arrival of the algal suspension to ensure that the condition of the algae did not change. *C.*

gracilis, *C. calcitrans*, and *Nannochloropsis* sp. were measured by using a nanoparticle size analyzer (SALD-7500 nano; Shimadzu Co.) and the mean particle sizes were 2.5, 2.4, and 1.3 µm, respectively. The Young's moduli were 91, 142, and 29 MPa, respectively, measured with a scanning probe microscope (Shimadzu Co., SPM-9700).

2.2. Ultrasonic treatment

Algal suspensions (100 mL) were placed in a stainless steel cylinder and sonicated by using an ultrasonic processor (Sonics & Materials, Inc., VC750) at 0.02 MHz (Fig. 1(a)) and then by a PZT ceramic disk-type transducer (Fuji Ceramics Co.) at 0.4, 1.0, 2.2, 3.3, and 4.3 MHz (Fig. 1(b)) for 10 min. Algae samples were taken at 0, 2, 4, 6, 8 and 10 min of sonication. The suspension temperature was kept at 15 °C using a water circulator (Cool Line CL301; Yamato Scientific Co., Ltd.). In all suspensions, the initial cell number was 10^7 cells/mL. To ensure reproducibility, all experiments were carried out in triplicate. The acoustic power P was 10 W and was measured by calorimetry [24]

$$P = mC_p\left(\Delta T/\Delta t\right)$$

(1),

where C_P is the specific heat capacity of water, m is the mass of water, ΔT is the increase in the temperature of the sonicated water, and Δt is ultrasonic irradiation time.

2.3. Analytical methods

Two analytical methods were used. The first method was to use the rate of algal cell disruption, calculated from the number of cells enumerated by hemocytometry. Cell counting was performed in triplicate and averaged. The reduction in algal cell numbers after sonication for *n* minutes ($CR_{n\,min}$) was calculated using the following equation:

$$CR_{n\min}[\%] = \left(CR_{0\min} - CR_{n\min}\right)/CR_{0\min} \times 100$$

(2),

where $CR_{n\,min}$ is the number of cells counted at *n* min and $CR_{0\,min}$ is the original number of cells at 0 min. Because chlorophyll a, which is present in the algae, has a peak optical absorbance at 680 nm, the second method for analyzing the condition of algal cells was to measure the peak height (PH) obtained by subtracting absorbance at 630 nm (Abs_{630nm}) from absorbance at 680 nm (Abs_{680nm})

$$PH = Abs_{680nm} - Abs_{630nm}$$

(3).

3. Results

3.1. Ultrasonic treatment of *C. gracilis*

Fig. 2 shows the absorbance spectra of *C. gracilis* suspensions before sonication (0 min) and after 10 min sonication at 2.3 MHz, with the wavelength plotted on the abscissa and the absorbance plotted on the ordinate. Upon ultrasonication, absorbance at longer wavelengths decreased, but the peak height at 680 nm increased. This decreased absorbance was possibly a result of the influence of Rayleigh scattering due to the algal cells, and the increased peak height was possibly caused by chlorophyll released from individual algae [15]. Therefore, we used peak height in this experiment.

Figs. 3 and 4 show the changes in cell reduction and peak height over time during sonication at the six frequencies of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz, at an acoustic power of 10 W. Cell reduction and peak height were calculated from Eqs. (2) and (3). At all frequencies, cell reduction and peak height increased with ultrasonication. At 2.2 MHz, approximately 100% of *C. gracilis* was destroyed at 2 min. In contrast, at 0.4 MHz, disruption of less than only 40% was obtained at 10 min, demonstrating the frequency dependence of ultrasonic disruption of *C. gracilis*. Fig. 5 shows the frequency dependence of cell reduction and peak height of *C. gracilis* suspensions upon sonication for 2 min at an acoustic power of 10 W. The most effective frequency was 2.2 MHz. Peak height was also in excellent agreement with cell reduction results.

3.2. Ultrasonic treatment of *C. calcitrans*

Fig. 6 shows the absorbance spectra of *C. calcitrans* suspensions before sonication (0 min) and after 10 min sonication at 3.3 MHz, with the wavelength plotted on the abscissa and the absorbance plotted on the ordinate. The trends in absorbance spectrum of *C. calcitrans* upon ultrasonication were the same as those seen for *C. gracilis*.

Figs. 7 and 8 show the results for changes in cell reduction and peak height over time during sonication at frequencies of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz at an acoustic power of 10 W. At all frequencies, cell reduction and peak height increased with ultrasonication. At 3.3 MHz, approximately 100% of *C. calcitrans* was at 2 min. In contrast, at 0.4 MHz, disruption of less than 40% was obtained at 10 min, demonstrating the frequency dependence of the ultrasonic disruption of *C. calcitrans*. Fig. 9 shows the frequency dependence of cell reduction and peak height of *C. calcitrans* suspensions upon sonication for 2 min at an acoustic power of 10 W. The most effective frequency was 3.3 MHz, a higher frequency than for *C. gracilis*. Peak height was also in excellent agreement with cell reduction results.

