



Analysis of dielectric properties of *Euglena gracilis* in the 5 kHz to 5 MHz range

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Analysis of dielectric properties of *Euglena*
gracilis in the 5 kHz to 5 MHz range

(5 kHz ~ 5 MHz におけるユーグレナの誘電解析)

LIN CHEN

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Summary

The overall objective of this research was to understand the physical nature of the dielectric properties of *Euglena gracilis* cells in suspension, and to evaluate the effect of extracellular toxicants to different compartments of the cell by the dielectric measurement as a non-invasive technique or a so-called “dielectric anatomy”. The cell suspension was confined in a parallel-plate condenser-type measuring chamber, and some characteristic parameters were extracted from the obtained data, such as characteristic frequencies, loss tangent, relaxation strength and so on. Based on these features, the observed dielectric behaviors were simulated with the empirical Cole-Cole equation and with theoretical models (single-shelled ellipsoidal cell models) to estimate dielectric parameters of the different compartments of the cell such as the cell membrane, the cytoplasm, and organelles. Cell suspensions were then challenged with some toxic substances to evaluate a possible application of dielectric spectroscopy for monitoring the quality of water.

In Chapter 1, I have summarized some of the background information that has been publicly available concerning this research. In Chapter 2, cell suspensions with graded cell densities between 10^5 and 10^6 cells/ml were prepared, and dielectric behavior of the suspensions were measured with HIOKI IM3570 impedance analyzer (Nagano, Japan) in the frequency range of 5 kHz - 5 MHz at 127 measuring points. A mutant strain of *E. gracilis* strain Z, that is artificially deprived of its chloroplasts by a prolonged treatment with streptomycin (strain SM-ZK) was used throughout this study, because of its simplicity in cellular architecture as compared with the wild-type strain. The obtained dielectric data in capacitance (C) and conductance (G) were converted to the relative permittivity (ϵ) and conductivity (κ) of the cell suspension by using cell constants that are

specific to chambers used for dielectric measurements. The frequency-dependent dielectric properties of *E. gracilis* cells in suspension showed that the permittivity decreased and the conductivity increased with the measuring frequency.

The frequency dependence of these dielectric parameters were simulated with the Cole-Cole equation, and with theories for a suspension of dielectric particles (Chapter 3) to correlate the observed dielectric behavior with the cell morphology and electrical characteristics of the cell components. The dielectric behavior of *E. gracilis* cells in suspension was examined at graded cell densities. This investigation was based on an idea to use dielectric spectrometry for the use of biological monitoring of water samples. The impedance analyzer allows us to measure the dielectric parameters in a wide frequency range, but quicker monitoring would be probable if we use only a limited number of frequency points at which dielectric dispersion is maximized. The second characteristic frequency (f_{C2} , determined as the vertex frequency value of the imaginary part of the complex conductivity κ''), for example, was found to show constant values independent of cell densities at around 220-290 MHz. On the contrary, the first characteristic frequency (f_{C1} , determined as the peak frequency value of the dielectric loss factor ϵ'') was positively correlated with the cell density. This result indicates that the degree of the second dielectric dispersion can be efficiently quantified by measuring the degree of dispersion as the imaginary part of the complex conductivity at this frequency range. Simulation with “ellipsoidal single-shell model” showed a good agreement with the experimental result, especially in the higher frequency range. The theory was based on an electrical model in which a conducting cytoplasm phase is enclosed by a thin less-conductive plasma membrane having an ellipsoidal shape suspended in a conducting medium in random orientation. When small ellipsoidal particles were postulated to be

included in the cell interior, the theoretical calculation gave a better fit to the experimental data especially in the low frequency range between 50 kHz and 100 kHz, but large gaps at frequency below 50 kHz did not disappear. The disagreement at low frequencies was regarded as the result of a large degree of electrode polarization that is not incorporated into the theoretical consideration. Nevertheless, this non-invasive technique allows us to make a rough estimate for the electric parameters of the plasma membrane and the cell interior. Namely, the relative permittivity and conductivity of the cytosol were estimated as 60-70 and 0.15-0.16 mS/m, irrespective of the possible inclusion of the organelles in the cytoplasm. Thickness of the plasma membrane, relative permittivity, and conductivity were estimated as 60 nm, 5, 0.001 - 0.0001 mS/m, respectively. As expected, the electric properties of the plasma membrane showed a highly resistant feature. The plasma membrane was estimated to be a very thick sheet with a thickness roughly 6 times as thick as the normal biological membrane, which is considered to be due to the complicated ridge-and groove configuration of the plasma membrane with thick underlying protein structure that is unique to the genus *Euglena*.

E. gracilis usually shows an elongated or cigar-shaped appearance, but characteristic “euglenoid movement” is occasionally evoked in response to various physical and chemical stimulants, resulting in transformation of the cell body into a globular appearance. Various kinds of toxic chemicals are known to induce rounding-up response of *Euglena*, especially those affecting the cell membranes such as chlorpromazine, which is known to be incorporated to the plasma membrane and induces various dysfunctions. In Chapter 4, *E. gracilis* was found to show a sensitive response in dielectric behavior to membrane-destructive toxicants such as HgCl₂, chlorpromazine and Triton X-100, suggesting the usefulness of this technique for water quality monitoring.

The normal (elongated) and chlorpromazine-treated (rounded) cells were subjected to the dielectric measurement at different cell densities. During the experiment, inhomogeneity of the cell suspension, which inevitably occurred due to the gravitaxis of *Euglena*, was found to strongly affect the measurement. Therefore, a perfusion system was constructed with a peristaltic mini-pump for avoiding possible sedimentation of *Euglena* in the measuring cell, in which a 3-ml cell suspension was continuously circulated through the measuring chamber at a constant rate. The dielectric behaviors of the both samples showed similar frequency dependencies. The dielectric behaviors observed for the both cell suspensions changed similarly with the measuring frequency, and the characteristic frequencies (f_{C1} and f_{C2}) were not affected by the cell density. However, most of the other dielectric parameters were dependent on the cell density, especially the peak values of the dielectric loss factor, the imaginary part of the complex conductivity, and the loss tangent all increased with increasing cell density. Finally, the possibility to use the method of dielectric spectroscopy as a novel tool for monitoring the safety of water was addressed. Since dielectric spectroscopy is a non-invasive technique in which multiple parameters can be used, it is suitable as a method of biomonitoring with a possibility to identify the name of the toxicant in the polluted water. At least, it is expected to be able to narrow the list of candidate toxicants, which might allow a prompt and appropriate action by the government authority that is responsible for the safety of the drinking water supply. *E. gracilis* cells were subjected to three different types of membrane-affecting chemicals (chlorpromazine, HgCl_2 , and Triton X-100). In the present research, I found different dielectric responses that are characteristic to three different toxic chemicals, even though they showed similar morphological and behavioral responses to *E. gracilis*. If we can make use of a good set of multiple dielectric parameters extracted from a wide frequency-

range data, it should provide us with a good starting point to identify toxicants in water samples.

Chapter 1: General introduction

Euglena is a genus of unicellular eukaryotes of the *Phylum Euglenozoa*, one of the first protists discovered by Antoni van Leeuwenhoek in the 17 century, and later named as *Euglena* by C. G. Ehrenberg (Buetow, 2011). Nowadays, more than 250 species have been recorded as members of the genus *Euglena*. In the wild environments, free living *Euglena* is easily found in pools, ponds and lakes, especially where the water is fresh and clean. They are also found in harsh conditions such as in the soil or marine environments (Ferreira et al., 2007), and even in marsh lands (Smith, 2001). Most of the species are photosynthesis with chloroplasts containing chlorophylls *a* and *b*, thus majority of *Euglena* species are green. *Euglena* cells are mostly in the spindle shaped, but sometimes seen spherical or ovoid, with the cell length varying from 10 μm (*Euglena minuta*) to 500 μm (*Euglena oxyuris*). Most genera are flagellated with one or two whip-shaped flagella, and an orange-red eyespot is located near the proximal part of the flagella (Lee et al., 2000). The pellicle of *Euglena* is highly differentiated and frequently bears spiraling strips (Grell, 1973).

E. gracilis is the first domesticated photosynthetic eukaryotic microorganism use in laboratories. It is easy to be cultivated, and can grow in various media. The generation times at about 25°C is about 25 h (Buetow, 2011). The cell size is approximately 50 μm $\times$ 10 μm (length $\times$ width) in a cigar-shaped appearance (Buetow, 1982) with a highly developed subcortical architecture (Watanabe and Suzuki, 2001). Owing to its labile nature of chloroplasts, *E. gracilis* can be bleached readily by a variety of reagents and changing culture conditions, resulting in the formation of permanently chloroplast-deprived strains. *E. gracilis* SM-ZK strain is a streptomycin-bleached white strain of *E.*

gracilis Z, which has lost its chloroplasts and also the activity of ribulose biphosphate carboxylase (Oda, et al., 1982). In the present research, this strain of *E. gracilis* was used, because its simpler cell architecture is desirable for theoretical considerations with dielectric shell models.

Dielectric spectroscopy is a non-invasive technology which provides a rapid and useful way in biological researches to examine electrical properties of living specimens in the radio-frequencies. This technique was introduced to collect information on the electrical properties of biological specimens from a single cell to a mass of complicated tissues and organs, in most of which Maxwell-Wagner mechanism is responsible for the observed dielectric response. The application of this technique includes intact yeast cell suspension (Asami et al., 1976), mouse lymphocytes and erythrocytes (Asami et al., 1989), the growth of *Saccharomyces cerevisiae*, *Escherichia coli*, *Perilla frutescens* (plant cells) and AFP-27 hybridoma cells (Matanguihan et al., 1994) as biological specimens. Human tissues have been examined by this technique as a tool for diagnosis, which include female breast tissues (Campbell & Land, 1992), lung tissues (Nopp et al., 1997), brain tissues (Schmid et al., 2003), glioma and surrounding tissues (Lu, et al., 1992), blood (Wang, et al., 2009), placenta, umbilical cord and the amniotic fluid (Peyman et al., 2011). It has also been used for detecting the behavior of plant tissues like *Crassula portulacea* (Broadhurst et al., 1987) and animal tissues such as porcine glands, gonads, and body fluid (Peyman & Gabriel, 2012a) porcine cerebrospinal tissues (Peyman et al., 2007), rabbit liver tissues (Smith et al., 1986), dog brain tissues (Foster et al.,), skeletal muscles of the frog (Ma et al., 2003) and the rat (Yong-peng et al., 2006), rat embryo and foetus (Peyman & Gabriel, 2012b). Other porcine organs were also reported as the target of this technique (Peyman, 2012).

In the People's Republic of China, the circumstances concerning the quality of water have been quite deteriorated. It is widely reported that 850 out of 1,200 rivers that have been examined are polluted. According to the data from 2,222 monitoring stations in the whole mainland, which covers 80% of the total area of China, including 7 major rivers such as Yangtze, Yellow, Liaohe, Songhua, Haihe, Liaohe, and Pearl Rivers, only 36.9% of the rivers and lakes can reach or are better than the standard of the surface water Grade III (adequate water source or acceptable water for swimming, namely, pH 6-9, COD $\leq$ 20 mg/L, BOD₅ $\leq$ 4mg/L, NH₃-N $\leq$ 1.0 mg/L and so on (MEP of P.R.China, 2002)). However, more than 70% of the lakes in these regions are reported to be eutrophicated (吴舜泽 et al, 2000).

Table 1-1 and Table 1-2 are reproduced from the Report on the State of the Environment in China, since 2005 to 2015 (MEP of P.R.China, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015), which summarize the data reported for 411 surface-water monitoring sections examined by the National Environmental Monitoring Center, P. R. China, to detect the quality of surface waters. As shown in Table 1-1, the seven major rivers have been examined, which shows that more than 30% of the examined points are still classified as intermediate pollution or worse. Table 1-2 also shows that many lakes are still in similar situations, and some of the lakes has prominent eutrophication problems in addition.