3.3. Ultrasonic treatment of *Nannochloropsis* sp.

Fig. 10 shows the absorbance spectra of *Nannochloropsis* sp. suspensions before sonication (0 min) and after 10 min sonication at 4.3 MHz with the wavelength plotted on the abscissa and the absorbance plotted on the ordinate.

Figs. 11 and 12 show the results for changes in cell reduction and peak height over time during sonication at frequencies of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz, at an acoustic power of 10 W. At all frequencies, cell reduction and peak height increased with ultrasonication. At 4.3 MHz, approximately 90% of *Nannochloropsis* sp. cells were disrupted at 10 min. In contrast, at 0.4 MHz, disruption of only about 10% was obtained at 10 min, indicating that *Nannochloropsis* sp. is more resistant to ultrasonication than *C. gracilis* and *C. calcitrans*. Fig. 13 shows the frequency dependence of cell reduction and peak height of *Nannochloropsis* sp. suspensions by sonication for 2 min at an acoustic power of 10 W, indicating that a frequency of 4.3 MHz is most effective in *Nannochloropsis* sp., which is higher than those for both *C. gracilis* and *C. calcitrans*. Peak height was also is in excellent agreement with cell reduction results.

4. Discussion

4.1. Direct effects without cavitation

The results of this study showed that algal disruption does occur upon ultrasonication. Although the direct (non-cavitation) effects of ultrasonication, such as radiation force and acoustic streaming, are considered one cause of algal disruption, the contribution of these direct effects to algal disruption are actually quite small, for three reasons.

First, the intrinsic acoustic impedance of algae is very close to that of water. Reflectance of a sound wave is expressed as the difference between the intrinsic acoustic impedances of the two media. Considering the ratio of the volume of the organized tissues of algal cells, the total acoustic impedance of algae is very close to that of the suspension. Hence, the reflectance of the sound wave upon striking the algae is approximately 0; that is, the algae is essentially transparent to sound waves.

Second, the size of algae is very small compared to the wavelength of the ultrasound. The wavelength of the highest frequency ultrasound, 4.3 MHz, in water is estimated to be 350 μm. This is 270 times greater than the 1.3 μm radius of a *Nannochloropsis* sp. cell. The scattering cross section depends on factors such as the size of the scatterer, a, wavelength of the ultrasound, λ, and acoustic impedance, more specifically, it is proportional to $(a/\lambda)^4$ when the acoustic impedance is sufficiently different. Therefore, we can presume that the ultrasound is not scattered by the algae.

Third, the ultrasonic disruption of algae has a threshold based on acoustic intensity. In this study, ultrasonic algal disruption at 3.3 MHz occurred with increasing acoustic intensity, as indicated in Fig. 14. Here, intensity increased with exposure time. Simultaneously, absorbance proportional to algae disruption was recorded. Sponer reported the dependence of the ultrasonic cavitation threshold on acoustic frequency, and found that the threshold acoustic pressure was about 0.2 MPa at a frequency of 3 MHz [25]. In our experiment, the absorbance began

increasing at an intensity of about 2.5 W/cm^2, corresponding to about 0.27 MPa in our ultrasonic reactor, indicating that algae disruption started when intensity exceeded the cavitation threshold. In addition, the acoustic spectra and visual checks by light scattering confirmed that cavitation bubbles were generated during every sonication experiment.

Hence, cavitation is required for ultrasonic algae disruption, rather than simply the direct effects of ultrasonication. Many other reports of the ultrasonic inactivation of microorganisms recognize cavitation as the cause of inactivation [7, 13–17, 23, 26–29].

4.2. Chemical effects

A chemical effect of acoustic cavitation is free radical reactions. Fig. 15 shows the frequency dependence of sonochemical efficiency (the number of reacted molecules per unit of ultrasonic energy) of the sonoreactor used in this study, measured by the KI method [24]. Experimental conditions were the same as for the algae: acoustic power of 10 W and temperature of 15 °C. The maximum efficiency was about 0.4 MHz, which differs from the result for the frequency dependence of algal disruption. Thus, we consider chemical effects to not be the main mechanism for the disruption of algal cells seen in this study.

4.3. Physical effects

The results of the experiment indicated that the disruption of the algae was dependent on ultrasound frequency. One possibility is the influence of oscillating bubble size caused by cavitation. A small stable bubble oscillating near an algae cell may be a source of excitation, causing vibrations. The resonance radius of a linearly oscillating bubble, R, in an ultrasonic field at frequency, f_{bubble}, can be determined from Minnaert's formula [30]

$$f_{bubble} = \frac{1}{2\pi R}\sqrt{\frac{3\gamma}{\rho}\left\{P_0 + \left(1 - \frac{1}{3\gamma}\right)\frac{2\sigma}{R}\right\}}$$

(4),

where $\gamma \approx 1.4$ is the specific heat of air, $P_0 \approx 10^5$ Pa is the ambient atmospheric pressure, $\sigma \approx 7.25$ N/m is the surface tension of the liquid, and $\rho \approx 1000$ kg/m^3 is the density of water. The resonance radius of a bubble in water was calculated at each of the frequencies used in the sonication. Results are shown in Table 1. The algal cell radius correlated roughly with the bubble resonance radius at each best frequency for disruption.

Another possibility is the effect of the mechanical resonance of the algal cells. The shell model was used to calculate the mechanical resonance frequencies and vibration modes of the algae. Such models have been used previously for bacteria and viruses by treating microorganisms as

spherical particles [31, 32]. The relative change in the area of an algal cell has a maximum at the resonance frequency given by [32]

$$f_{cell} \approx \frac{1}{2\pi} \sqrt{\frac{K_A}{\rho a^3}}$$

(5),

where K_A is the surface area modulus, $\rho \approx 1000$ kg/m³ is the density of the cell, and a is the mean particle radius of cell. The surface area modulus, K_A, is estimated from Young's modulus E measured by scanning probe microscope by using force curve mode as

$$K_A = \frac{Eh}{2(1-\mu)}$$

(6),

where $\mu \approx 0.5$ is Poisson's ratio of algae and h is its cell wall thickness, which amounts to approximately 5% of the cell size. Table 2 shows calculated resonance frequencies, best frequencies for disruption, and the physical properties of the algae. For the three types of cells, there was a positive relationship between calculated resonance frequency and the observed best disruption frequency; the two values differed, but the same ordering was observed. This may be due to the method used for measurement of the Young's modulus. Zinin et al. reported that the value of the resonance frequency varies according to the method used to measure Young modulus [31, 32].

Ultrasonic algae disruption has been reported previously by several groups. We reported that high-frequency sonication (1.146 MHz) is more effective than conventional low-frequency sonication (0.02 MHz) for the disruption of *C. concordia* and *D. salina* cells [17]. Miller et al. reported that when portions of a *Hydrodictyon reticulatum* colony (a green alga) were exposed to a high-amplitude 1 MHz ultrasonic standing wave, cells were typically destroyed by cavitation in the surrounding water [19]. Hao et al. sonicated a cyanobacteria suspension at different frequencies (0.02, 0.2, and 1.7 MHz) at the same acoustic power, reporting that the higher frequency of 1.7 MHz was weaker than 0.02 MHz for generating cavitation, but had more effective inhibition because it was nearer to the resonance frequency of gas vesicles [32]. Ma et al. concluded that 0.15 MHz was the most effective sonication frequency for removing *Microcysti*s among those tested (0.02, 0.15, 0.41, and 1.7 MHz) given the same acoustic power [34]. There are also various reports regarding the mechanical behavior of microorganisms and cells. Using an atomic force microscope, Pelling et al. demonstrated that the cell wall of living *Saccharomyces cerevisiae* (baker's yeast) exhibits local temperature-dependent nanomechanical motion at characteristic frequencies, [35]. Wamel et al. used microscopy and a high-speed camera to show that microbubble expansion of 100% resulted in a 2.3 μm displacement of the cell membrane [36]. Other research groups have reported that mechanical effects, including

shear force, microjets, and microstreaming, induce cell disruption [37, 38]. Gao et al. have confirmed that the inactivation of microorganisms is due to shear forces generated by collapse of cavitation bubbles [39, 40]. Given this background and our own experimental results, we consider that physical effects are responsible for algae disruption under ultrasonication in the present study.

5. Conclusions

In this study, the effects of ultrasonication on suspensions of *C. gracilis*, *C. calcitrans*, and *Nannochloropsis* sp. were investigated at frequencies of 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz at the same acoustic power of 10 W. The results showed that reduction in algal numbers were frequency-dependent, and frequencies of 2.2, 3.3, and 4.3 MHz were found to be most effective for disrupting *C. gracilis*, *C. calcitrans*, and *Nannochloropsis* sp., respectively. Upon considering the possible direct and chemical effects of ultrasonication, it is likely that cavitation bubbles are necessary for algal disruption, but that free radical reactions were not involved. In addition, mechanical resonance frequencies were estimated by a shell model, taking into account elastic properties. Taken together, the results demonstrate that the suitable disruption frequency for each algal species were associated with the cell's mechanical properties.