From these data, the situation appears to be improving in recent years. However, almost half of the monitoring stations reveal that the water qualities are not in the suitable state. Moreover, we should pay more attention to the water quality of the lakes, since lakes are usually unstable in condition. Thus, the situation of the surface water quality in China is still fairly serious, and the development of the technologies for managing the

safety of the surface water is an urgent matter. The highly-sensitive and cost-efficient technique for water quality monitoring is one of such techniques needed for the harmonized development of the healthy water environment and the national economy.

In Kobe, Japan, where I am living, the water works of the city inspects the quality of tap water, being based on the issued regulation by Ministry of Health, Labor and Welfare, Japan. It is usually carried out once in every month for general bacteria, organic compounds, pH, and taste. Some kind of heavy metals is examined only once in every 3 months by ICP mass spectrometry. A continuous biological monitoring of water is also in operation at most of the large water purifying plants and water intake points, usually using goldfish. Although the biological monitoring using fish is not always sensitive enough to detect toxic materials introduced to the water resource, it is an effective procedure to reassure the public that the safety of the drinking water of the area is continuously guaranteed. In order to meet the full range of security, in reality and in name, a more sensitive and specific technique of biomonitoring is highly demanded in modern societies.

Therefore, I have aimed to conduct this research in an attempt to develop a rapid, real-time, long-term and low-cost technique for fresh water monitoring system. Recently, *E. gracilis* has been reported as a sensitive indicator to monitor the quality of waste water (Ahmed & Häder, 2010; Azizullah et al., 2013; Azizullah et al., 2012; Trenfield et al., 2012), which inspired a combining use of *Euglena* as a monitoring organism and dielectric spectroscopy as a measuring technique.

In my dissertation, I observed the dielectric behavior of *E. gracilis* SM-ZK in suspension in the range of wide frequencies between 1 kHz and 5 MHz in the culture media by using an impedance analyzer, and analyzed the data to extract some characteristic parameters such as characteristic frequencies, loss tangent, relaxation

strength and so on. Based on these features, the observed dielectric behavior of the cell suspension was simulated with dielectric theories for a suspension of ellipsoidal particles to correlate the observed dielectric behavior with the cell morphology and electrical characteristics of the cell.

Table 1-1 The quality of water in seven major rivers in China

(MEP of P. R. China, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015)

Year Grade ¹⁾	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
I - III (%)	41	40	49.9	55.0	57.3	59.9	61.0	68.9	71.7	71.2	72.1
IV - V (%)	32	32	26.5	24.2	24.3	23.7	25.3	20.9	19.3	19.8	19.0
Worse than V (%)	27	28	23.6	20.8	18.4	16.4	13.7	10.2	9.0	9	8.9

1) Water of grades I-III is suitable for drinking, grade IV is for industrial and recreational use, and grade V is suitable only for agricultural use.

Table 1-2 The quality of water in major lakes in China

(MEP of P. R. China, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015)

Year Grade ¹⁾	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
I - II (%)	7	7	7.1	14.3	3.9	3.8	19.2	29.0	27.9	29.0	29.0
III (%)	21	22	21.4	7.1	19.2	19.2	23.1	32.3	32.8	32.2	40.3
IV (%)	11	4	14.3	21.4	23.1	15.4	34.6	25.8	26.2	24.2	16.1
V (%)	18	19	17.9	17.9	19.2	23.1	15.4	1.7	1.6	6.5	6.5
Worse than V (%)	43	48	39.3	39.3	34.6	38.5	7.7	11.3	11.5	8.1	8.1

1) Water of grades I-III is suitable for drinking, grade IV is for industrial and recreational use, and grade V is suitable only for agricultural use.

Chapter 2: Dielectric behavior of *Euglena gracilis* SM-ZK at graded cell densities

2.1. Introduction

The dielectric response of *Euglena gracilis* cells in the AC electric field has not been studied before. As a simple unicellular protist belonging to the supergroup Excavata, *Euglena gracilis* is regarded as a useful organism to investigate on the evolution and diversity of eukaryotic cells, and has been effectively used as a material for a variety of biological research area. Another advantage is its simplicity in the culture method. In this part of the research, as the first step for the further analysis, I investigated the cell's response to the electric field in the wide range of increasing frequencies by an impedance analyzer using a grading densities of *E. gracilis* SM-ZK cells. The physiological implication for each characteristic dielectric parameter for the cell suspension is shown in Table 2-1.

2.2. Material and Methods

2.2.1. Cell suspension gradient concentration preparation

A wild type of *E. gracilis* strain Z (NIES-48) was obtained from the National Institute for Environmental Studies, Japan. *Euglena gracilis* SM-ZK strain was obtained through the courtesy of Professor Y. Nakano of Osaka Prefecture University. SM-ZK is a streptomycin-bleached mutant strain derived from *Euglena gracilis* Z, which is permanently deprived of chloroplasts (Oda et al., 1982).

2.2.2. Cell culture

The culture medium (“*Chlorogonium* medium”) consisted of 0.1% sodium acetate trihydrate (Nacalai Tesque, Japan), 0.1% polypepton (Nihon Pharmaceutical, Japan), 0.2% tryptone (Nacalai Tesque, Japan), 0.2% yeast extract (Bacto, USA) and 0.001% calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Nacalai Tesque, Japan). After the cells were cultured at 25°C for 7 days, they were efficiently concentrated with two layers of membrane filters, one was an 11- μm nylon mesh filter and the other was a hydrophilic PTFE filter with 10 μm pores (Omnipore, Ireland) placed on the bottom. Cells accumulated on top of the filters were used for measurement after the cell density was adjusted to 5×10^5 cells/ml.

2.2.3. Dielectric measurement

The dielectric measurement system was composed of a measuring chamber and an impedance analyzer. The measuring chamber was of a parallel-plate capacitor type, as shown in Fig. 2-1, made up with an acrylic glass tube (7.5 and 12.5 mm in inner and outer diameters, respectively), and a pair of Pt disks as parallel electrodes (diameter, 10.0 mm; thickness, 0.1 mm; cell constant, 5.1×10^{-14} F; placed 8.4 mm apart,) coated with Pt-black for reducing the electrode polarization (Bordi et al, 2001; Ilic et al., 2000; Schwan, 1968; Stoneman et al., 2007). Volume capacity of the chamber was 0.391 ml, and two holes of 1 mm in diameter were placed on the top of the chamber for sample injection. The dielectric response was measured with an impedance analyzer (IM3570, HIOKI E. E. Corporation, operated at 1 V) in the frequency range from 4 Hz to 5 MHz. Since dielectric data at frequencies under 5 kHz are usually regarded to include large amount of artefact due to electrode polarization, only the data between 1 kHz to 5 MHz at 127 different frequency points were used for further analysis, and the data at frequency below 1 kHz

were used for the correction of electrode polarization. Measurements were carried out at 25 ± 1 °C, and a single series of measurements took about 50 sec. The measuring chamber was set at a fixture (SMD TEST FIXTURE 9263, HIOKI E. E. Corporation) and attached to the impedance analyzer as shown in Fig. 2-2.

2.3. Results

Euglena is one of the non-excitabile cells which does not produce action potentials in the applied electric field, but a passive polarization is provoked that can be monitored by an impedance analyzer. The passive polarization of the living cells is regarded as the result of microscopic motions of electric charges in the macroscopic electric field in and around the biological cells. Frequency domain impedance analysis is employed for the study in non-excitabile cells for measuring the relative permittivity and the conductivity of the sample cell suspension, in which the relative permittivity is regarded as a measure of the ability of biological materials to store electromagnetic energy, and conductivity is regarded as a measure of the conduction of electromagnetic energy in the electric field.

The suspension of *E. gracilis* SM-ZK is a heterogeneous dielectric system, being composed of three main dielectric phases, namely the extracellular fluid, the cell membrane, and the cytoplasm. When AC field is applied to the cell suspension, charges are discontinuously accumulated on the interface of the different phases, whose migration is inhibited by the cell membrane by the Maxwell-Wanger effect.

Fig. 2-3 and 2-4 show the dielectric behavior of *E. gracilis* cells in suspension. Frequency dependence of relative permittivity ϵ (Fig. 2-3) and conductivity κ (Fig. 2-4)

are shown. With the increase in frequency, the relative permittivity decreased and conductivity rose. The complex relative permittivity (ε^*) and the complex conductivity (κ^*) were calculated by the following equations,

$$\varepsilon^* = \varepsilon - \frac{j\kappa}{\omega\varepsilon_v} = \varepsilon' - j\varepsilon'' \quad (\text{Eq. 2-1})$$

$$\kappa^* = \kappa + j\omega\varepsilon\varepsilon_v = \kappa' + j\kappa'' \quad (\text{Eq. 2-2})$$

where j is $\sqrt{-1}$, ω is the angular frequency, and ε_v is the permittivity of free space.

Every frequency band works on the different elements of the cell. In the low frequency range, the relative permittivity is higher and the conductivity is in lower, which are ascribed to the high inclusion and low conductivity of blocking substances to the passage of the electric current. As the current cannot pass through the cell at lower frequencies, the effective AC field is only restricted to the external phase, in other words the relative permittivity and the conductivity at lower frequencies represent the dielectric characteristics of the external fluid. In higher frequencies, the relative permittivity and the conductivity reach to minimum (ε_h) and maximum (κ_h) values, respectively. In the high frequency range, the cell becomes a kind of “shorted” situation, in which cell interior becomes exposed to the AC field. The cell membrane is not a hindrance to the electric current any more, and the dielectric parameters represent a combined figure of the extracellular and intracellular fluids.

As summarized in Table 2-2, two major dielectric relaxations were detected in the suspension of *Euglena* cells. The first one at the frequency f_{C1} is considered to be the relaxation at the interface between the extracellular fluid and the outer surface of the

plasma membrane, and the second one at the frequency f_{C2} , is considered to be ascribed to the relaxation at the interface between the inner surface of the plasma membrane and the cell interior. The frequency dependence of the imaginary part of the relative permittivity ε'' is shown in Table 2-2, in which the first characteristic frequencies f_{C1} was determined by reading the vertex frequency of the curve, and listed in Table 2-2 at different cell densities. In Fig. 2-6 the frequency dependencies of the imaginary parts of the conductivity κ'' is shown. From this graph, the second characteristic frequency f_{C2} was determined. Fig. 2-7 is the frequency dependence of the dielectric loss tangent, also determined at different cell densities.

2.4. Discussion

The dielectric behavior of *E. gracilis* cells in suspension was examined at graded cell densities. This investigation was based on an idea to use dielectric spectrometry for the use of biological monitoring of water samples. The impedance analyzer allows us to measure the dielectric parameters in a wide frequency range, but quicker monitoring would be probable if we use only a limited number of frequency points at which dielectric dispersion is maximized. For this purpose, measurements were carried out at different cell densities to evaluate the dependency of the characteristic frequencies (f_{C1} and f_{C2}) at which dielectric dispersions occur. As summarized in Table 2-2, the second characteristic frequency f_{C2} was found to be almost constant throughout the whole range of cell densities except for the lowest one at 1×10^5 cells/ml. As shown in Fig. 2-6, the vertex frequencies (ie, f_{C2}) stays almost constant at 220-290 MHz, indicating that the degree of the second

dielectric dispersion can be efficiently quantified by measuring the degree of dispersion as the imaginary part of the complex conductivity.

Table 2-1 Implications of each characteristic parameter of dielectric properties.