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Figure captions

Figure 1 Experimental apparatus for sonication at 0.02 (a), and 0.4, 1.0, 2.2, 3.3 and 4.3 MHz (b).

Figure 2 Absorbance spectra of *Chaetoceros gracilis* suspensions before sonication (0 min) and after 10 min sonication at 2.2 MHz.

Figure 3 Cell reduction of *C. gracilis* over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 4 Peak height of absorbance of *C. gracilis* over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 5 Frequency dependence of cell reduction (■) and peak height (□) in *C. gracilis.*

Figure 6 Absorbance spectra of *Chaetoceros calcitrans* suspensions before sonication (0 min) and after 10 min sonication at 3.3 MHz.

Figure 7 Cell reduction of *C. calcitrans* over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 8 Peak height of absorbance of *C. calcitrans* over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 9 Frequency dependence of cell reduction (▲) and peak height (△) in *C. calcitrans.*

Figure 10 Absorbance spectra of *Nannochloropsis* sp. suspensions before sonication (0 min) and after 10 min sonication at 3.3 MHz.

Figure 11 Cell reduction of *Nannochloropsis* sp. over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 12 Peak height of absorbance of *Nannochloropsis* sp. over time during sonication at an acoustic power of 10 W at 0.02, 0.4, 1.0, 2.2, 3.3, and 4.3 MHz.

Figure 13 Frequency dependence of cell reduction (◆) and peak height (◇) in *Nannochloropsis* sp.

Figure 14 Association between absorbance (proportional to algae disruption) and acoustic intensity during sonication at 3.3 MHz in *C. calcitrans*.

Figure 15 Frequency dependence of sonochemical efficiency as measured by KI method at an acoustic power of 10 W.

Table 1

Frequency [MHz]	Affected algae	Algae radius [μm]	Bubble radius [μm]
0.02			163
0.4			8.66
1.0			3.72
2.2	*C. gracilis*	2.5	1.87
3.3	*C. calcitrans*	2.3	1.34
4.3	*Nannochloropsis* sp.	1.3	1.08

Calculated resonance radius of a bubble in water at the different frequencies used in the sonication, and average radius of an algal cell of each species examined.

Table 2

Algae	Young modulus [MPa]	Resonance frequency [MHz]	Best frequency [MHz]
C. gracilis	91	4.03	2.2
C. calcitrans	142	4.39	3.3
Nannochloropsis sp.	29	4.85	4.3

Calculated resonance frequencies of each type of algae and best frequencies for algae disruption.

Ultrasonic
Processor
(0.02 MHz)

Stainless-steel
Cylinder

Temperature
controlled

Disk-type
Transducer
(0.4, 1.0, 2.2,
3.3, 4.3 MHz)

Haemocytometer

Spectrophotometer

Function Generator

Power Amplifier
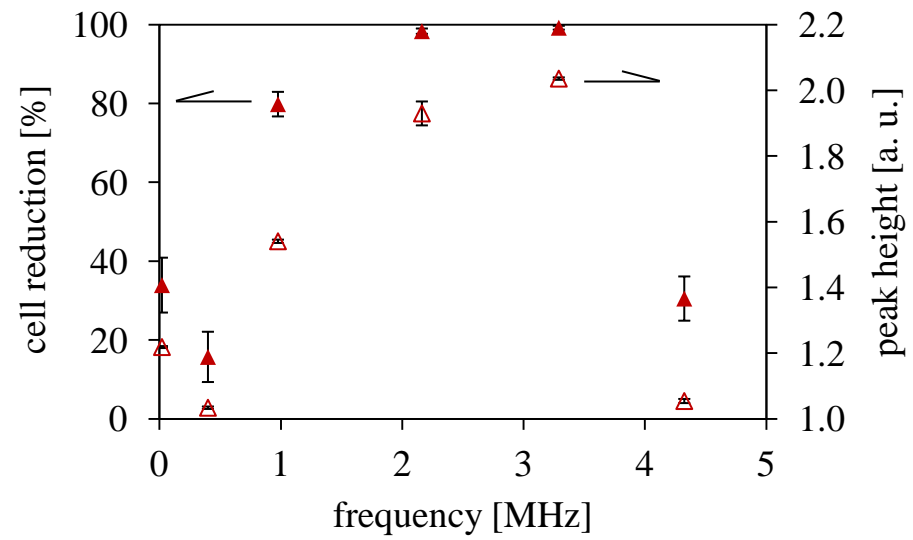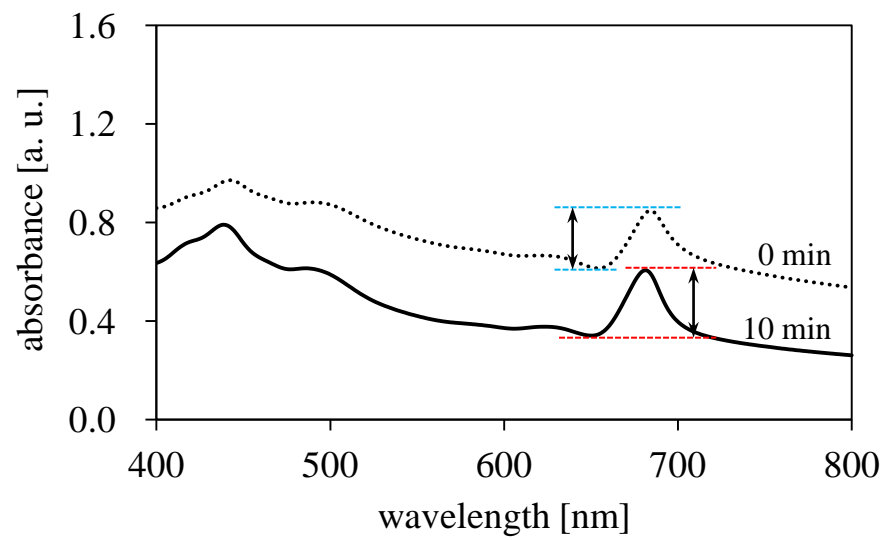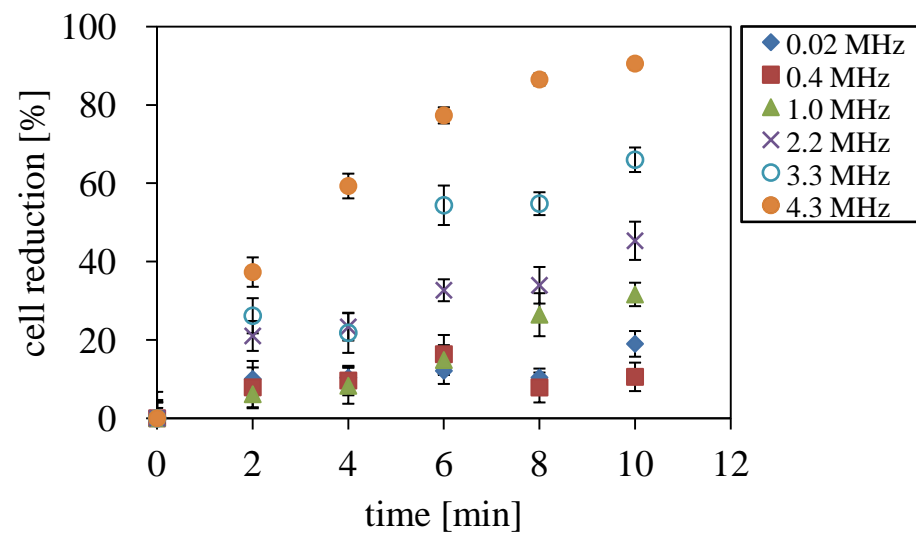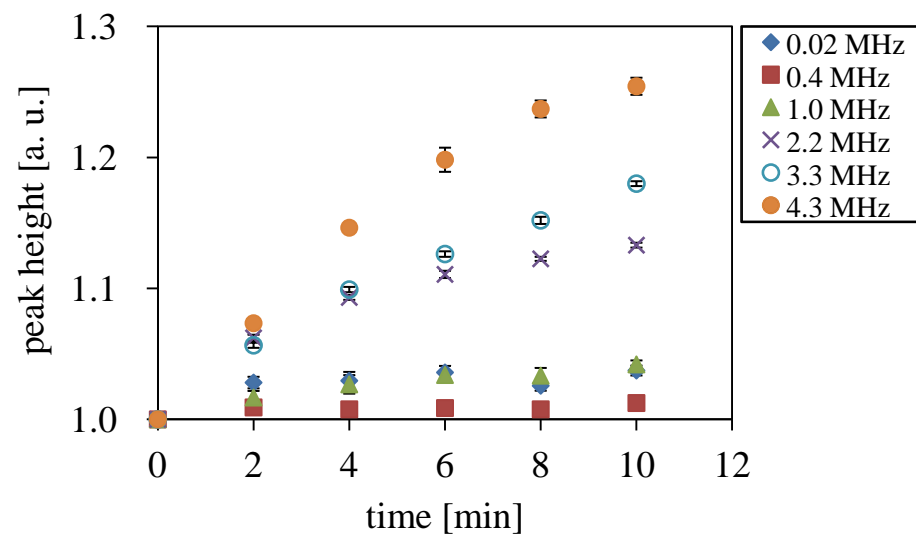
(a)