Parameters	Symbol	Implications
Low frequency limit of permittivity	ϵ_L	Insulation or non-conductive properties of external phase of cell
High frequency limit of permittivity	ϵ_h	Insulation or non-conductive properties of inner phase of cell
Permittivity increment	$\Delta\epsilon=\epsilon_L-\epsilon_h$	Insulation performance gap of external and inner phases
Low frequency limit conductivity	κ_L [S/m]	Conductive property of external phase of cell
High frequency limit conductivity	κ_h [S/m]	Conductive property of external phase of cell
Conductivity increment	$\Delta\kappa=\kappa_h-\kappa_L$	Conductive property gap of external and inner phases
First characteristic frequency	f_{C1} [Hz]	The frequency at which polarization relaxation at the interface between the plasma membrane and the extracellular space occurs
Second characteristic frequency	f_{C2} [Hz]	The frequency at which polarization relaxation at the interface between the plasma membrane and the intracellular space occurs
Peak of dielectric loss factor	ϵ''_{max}	Greatest loss-induced dipole at the interface between the plasma membrane and the extracellular space at the frequency f_{C1}
Peak of conductivity imaginary	κ''_{max}	Dipole induced as the greatest change in the imaginary part of the conductivity at the interface between the cell membrane and the intracellular space at the frequency f_{C2}
Loss factor tangent	$\text{tg}\delta_{max}$	Maximum ratio of loss of energy and stored energy

Table 2-2 Characteristic parameters of the control (elongated) *E. gracilis* SM-ZK suspension at different cell densities.

Parameter	Symbol	Cell density (cells/ml)									
		1×10 ⁵	2×10 ⁵	3×10 ⁵	4×10 ⁵	5×10 ⁵	6×10 ⁵	7×10 ⁵	8×10 ⁵	9×10 ⁵	10×10 ⁵
Low frequency limit of permittivity	ε_L	305.49	308.82	319.22	325.47	330.58	340.25	343.20	352.34	352.85	354.97
High frequency limit of permittivity	ε_h	8.42	8.46	8.50	8.64	8.66	8.78	8.81	8.89	8.91	8.98
Relaxation strength	$\Delta\varepsilon=\varepsilon_L-\varepsilon_h$	297.06	300.35	310.72	316.83	321.92	331.46	334.39	343.44	343.94	345.99
Low frequency limit conductivity	κ_L [S/m]	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
High frequency limit conductivity	κ_h [S/m]	0.16	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.16
Conductivity increment	$\Delta\kappa=\kappa_h-\kappa_L$	2.28E-03	3.12E-03	3.85E-03	4.73E-03	4.73E-03	6.34E-03	6.80E-03	7.55E-03	8.21E-03	8.79E-03
First characteristic frequency	f_{C1} [Hz]	13,083	13,083	13,084	13,084	13,084	13,084	13,825	14,607	15,434	21,475
Second characteristic frequency	f_{C2} [Hz]	336,860	285,550	270,250	270,250	270,250	270,250	225,780	225,780	225,780	225,780
Peak of dielectric loss factor	ε''_{max}	41.786	42.454	43.979	45.329	46.471	48.467	49.303	50.998	52.329	55.300
Peak of conductivity imaginary	κ''_{max}	8.03E-04	9.88E-04	1.24E-03	1.55E-03	1.76E-03	2.09E-03	2.24E-03	2.49E-03	2.70E-03	2.81E-03
Loss factor	$\text{tg}\delta_{max}$	0.83	0.78	0.81	0.86	0.91	0.98	1.01	1.06	1.10	1.13

The low-frequency limit values (ε_L and κ_L) were determined as the values obtained at 5 kHz, and the high-frequency values (ε_h and κ_h) were determined at 5 MHz. The first and second characteristic frequencies were determined by reading the vertex frequency of the curves shown in Fig. 2-5 and Fig. 2-6 ε''_{max} , κ''_{max} , and $\text{tg}\delta_{max}$ were determined from Fig. 2-5, Fig. 2-6 and Fig. 2-7, respectively.

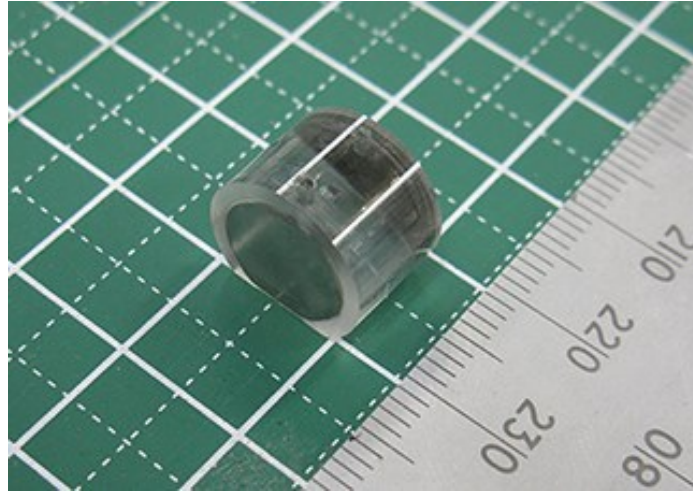


Fig. 2-1. The measuring chamber used in the experiments in Chapter 2.



Fig. 2-2. The measuring chamber set on the test fixture 9263 that is attached to the impedance analyzer HIOKI IM3570.

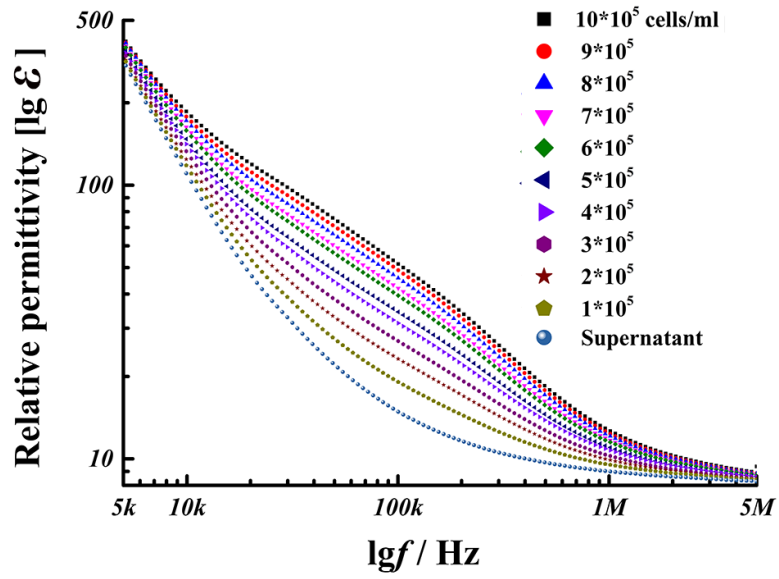


Fig. 2-3. Frequency-dependence of the relative permittivity of *E. gracilis* SM-ZK in suspension as determined at different cell densities.

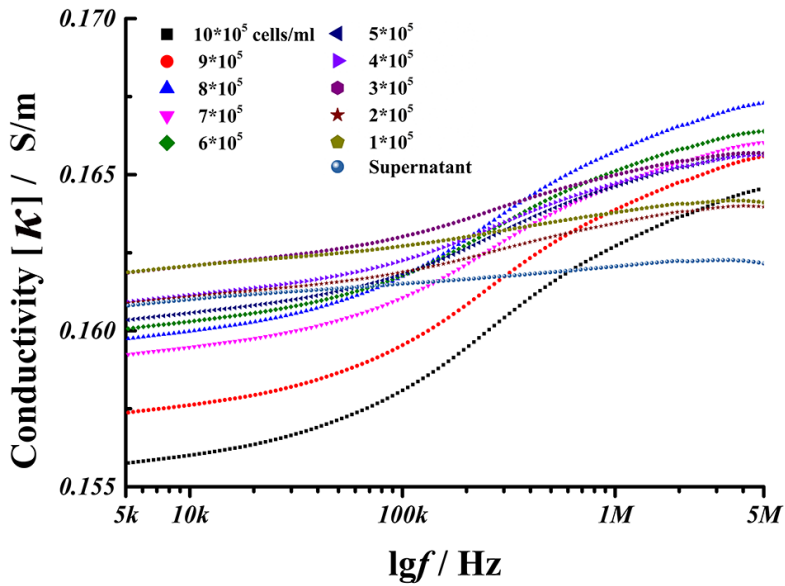


Fig. 2-4. Frequency-dependence of the conductivity of *E. gracilis* SM-ZK in suspension as determined at different cell densities.

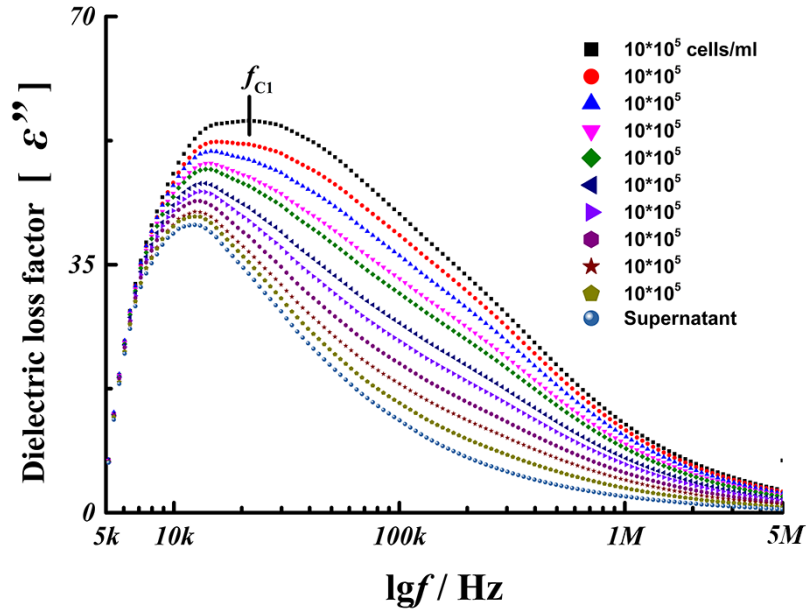


Fig. 2-5. Frequency-dependence of the dielectric loss factor of *E. gracilis* SM-ZK in suspension as determined at different cell densities.

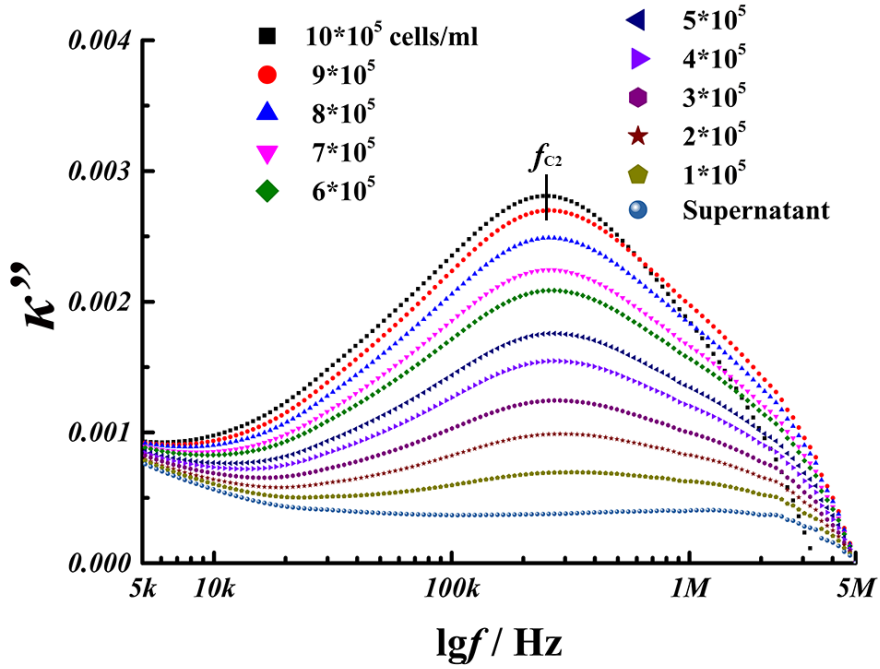


Fig. 2-6. Frequency-dependence of the imaginary part of the complex conductivity of *E. gracilis* SM-ZK in suspension as determined at different cell densities.

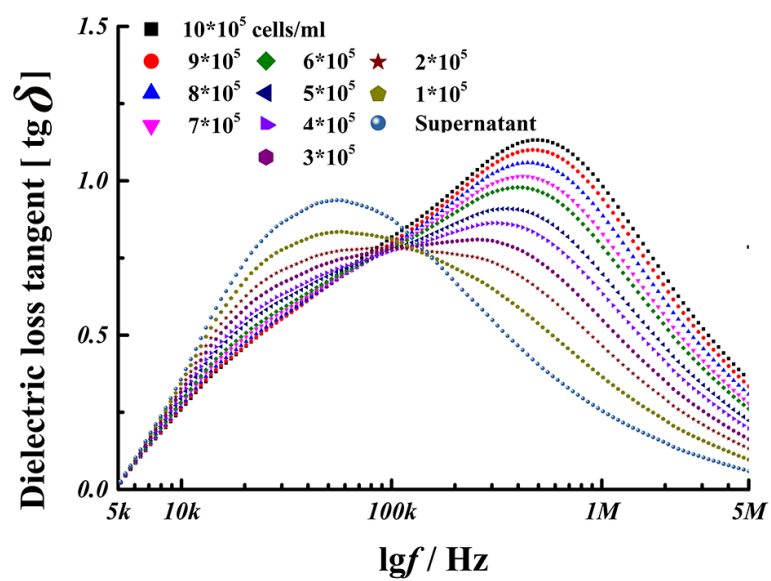


Fig. 2-7. Frequency-dependence of the dielectric loss tangent of the control (elongated) *E. gracilis* SM-ZK in suspension as determined at different cell densities.

Chapter 3: Simulation of dielectric behavior of *Euglena gracilis* with Cole-Cole equation

3.1. Introduction

In the middle of the 18th century, Luigi Galvani discovered a specific electrical phenomenon in the heart and the frog nerve, which he called “animal electricity” (Bresadola, 1998; Galvani, 1791). The bioelectricity of living tissues, such as muscle, was shown for the first time by Bois-Reymond in the 19th century (Finkelstein, 2006). As time went on, alternating current (AC) field was employed for investigating the electric properties of biological cells. In the early 20th century, Höber made an analysis of blood cells at high and low frequencies (Höber, 1910, 1912). Fricke and Curtis investigated yeast (Fricke & Curtis, 1934a), red blood cells (Fricke & Curtis, 1934b), and leucocytes (Fricke & Curtis, 1935) in capacities and resistances at full-band radio waves. Cole made a spherical model (Cole, 1928), and applied the theory to investigate the electric impedance of *Hippoonoe* eggs (Cole, 1935) and *Arbacia* eggs (Cole & Cole, 1936). Improved method and cell models were further created for expanded studies in a variety of cell systems as well (Cole, 1968). Not only the cells but also tissues were systematically studied, including plant tissues such as potato and alfalfa tissues (Hayden et al., 1969), cucumber (Inaba et al., 1995) and Scots pine needles (Zhang et al., 1995).

In this part of my dissertation, I examined the electrical properties of cell suspension with an attempt to simulate the observed dielectric behavior with the theoretical equation, namely an empirical Cole-Cole equation (Cole & Cole, 1941; Cole, 1942) with consideration in relation to the cell shape change induced by the addition of chlorpromazine, a membrane-targeting reagent that is incorporated into the plasma

membrane. As a non-excitabile cell, *E. gracilis* changes in shape by a variety of physical and chemical stimulants (Daiker et al , & Häder, 2010; Lonergan, 1985).

As described in the previous chapters, the dielectric properties of cells includes capacity and conductivity, first reported in 1890 in investigating living cells' resistance within an electric field (Fricke, 1933). When an electrical current flows through a cell, the polarization occurs, while in the AC field the cell membrane is deemed to be an insulator at low frequency (Kyle et al., 2004). With the increase in frequency, the current will pass through the cell membrane, as illustrated in Fig. 3-1. If the frequency goes up to the GHz range, even the characteristic of DNA in the nucleus (Hölzel, 2009) and smaller molecules (Gařduk & McConnell, 1997) can be detected.

Cole-Cole equation of complex permittivity constant (Cole & Cole, 1941, 1942) is widely used to analyze the empirical electric properties of materials and liquids, which is showed in Eq.3-1.

$$\varepsilon^* = \varepsilon - \frac{j\kappa}{\omega\varepsilon_v} = \varepsilon' - j\varepsilon'' \quad (\text{Eq. 3-1})$$

where ε^* is complex permittivity constant, κ is conductivity, ε is permittivity constant, ε_v is the permittivity constant of vacuum, j is the unit of imaginary part presents in Eq. 3-2, ω is angular frequency as in Eq. 3-3, f is frequency of applied electric field, ε' and ε'' are the real and imaginary parts of ε^* .

$$j = \sqrt{-1} \quad (\text{Eq. 3-2})$$

$$\omega = 2\pi f \quad (\text{Eq. 3-3})$$

Cole-Cole equation is shown in Eq. 3-4.

$$\varepsilon^* = \varepsilon_h + Af^{-m} + \frac{\Delta\varepsilon_1}{1+\left(j\frac{f}{f_{C1}}\right)^{\beta_1}} + \frac{\Delta\varepsilon_2}{1+\left(j\frac{f}{f_{C2}}\right)^{\beta_2}} + \frac{\kappa_l}{j\omega\varepsilon_v} \quad (\text{Eq. 3-4})$$

where ε^* is the complex permittivity constant shown in Eq. 3-1, Af^m represents the experimentally-definable electrode polarization with constants A and m , f is frequency, $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ are dielectric increment of the 1st and 2nd dielectric relaxations, β_1 and β_2 represent the 1st and 2nd dielectric relaxation distribution time ($0 < \beta < 1$), the 1st and 2nd characteristic frequencies are expressed as f_{C1} and f_{C2} , respectively, ε_h , κ_l are the high-frequency limit value of relative permittivity and low-frequency limit value of conductivity, respectively.

For convenience in calculation, I defined A_1 , A_2 , B_1 , B_2 , C_1 and C_2 as shown in the following equations.

$$A_1 = \cos\left(\beta_1 \frac{\pi}{2}\right) f^{\beta_1} + f_{C1}^{\beta_1} \quad (\text{Eq. 3-5})$$

$$A_2 = \cos\left(\beta_2 \frac{\pi}{2}\right) f^{\beta_2} + f_{C2}^{\beta_2} \quad (\text{Eq. 3-6})$$

$$B_1 = f_{C1}^{\beta_1} \Delta \varepsilon_1 \quad (\text{Eq. 3-7})$$

$$B_2 = f_{C2}^{\beta_2} \Delta \varepsilon_2 \quad (\text{Eq. 3-8})$$

$$D_1 = \sin\left(\beta_1 \frac{\pi}{2}\right) f^{\beta_1} + f_{C1}^{\beta_1} \quad (\text{Eq. 3-9})$$

$$D_2 = \sin\left(\beta_2 \frac{\pi}{2}\right) f^{\beta_2} + f_{C2}^{\beta_2} \quad (\text{Eq. 3-10})$$

With Eq. 3-5 to Eq. 3-10, the relative part of the relative permittivity in Eq. 3-4 is expressed as:

$$\varepsilon' = \Lambda f^{-m} + \frac{A_1 B_1}{A_1^2 + D_1^2} + \varepsilon_h + \frac{A_2 B_2}{A_2^2 + D_2^2} \quad (\text{Eq. 3-11})$$

and the imaginary part as the following equation.

$$\varepsilon'' = \frac{B_1 D_1}{A_1^2 + D_1^2} + \frac{\kappa_l}{\omega \varepsilon_v} + \frac{B_2 D_2}{A_2^2 + D_2^2} \quad (\text{Eq. 3-12})$$

In the present study the dielectric behavior of *Euglena gracilis* SM-ZK as obtained

in Chapter 2 was simulated with the Cole-Cole equation to estimate dielectric parameters employed in the Cole-Cole simulation.

To correlate the dielectric behavior of the *Euglena* cell in suspension with electrical parameters of the cell, a theoretical analysis was also carried out with “ellipsoidal-shell models” for membrane-bounded ellipsoidal shells which explains dielectric relaxation of cell suspensions due to interfacial polarization that occurs at the boundaries between materials of different electrical properties (Asami, 2002b; M. Watanabe et al., 1991). To validate the applicability of the dielectric theory to living *Euglena* cells in suspension, three different models were examined as shown in Fig. 3-1, Fig. 3-2 and Fig. 3-3. The first model (Model 1) is a suspension of ellipsoidal shells that contain a conductive and homogeneous interior fluid. The ellipsoids are in random orientations. The second model (Model 2) is also a suspension of ellipsoidal shells but contain a single type of ellipsoidal inclusion, supposing the presence of organelles in the cell. The third model (Model 3) has the most complicated cell architecture, comprising a mixture of three different types of ellipsoidal inclusions, envisaging a variety of organelles suspended in the cytosol. Parameters used for each model are determined through trials and errors, as described above, by fitting the simulated curve to the experimentally obtained data to minimize the total sum of the residuals.

3.2. Material and Methods

3.2.1. Preparation of cell suspension

Cell suspensions of *E. gracilis* strain SM-ZK were prepared as described in Chapter 2 at three different cell densities (1×10^5 , 5×10^5 , and 1×10^6 cells/ml).

3.2.2. Impedance measurement

The same measuring chamber was used in this experiment as described in the previous chapter, and the measurement system was also same as in Chapter 2.

3.2.3. Simulation with Cole-Cole equation

For fitting the Cole-Cole equation to the experimentally-obtained data, residual errors were calculated individually for ε , κ , ε'' , and κ'' as shown in Eq. 3-13, Eq. 3-14, Eq. 3-15, Eq. 3-16, respectively. The parameters for the Cole-Cole equation (ie, A , m , ε_h , $\Delta\varepsilon_1$, $\Delta\varepsilon_2$, f_{c1} , f_{c2} , and κ_l) were determined by trials and errors to minimize the total sum of the four residual errors.

$$R(\varepsilon) = \frac{100}{n} \sqrt{\frac{\sum_{i=1}^n (\varepsilon_{exp} - \varepsilon_{theory})^2}{(\varepsilon_{max,exp} - \varepsilon_{min,exp})^2}} \quad (\text{Eq. 3-13})$$

$$R(\kappa) = \frac{100}{n} \sqrt{\frac{\sum_{i=1}^n (\kappa_{exp} - \kappa_{theory})^2}{(\kappa_{max,exp} - \kappa_{min,exp})^2}} \quad (\text{Eq. 3-14})$$

$$R(\varepsilon'') = \frac{100}{n} \sqrt{\frac{\sum_{i=1}^n (\varepsilon''_{exp} - \varepsilon''_{theory})^2}{(\varepsilon''_{max,exp} - \varepsilon''_{min,exp})^2}} \quad (\text{Eq. 3-15})$$

$$R(\kappa'') = \frac{100}{n} \sqrt{\frac{\sum_{i=1}^n (\kappa''_{exp} - \kappa''_{theory})^2}{(\kappa''_{max,exp} - \kappa''_{min,exp})^2}} \quad (\text{Eq. 3-16})$$

In the equations shown above, ε is the real part of the complex permittivity, and κ is the real part of the complex conductivity, while ε'' and κ'' represent the imaginary parts of the complex relative permittivity and that of the complex conductivity, respectively. The subscript max, min, exp and theory represent the value of maximum, minimum, experiment and theory.

3.3 Results and discussion

As shown in Fig. 3-4 to Fig. 3-8, dielectric behavior of *Euglena* cells in suspension was well fitted with the Cole-Cole empirical equation, except for the frequency range below 10 kHz. The estimated characteristic frequencies are summarized in Table 3-1. Among the estimated values, the characteristic frequencies f_{c1} and f_{c2} are similar to those determined in the previous chapter by reading the vertex frequencies of ε'' and κ'' . In particular, f_{c2} was determined as about 250 MHz, which is in good agreement with the value determined in Chapter 2 (220-290 MHz). One of the advantages of using Cole-Cole simulation is that we can make a precise estimation of the lower limit value of relative permittivity and conductivity at low frequencies (ε_L and κ_L , respectively) where the sample impedance is usually obscured by the presence of a large amount of the electrode polarization. The arbitrary choice of the frequency (5 kHz in Chapter 2) to estimate the lowest limit values of ε_L and κ_L is also a problem, as ε and κ are not stable in this frequency range. In fact, the permittivity increment ($\Delta\varepsilon$) seems to be overestimated by the conventional method in Chapter 2, as it was estimated as 343 at 1×10^6 cells/ml, while it was calculated by Cole-Cole simulation as 233 (Table 3-1).