(b)
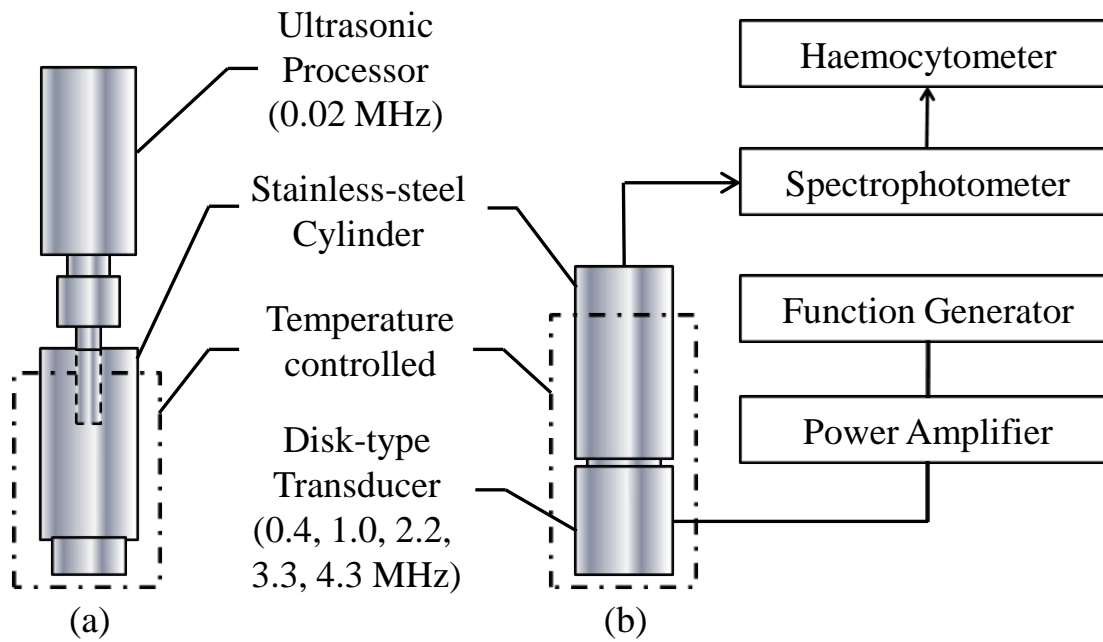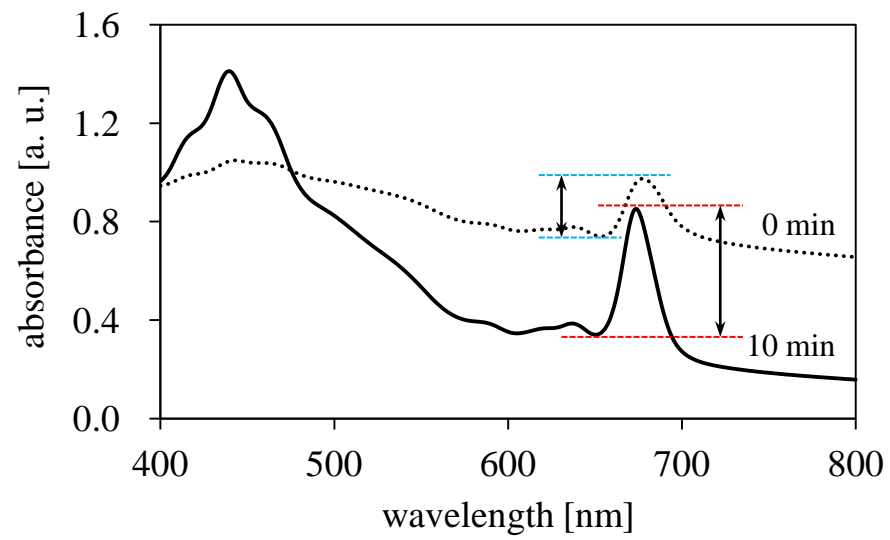
Fig. 1