Although the Cole-Cole simulation provides a good measure for characterizing the dielectric behavior of biological specimens, it is not useful for correlating the observed dielectric behavior the physiological and morphological parameters of the cell. Therefore I have applied a method of “shell model analysis”, a simulation based on the theory of dielectric dispersion of a suspension of randomly-oriented shelled ellipsoids. The theory was created by Asami and his co-workers (Asami, 2002a), and successfully applied to the suspension of *E. coli* cells (Asami et al., 1980a, 1980b; Asami & Yonezawa, 1995). In this

model, one can include multiple phases of shelled ellipsoids (implying the nucleus and various organelles) in the main ellipsoids (the cell body), with which we can estimate the electric parameters of each compartment of the cells, from the plasma membrane down to the interior of the very small organelles, if precise analysis is available with high precision. Fig. 3-9 and Fig. 3-10 are the result of simulation with three different shell-models (1, 2 and 3) with different intracellular inclusions, and best-fit parameters determined for these models are listed in Table 3-2, Table 3-3 and Table 3-4, respectively. The curve-fitting was not quite successful, and large gaps between theory and experiment persisted at low frequencies below 100 kHz. When small organelles were included in the cytoplasm, as in the model 2 and 3, curve-fittings became improved between 50 kHz and 100 kHz, but large gaps at frequency below 50 kHz did not disappear. The disagreement at low frequencies is mainly because of the fact that a large degree of electrode polarization is not incorporated into the theoretical consideration. Nevertheless, I succeed in making a rough estimate of the electric parameters of the plasma membrane and the cell interior as listed in Table 3-2, Table 3-3 and Table 3-4. Namely, the relative permittivity and conductivity of the cytosol were estimated as 60-70 and 0.15-0.16 mS/m, irrespective of the possible inclusion of the organelles in the cytoplasm. Thickness of the plasma membrane, relative permittivity, and conductivity were estimated as 60 nm, 5, 0.001 - 0.0001 mS/m, respectively. The electric properties of the plasma membrane is, as expected, shows a highly resistant membrane with a thickness of roughly 6 times as thick as the normal biological membrane. Since the plasma membrane of *Euglena* is not a smooth sheet but an undulating membrane with ridge-and-groove configuration (Suzaki & Williamson, 1986) connected with a thick undercoat called pellicular strip, the

thickness of the plasma membrane estimated by the theoretical simulation might be the consequence of such a complicated cell surface structure.

Table 3-1 Characteristic parameters of normal group experiment data and Cole-Cole equation simulation data

Parameter	Symbol	Cell density		
		1×10^5	5×10^5	10×10^5
Low frequency limit of permittivity	ε_L	133.18	132.29	242.60
High frequency limit of permittivity	ε_h	8.03	8.49	9.60
Relaxation strength	$\Delta\varepsilon = \varepsilon_L - \varepsilon_h$	125.15	123.81	233.00
Low frequency limit conductivity	κ_L [S/m]	0.16	0.16	0.16
High frequency limit conductivity	κ_h [S/m]	0.16	0.17	0.16
Conductivity increment	$\Delta\kappa = \kappa_h - \kappa_L$	2.10×10^{-03}	4.85×10^{-03}	7.74×10^{-03}
First characteristic frequency	f_{c1} [Hz]	12,383	17,230	22,690
Second characteristic frequency	f_{c2} [Hz]	336,830	255,780	255,780
Peak of dielectric loss factor	ε''_{max}	38.91	41.34	58.67
Peak of conductivity imaginary	κ''_{max}	8.29×10^{-04}	1.76×10^{-03}	2.79×10^{-03}
Loss factor	$\text{tg}\delta_{max}$	0.82	0.91	1.04

Parameters were determined by fitting the values calculated with the Cole-Cole equation to the experimental data as shown in Fig. 3-1 to Fig. 3-5, by reducing the total sum of the residuals calculated with Eq. 3-13 to 3-16.

Table 3-2 Best-fit phase parameters for Model 1

Most external phase	ε	5
	κ (S/m)	0.1351
Volume fraction (%)		3.166
Inner phase	ε	65
	κ (S/m)	0.16
Cell size (Semiaxis, μm)	a	20
	b	6
	c	7
	thickness (nm)	60
Shell	ε	5
	κ (S/m)	0.0001

Parameters were determined by fitting the theoretical calculation with the Model 1 to the experimental data as shown in Fig. 3-5 and Fig. 3-6, by reducing the total sum of the residuals calculated with Eq. 3-13 to 3-16.

Table 3-3 Best-fit phase parameters for Model 2

Most external phase					
ε		5			
κ (S/m)		0.1345			
Phase 1			Phase 2 (in Phase 1)		
Volume fraction (%) in the whole space		3.166	Volume fraction (%) in Phase 1		
			10		
Inner phase	ε	70	Inner phase	ε	80
	κ (S/m)	0.15		κ (S/m)	0.8
Size (semi-axis, μm)	a	50	Size (semi-axis, μm)	a	1
	b	6		b	1
	c	7		c	1
	thickness (nm) 60			thickness (nm) 20	
Shell	ε	7	Shell	ε	7
	κ (S/m)	0.00001		κ (S/m)	0.000001

Parameters were determined by fitting the theoretical calculation with the Model 2 to the experimental data as shown in Fig. 3-5 and Fig. 3-6, by reducing the total sum of the residuals calculated with Eq. 3-13 to 3-16.

Table 3-4 Best-fit phase parameters for Model 3

Most external phase											
ε		4.6									
κ (S/m)		0.1348									
Phase 1			Phase 2			Phase 3			Phase 4		
Volume fraction in the whole space (%)		3.166	Volume fraction in Phase 1 (%)		10	Volume fraction in Phase 1 (%)		2	Volume fraction in Phase 1 (%)		20
Inner phase	ε	70	Inner phase	ε	600	Inner phase	ε	300	Inner phase	ε	70
	κ (S/m)	0.15		κ (S/m)	0.8		κ (S/m)	0.17		κ (S/m)	0.16
Size (semi-axis, μm)	a	50	Size (semi-axis, μm)	a	1	Size (semi-axis, μm)	a	0.5	Size (semi-axis, μm)	a	1.1
	b	6		b	1		b	0.5		b	1
	c	7		c	2		c	0.5		c	1.1
Shell	thickness (nm)	60	Shell	thickness (nm)	20	Shell	thickness (nm)	20	Shell	thickness (nm)	10
	ε	7		ε	12		ε	100		ε	10
	κ (S/m)	0.00001		κ (S/m)	0.000001		κ (S/m)	1E-07		κ (S/m)	0.001

Parameters were determined by fitting the theoretical calculation with the Model 3 to the experimental data as shown in Fig. 3-5 and Fig. 3-6, by reducing the total sum of the residuals calculated with Eq. 3-13 to 3-16.

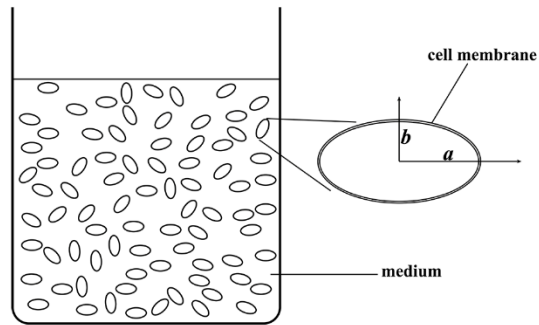


Fig. 3-1. Model A: dispersion system of shelled ellipsoids.

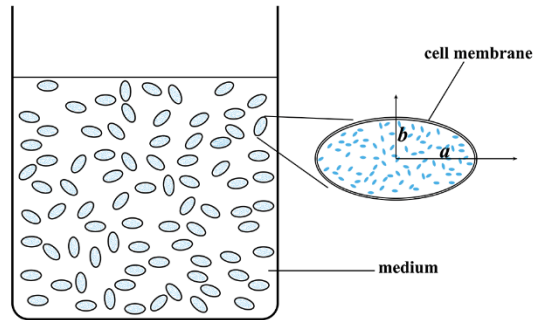


Fig. 3-2. Model B: dispersion system of shelled ellipsoids with one type of inclusions.

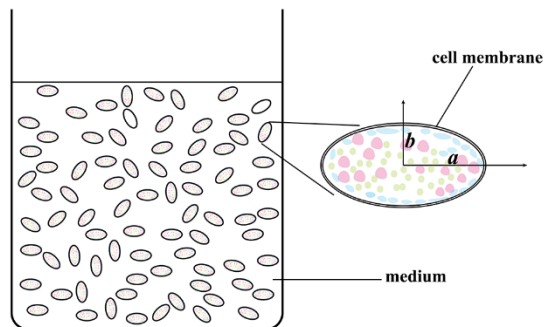


Fig. 3-3. Model C: dispersion system shelled ellipsoids with three types of inclusions envisaging different organelles with different sizes.

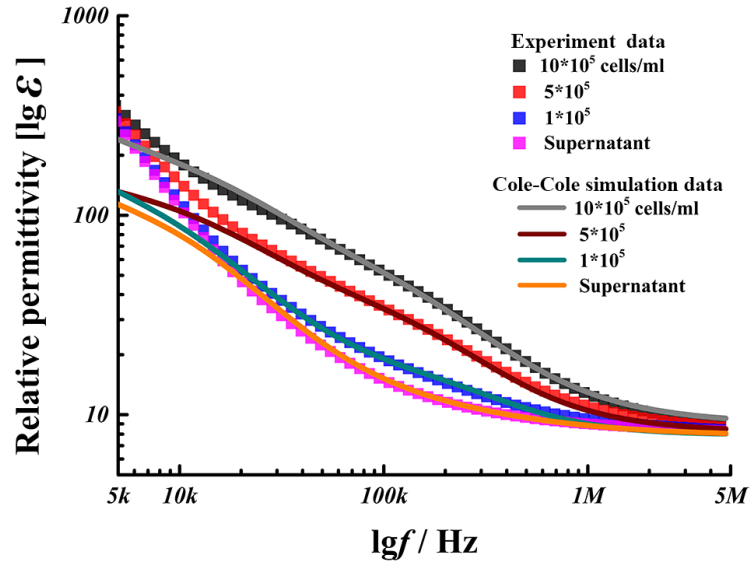


Fig. 3-4. Cole-Cole simulation on relative permittivity to the experimental data measured for the suspensions of *E. gracilis* SM-ZK cells in three concentration cell densities.

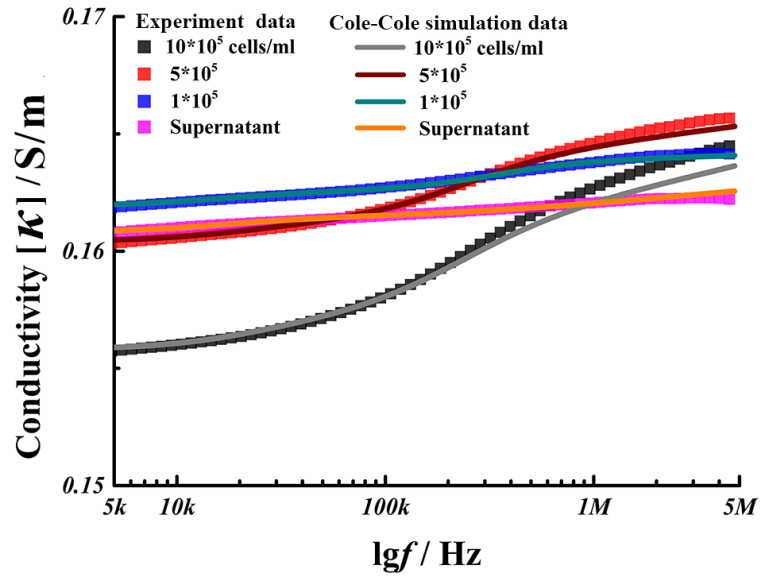


Fig. 3-5. Cole-Cole simulation on conductivity to the experimental data measured for the suspensions of *E. gracilis* SM-ZK cells in three concentration cell densities.

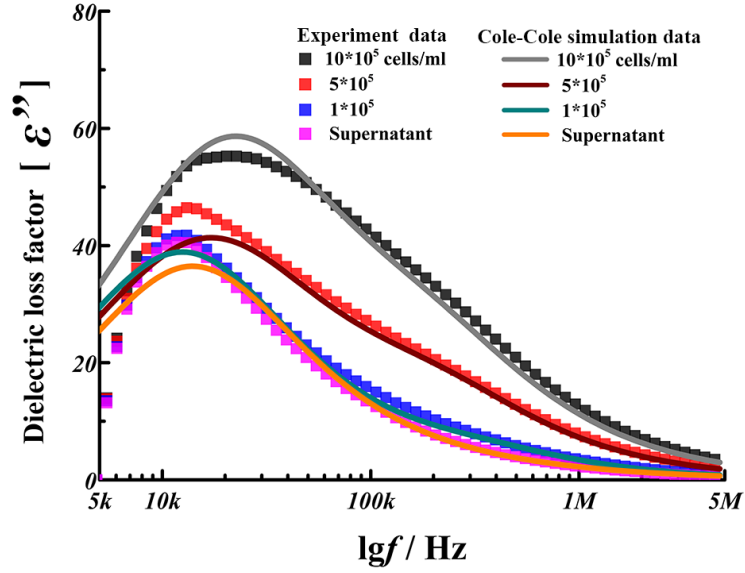


Fig. 3-6. Cole-Cole simulation on dielectric loss factor to the experimental data measured for the suspensions of *E. gracilis* SM-ZK cells in three concentration cell densities.

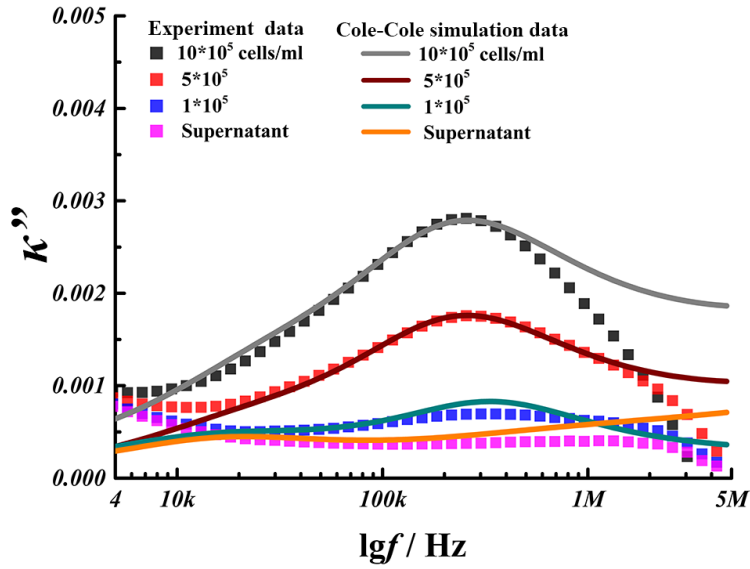


Fig. 3-7. Cole-Cole simulation on imaginary part of the complex conductivity to the experimental data measured for the suspensions of *E. gracilis* SM-ZK cells in three concentration cell densities.

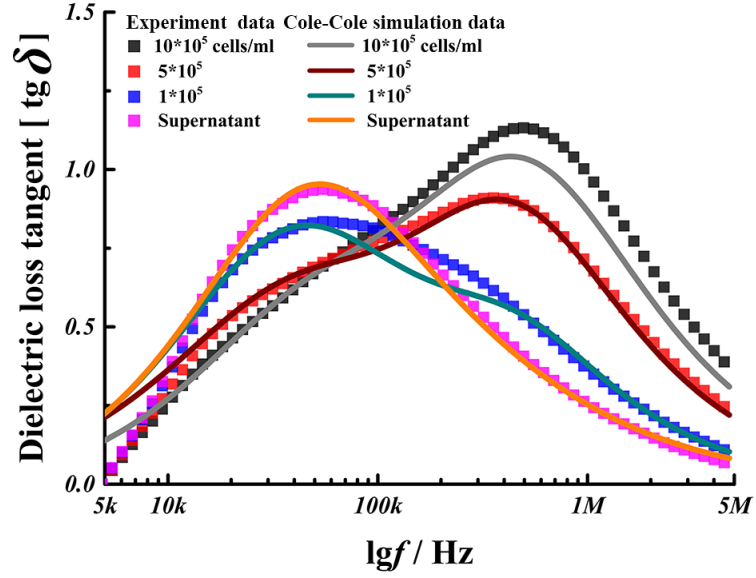


Fig. 3-8. Cole-Cole simulation on loss tangent to the experimental data measured for the suspensions of *E. gracilis* SM-ZK cells in three concentration cell densities.

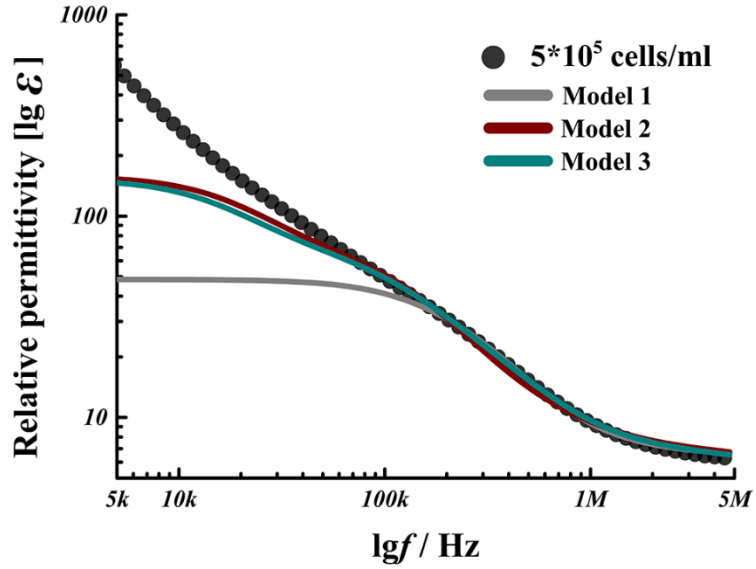


Fig. 3-9. Simulation of frequency dependence of relative permittivity of the cell suspension of *E. gracilis* (5×10^5 cells/ml) with three different dielectric models.

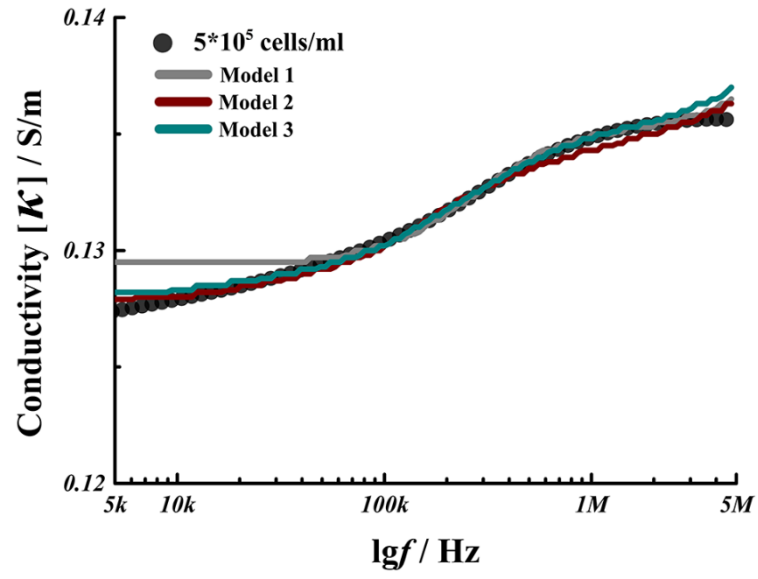


Fig. 3-10. Simulation of frequency dependence of conductivity of the cell suspension of *E. gracilis* (5×10^5 cells/ml) with three different dielectric models.

Chapter 4: Dielectric response of *Euglena gracilis* to chemical stimulation as a possible tool for monitoring water quality

4.1. Introduction

Identification of toxic substances in various samples, such as drinking water or food specimens, is exceedingly important, but rapid and sensitive analytical methods by using chemical procedures require time-consuming and labor-intensive sample preparations (Thomas et al., 1999). Therefore, biomonitoring approaches have been developed to rapidly identify toxicity using living organisms such as microorganisms (Woutersen et al, 2011), animals (Green et al., 2003; Kang et al., 2009), unicellular algae (Millán de Kuhn et al., 2006), and protists (Apostol, 1973) as biomonitors. Although biomonitoring allows an integrated evaluation of cumulative effects of various contaminants, it is not possible to identify the name of chemical substances contained in the polluted water. Tahedl and Häder (Tahedl & Häder, 1999, 2001) reported an approach for monitoring water quality by using the motile unicellular flagellate *Euglena gracilis* as a monitoring organism. They developed an elaborate system to determine six different movement parameters including motility, swimming velocity, and gravitactic orientation, and showed that different chemicals affect different parameters (Tahedl & Häder, 1999). *E. gracilis* is suitable as a biomonitor organism, since 1) methods for axenic mass cultivation has been established (Ruiz et al, 2004), 2) free-swimming motility ensures a long-lasting homogeneous cell suspension that is required for stable measurement, and 3) the cells have a uniform and symmetrical morphology that changes in response to various environmental factors (Suzaki & Williamson, 1986). Dielectric spectroscopy is a non-invasive technique by which multiple electrical and morphological parameters of the living cells in suspension can be obtained over a wide frequency range (Asami et al., 1976, 1989). In this paper, we

have applied this method to *E. gracilis*, as an attempt to evaluate the possibility that different multiple parameters obtained by this technique might be useful to quickly unveil the chemical composition of unknown water samples.

4.2. Material and methods

4.2.1. Cells

Euglena gracilis SM-ZK strain was used as in the previous chapters, and cell suspension was prepared as described at 5×10^5 cells/ml.

4.2.2. Dielectric measurement

The dielectric measurement system was almost same as described before, but for avoiding sedimentation of *Euglena* cells that is likely to be seen as a cause of unstable data acquisition, we designed a perfusion system with a peristaltic pump (AC-2120, ATTO, Japan), in which the 3-ml cell suspension was circulated continuously through the measuring chamber at a rate of 4.2 ml/min (Fig. 4-1).

4.2.3. Transmission electron microscopy

TEM was applied for ultrastructure investigation after toxic chemicals treated *E. gracilis* cells were centrifuged at 1,000 rpm for 5 min. Cells were pre-fixation in 2.5% glutaraldehyde (Electron Microscopy Sciences, US) solution with 50 mM cacodylate buffer (pH 7.0), and suspended well for 30 seconds in room temperature; then, centrifugation was carried out in 1,000 rpm of 5 min, collected the pellet contrasted with 2% OsO₄ (Heraeus, Germany) for 30min. After resin and serial dehydration with ethanol solutions, *Euglena* cells were embedded in Spurr resin (Polysciences, Inc., US) at 70°C

for 8 hrs. 70 nm ultrathin sections were collected on one slot grid mesh (Nisshin EM, Japan) by a Leica EM UC7 ultra-microtome. Then stained with EM Stainer (Nisshin EM, Japan) and 38 mM lead citrate (Nacalai, Japan) for 10 min, respectively. Investigation was carried on H-7100 electron microscopy (HITACHI, Japan) and images was taken by C4742-95 digital camera (HAMAMATSU, Japan).

4.2.3. Theoretical analysis

To correlate changes in dielectric behaviors of the *E. gracilis* cell suspension with possible changes in electrical parameters of the cell, a theoretical analysis was carried out with a “single-shell model” as described in Chapter 3 by using a computer program (Asami, 2002b; M. Watanabe et al., 1991).