Fig. 2
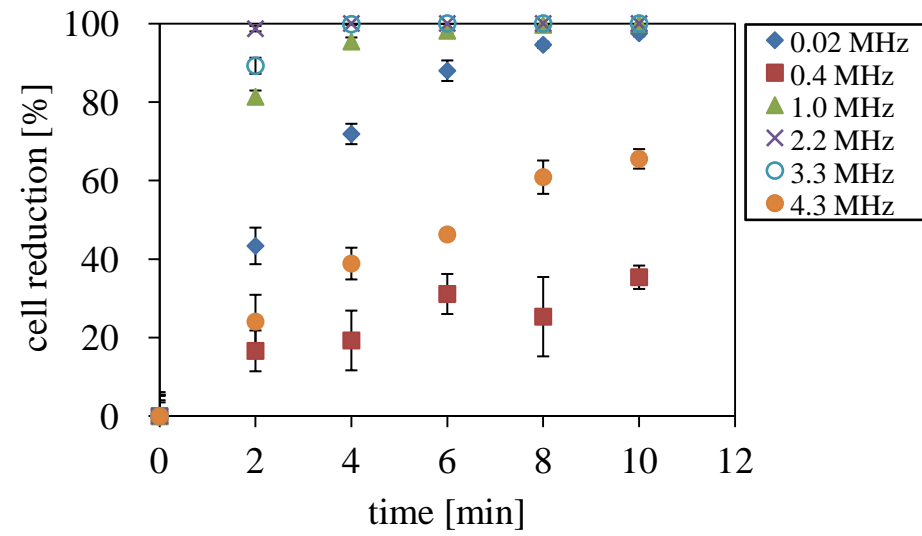
Fig. 3
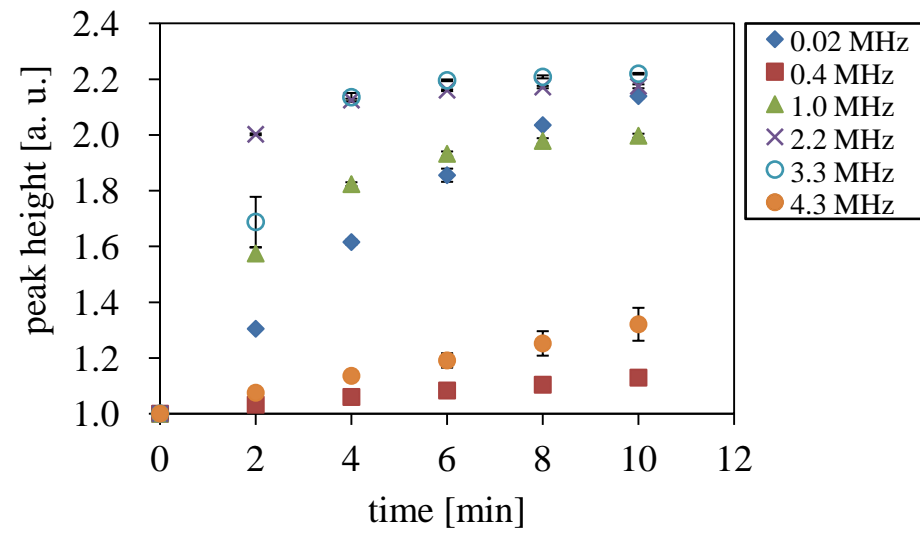
Fig. 4
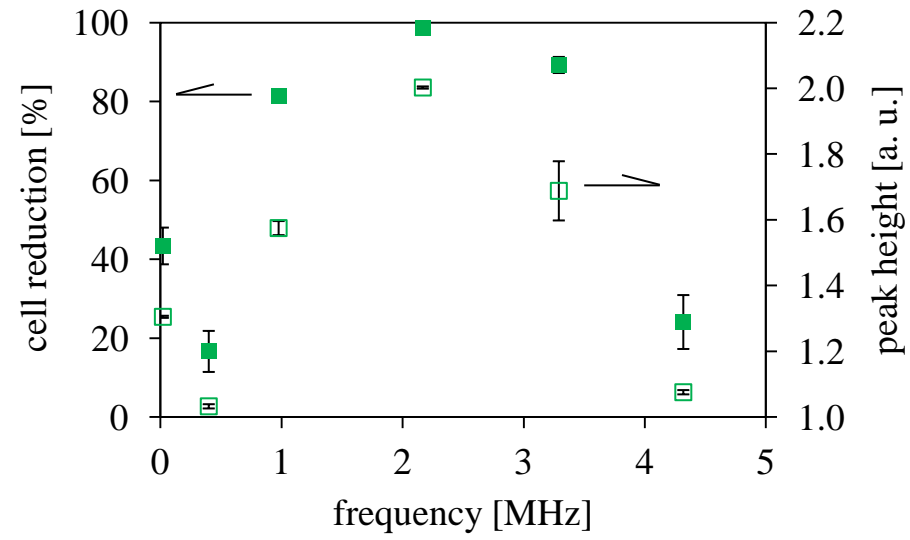
Fig. 5
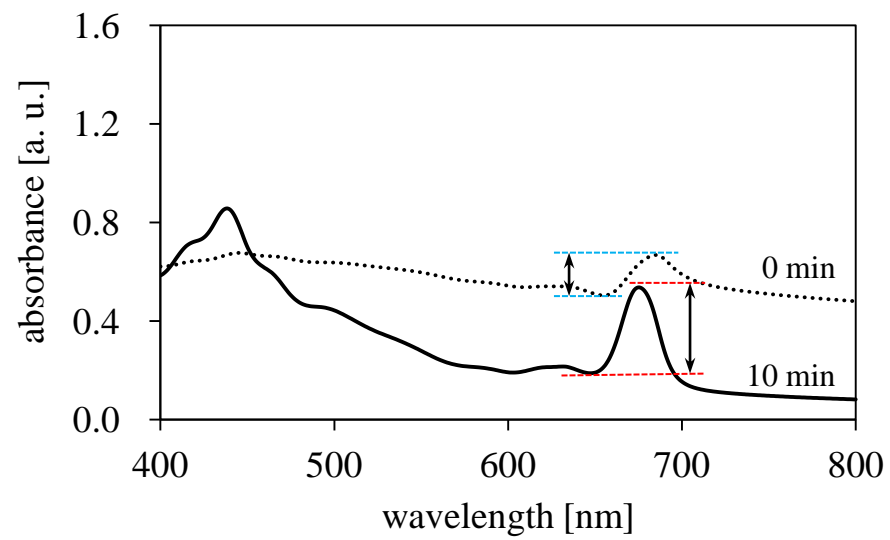