4.3. Results

4.3.1. Effects of toxic chemicals to the dielectric behavior

Three different membrane-affecting chemicals were used to compare their effects on the dielectric behavior of *E. gracilis* cells in suspension. As shown in Fig. 4-2, all chemicals showed an inhibitory effect on cell swimming, and induced rounding up of the cell. Each chemical, however, showed different effects on both relative permittivity and conductivity of the cell suspension as shown in Fig. 4-6, Fig. 4-7 and Fig. 4-8. Chlorpromazine reduced the permittivity at around 100 kHz range, which indicates that the dielectric dispersion caused by the cells was reduced (Fig. 4-6A). On the contrary, conductivity of the cell suspension was increased over the whole range of frequencies, especially in the lower frequencies. HgCl_2 induced only a little elevation in permittivity

(Fig. 4-7A), while it strongly suppressed the conductivity of the cell suspension in the whole frequency range (Fig. 4-7B). The effect of Triton X-100 was different from either that of chlorpromazine or HgCl₂. It reduced the permittivity of the cell suspension as in the chlorpromazine-treated cells (Fig. 4-8A), but the conductivity decreased in the whole range, especially in higher frequencies (Fig. 4-8B). The results were summarized in Table 4-1, showing that different chemicals provoked different effects on the dielectric behavior of cell suspension, even though morphological and motility changes are similar.

4.3.2. *Euglena* cells response to toxic chemicals in TEM

Transmission electron microscopy was applied for investigating the intracellular morphology alternation of *E. gracilis* after toxic chemicals treated. Fig. 4-3, Fig. 4-4 and Fig. 4-5 show the morphological changes of *E. gracilis* cells in the plasma membrane, mitochondria and the nuclear membrane, respectively. As shown in Fig. 4-1 A-D, no obvious differences were detected in the pellicular membranes after treatment with the toxic chemicals. However, in mitochondria and the nuclear membranes, the membrane structures were deteriorated when the cells were treated with chlorpromazine or Triton X-100. On the contrary, mitochondrial and nuclear membranes looked intact in the specimens treated with HgCl₂ (Fig. 4-3C and 4-5C).

4.3.3. Theoretical consideration

To understand the causes of changes in dielectric behavior of *E. gracilis* cells in suspension, *in silico* analysis was performed with a computer program developed for simulating the dielectric behavior of living cells. As shown in Fig. 4-9, the theory is based on an electrical model in which a conducting cytoplasm phase is enclosed by a thin less-conductive plasma membrane. Although it is an over-simplified model, it was

successfully applied to explain the dielectric behavior of the living cells such as human erythrocytes (Irimajiri et al., 1996). When the conductivity of the outer medium (κ_a) was changed, vertical parallel displacement of the curve occurred (dashed dotted lines in Fig. 4-9). A rise in κ_m resulted in the elevation of the conductivity of the suspension only at lower frequencies (broken lines in Fig. 4-9). On the contrary, when smaller κ_i was used, the curve shifted downward but only at higher frequencies (dotted lines in Fig. 4-9).

4.4. Discussion

Dielectric spectroscopy is a non-invasive technology that provides a rapid and useful way of biological research to analyze electrical properties of living cell suspensions (Asami et al., 1976, 1989) and tissues (Nopp et al., 1997; Schmid et al., 2003; Wang et al., 2009), and also to monitor the growth of cultured cells (Matanguihan et al., 1994). In this study, we observed the dielectric behavior of *E. gracilis* in suspension in the range of frequency between 1 kHz and 5 MHz for detecting possible changes due to the presence of toxic chemical compounds in the surrounding medium. Characteristic changes in the dielectric behavior were induced by chlorpromazine, HgCl_2 , and Triton X-100, which indicate that the effect of different chemicals might have been distinguished by different dielectric compartments of the cell.

As demonstrated in Fig. 4-9, increase in conductivity by chlorpromazine may be explained by the raise in conductivity of the extracellular medium (κ_a) by a possible leaking of intracellular electrolytes toward outside, as theoretically predicted by the alternate long and short dash line in Fig. 4-9, curve B. The larger increase in conductivity

at lower frequencies as compared with those at higher frequencies might be ascribed to the additional increase of κ_m , which is also predicted to occur as shown in Fig. 4-9, curve C. Chlorpromazine is a membrane-affecting positively-charged molecule that has an effect to enhance permeability to water (Fang & Iwasa, 2007) and various electrolytes (Seeman et al., 1970; Wilson et al., 1983). Although plasma membrane damage was not observed, deterioration in mitochondria and nuclear membranes leaked the contents into cytoplasm enhanced the ion density in cytoplasm, which is probably the reason of conductivity raise in Fig. 4-6B. These effects of this drug are in good agreement with the apparent dielectric changes observed in the present study.

The main cellular target of inorganic mercury is also regarded as the plasma membrane, by influencing transport of water and electrolytes (Ballatori et al., 1988; Patel & Markx, 2008). The cells ultrastructure did not show any obvious damage in Fig. 4-3C, Fig. 4-4C and Fig. 4-5C, however, the decrease in conductivity by the treatment with HgCl_2 as shown in Fig. 4-7B may be the result of decrease in the medium conductivity (κ_a), but an alternative explanation may be the possible increase in the volume fraction of the cells with concomitant decrease in the cytoplasmic conductivity (κ_i), as shown in Fig. 4-9, curve E. At this moment, it is difficult to distinguish these two possible mechanisms, and strong inhibitory effect of mercury to various cellular enzymes should also be considered (Ballatori et al., 1988).

Triton X-100 is a nonionic detergent that is probably the most commonly used permeabilization agent for biological researches. It has a strong effect on the living cell membranes by destructing the compactness and integrity of the lipid membranes (Koley & Bard, 2010). The decrease in conductivity of the cell suspension as shown in Fig. 4-8B, especially notable in the high frequency range, may be related with the damages on the

mitochondrial (Fig. 4-4D) and the nuclear membranes (Fig. 4-5D) Such deterioration of the cellular organelles might have caused a release of the contents into the cytoplasm and eventually also toward the cell exterior, which may explain a decrease of the extracellular conductivity (κ_a) with concomitant decrease in the intracellular conductivity (κ_i) as shown in Fig. 4-9, curve D.

Table 4-1 Effects of exposure to toxic substances on morphology, swimming motility, and dielectric parameters of *E. gracilis* in suspension.

	Chlorpromazine (1 mM)	HgCl₂ (1 mM)	Triton X-100 (0.1%)
Cell shape	partially rounded	rounded-up	partially rounded
Swimming	inhibited	inhibited	inhibited
Relative permittivity of cell suspension	decreased	slightly increased	decreased
Conductivity of cell suspension	increased	decreased	decreased

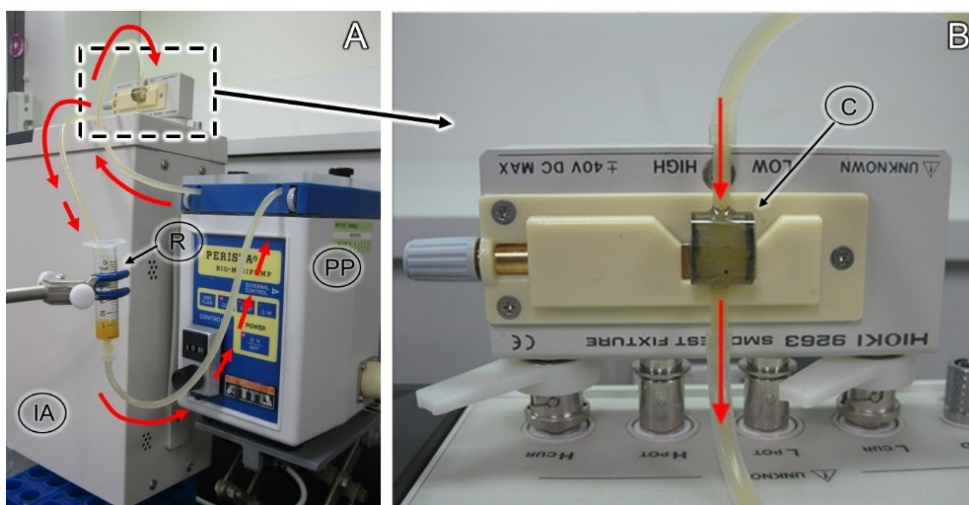


Fig. 4-1. A micrograph of the dielectric measurement system (A) and an enlarged picture of the measuring chamber on a fixture connected to the impedance analyzer (B). The system was composed of a parallel-plate capacitor type measuring chamber (C) connected to an impedance analyzer (IA) and a perfusion system with a peristaltic pump (PP). The sample cell suspension was circulated continuously through the measuring chamber in a direction shown with arrows. A reservoir (R) was placed in the flow passage, to which test chemicals were introduced.

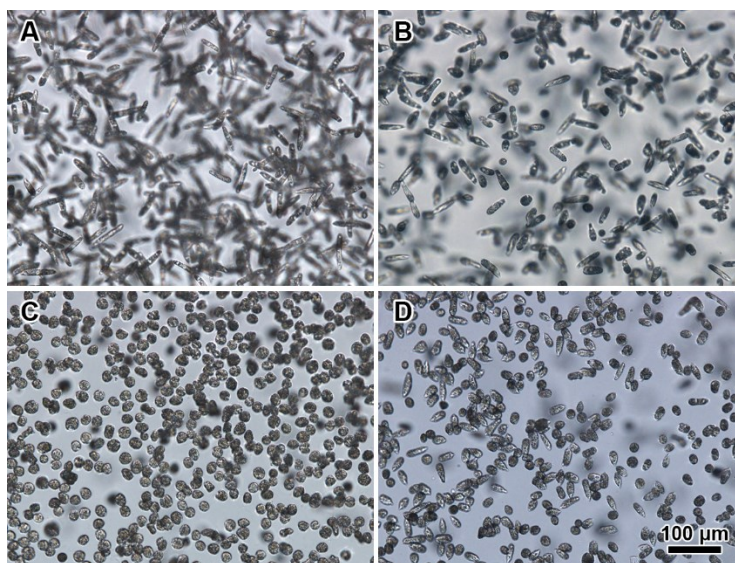


Fig. 4-2. Light micrographs of *E. gracilis* showing control cells (A), cells treated with 1 mM chlorpromazine (B), 1 mM HgCl₂ (C) , and 0.1% Triton X-100 (D). Swimming motions of the cells ceased by the additions of these chemicals, and the cells became rounded-up completely by either chlorpromazine or HgCl₂, or partially rounded by Triton X-100. Pictures in B-D were taken 5 min after the addition of the chemicals.

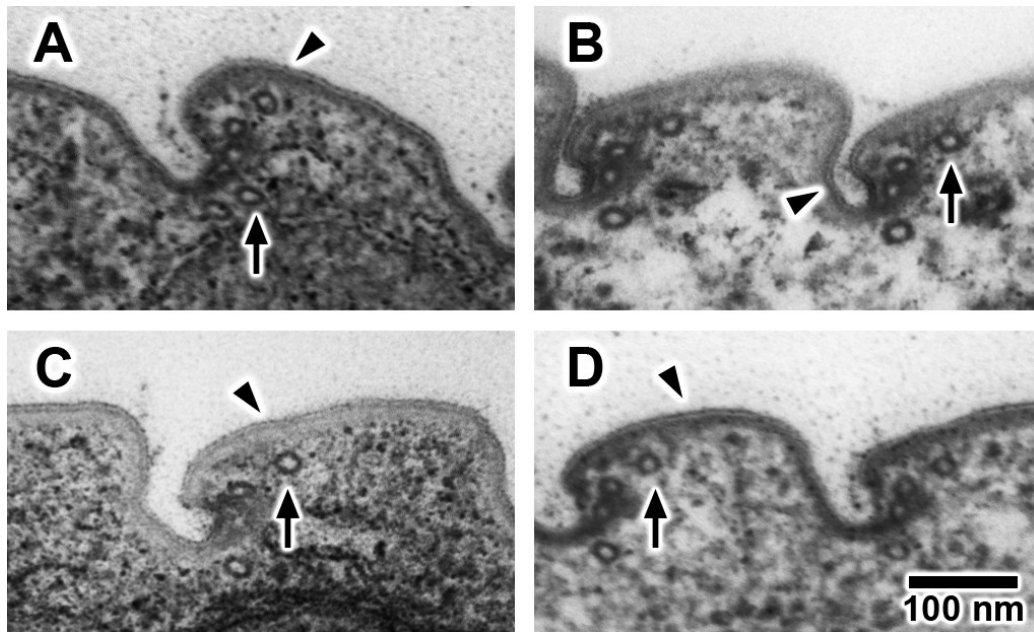


Fig. 4-3. Transmission electron microscopy images of the plasma membranes of *E. gracilis*, showing control cells (A), cells treated with 1 mM chlorpromazine (B), 1 mM HgCl₂ (C), and 0.1% Triton X-100 (D). No obvious damages were observed in the plasma membrane, with intact unit membrane structures (arrowheads) and microtubules (arrows).