Fig. 6
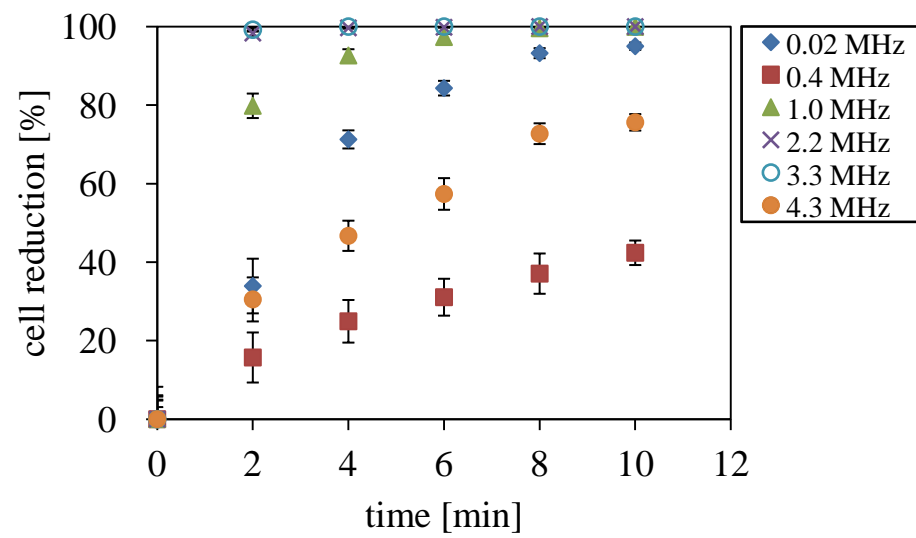
Fig. 7
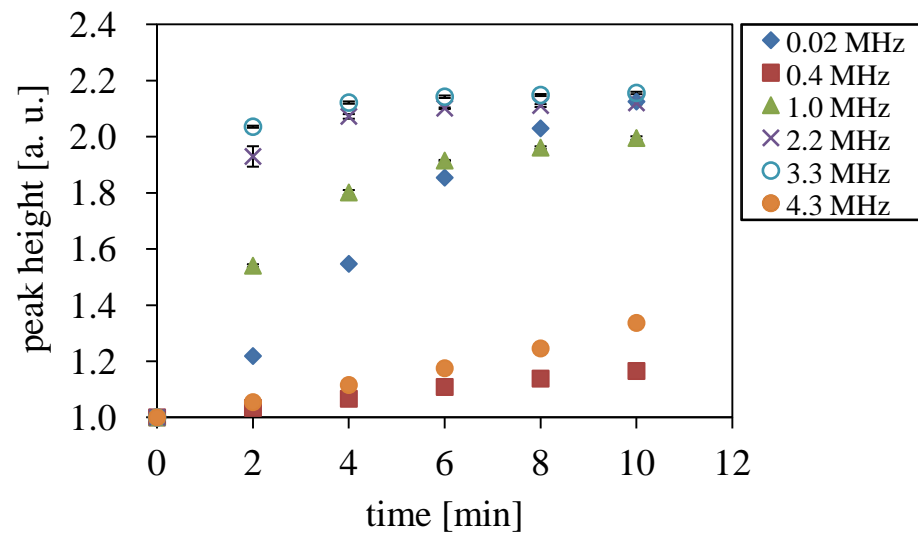
Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

Fig. 15