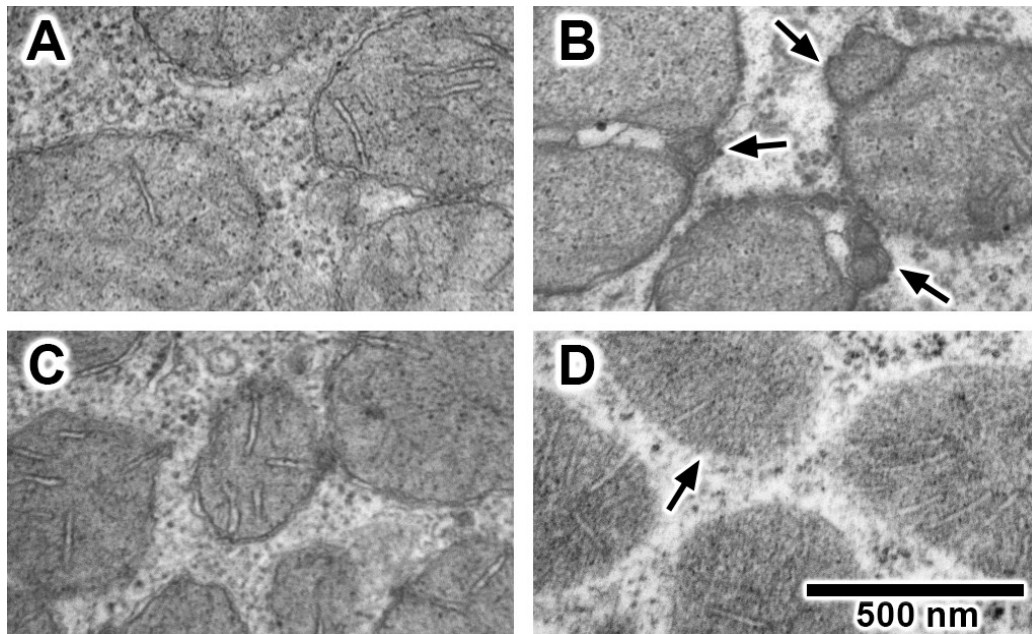


Fig. 4-4. Transmission electron microscopy images of *E. gracilis* mitochondria, showing a control cell (A), cells treated with 1 mM chlorpromazine (B), 1 mM HgCl₂ (C), and 0.1% Triton X-100 (D). Mitochondria in B were deformation and membranes appears to be fused (arrows). No significant alternation was observed in the HgCl₂-treated cell. In the Trion X-100-treated cell, the mitochondria membrane was completely missing (arrow).

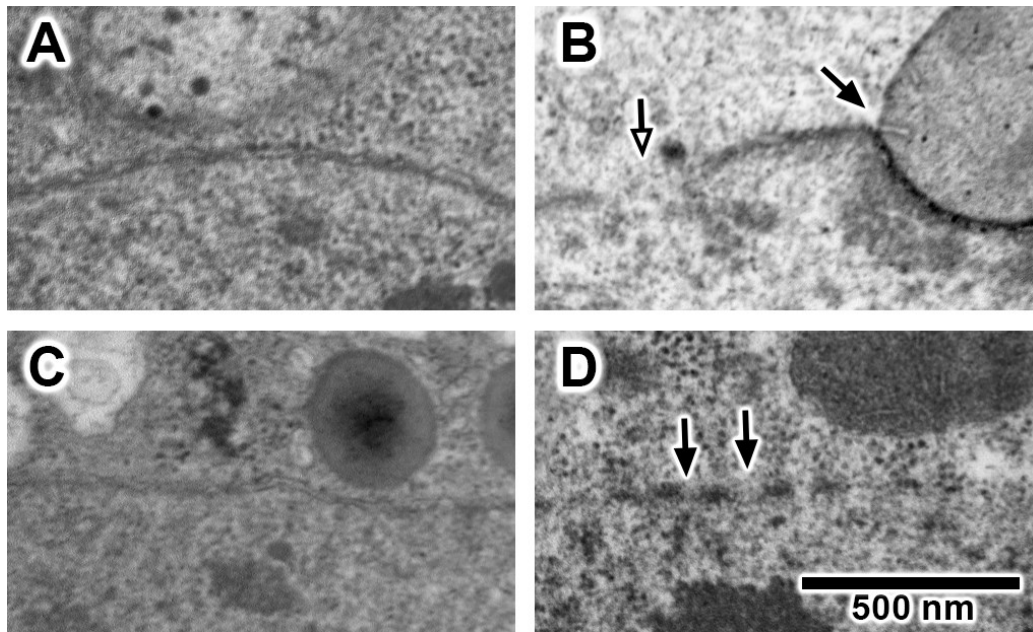


Fig. 4-5. Transmission electron microscopy images of *E. gracilis*, showing the nuclear membranes in a control cell (A), cells treated with 1 mM chlorpromazine (B), 1 mM HgCl₂ (C), and 0.1% Triton X-100 (D). In the chlorpromazine-treated cell, the nuclear membrane was not seen as a continuous structure, showing a drastic breakage of the membrane (open arrow). Fusion or tight attachment was also observed between a mitochondrion and the nuclear membrane (arrow). An apparently intact nuclear membrane structure was observed even after treatment with HgCl₂ (C). In Triton X-100-treated cells, a discontinuity of the nuclear membrane was observed with wide openings as shown with arrows in D.

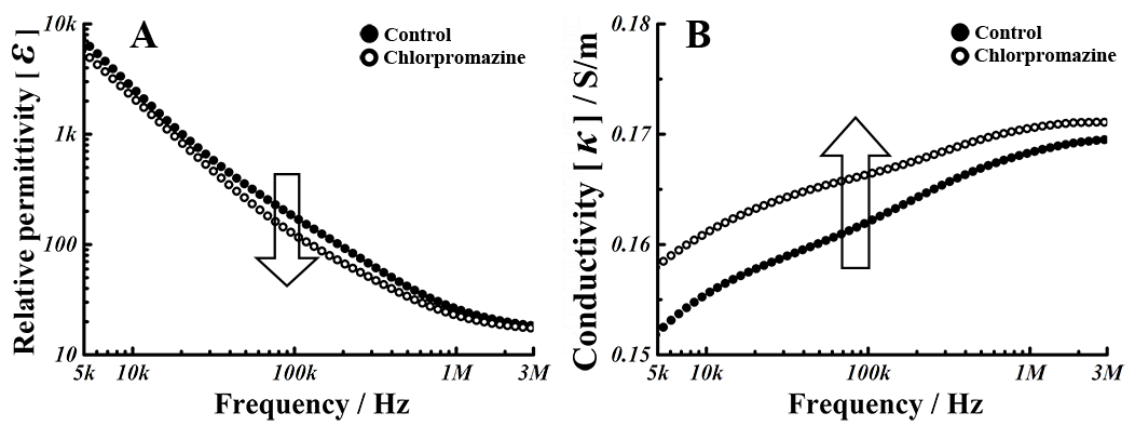


Fig. 4-6. Frequency dependence of relative permittivity ϵ (A) and conductivity κ (B) of *E. gracilis* in suspension. Control cells (closed circles) and those measured about 5 min after the addition of chlorpromazine at the final concentration of 1 mM (closed circles) were shown. Arrows indicate changing direction induced by the addition of the drug.

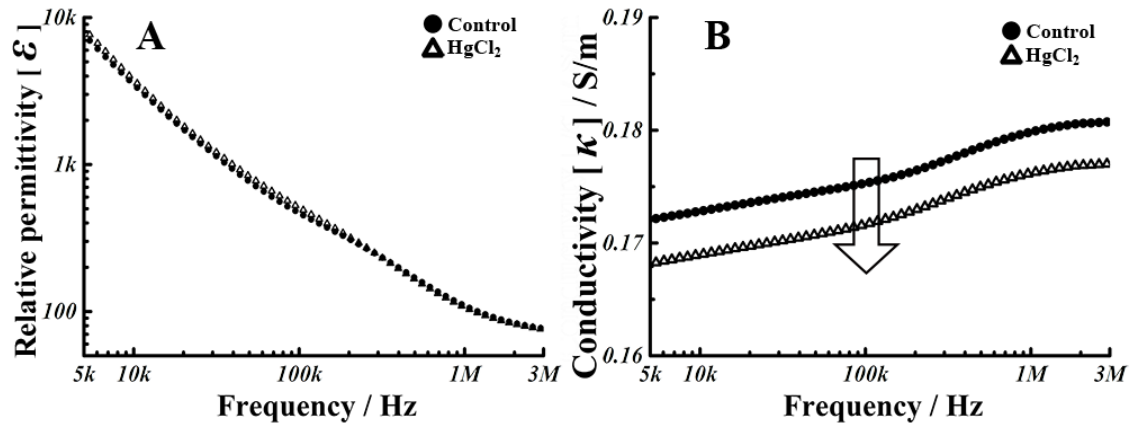


Fig. 4-7. Frequency dependence of relative permittivity ϵ (A) and conductivity κ (B) of *E. gracilis* in suspension. Control cells (closed circles) and those measured about 5 min after the application of HgCl₂ at the final concentration of 1 mM (open triangles) were shown. An arrow indicates changing direction induced by the treatment. Only a slight increase in relative permittivity was observed, while the conductivity was decreased in the whole range of frequencies.

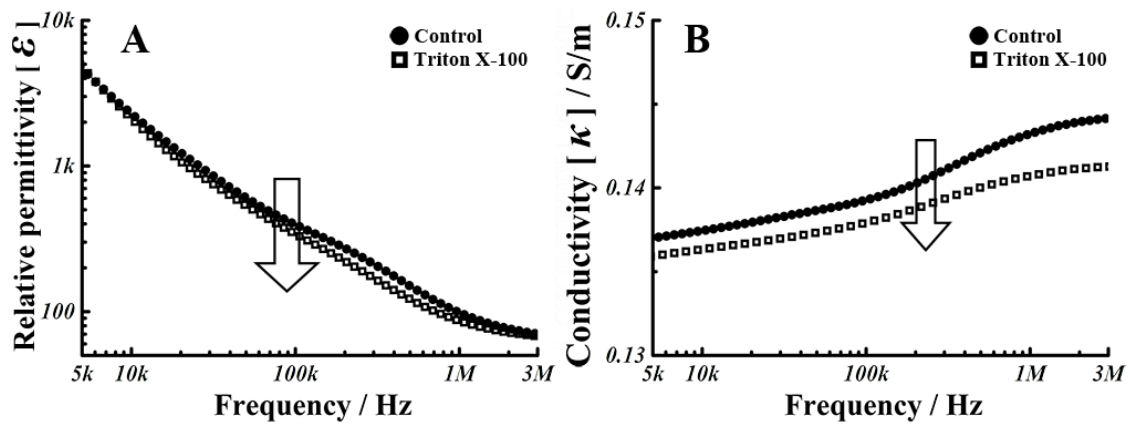


Fig. 4-8. Frequency dependence of relative permittivity ϵ (A) and conductivity κ (B) of *E. gracilis* in suspension. Control cells (closed circles) and those measured about 5 min after the application of Triton X-100 at the final concentration of 0.1% (closed circles) were shown. Arrows indicate changing directions induced by the treatment.

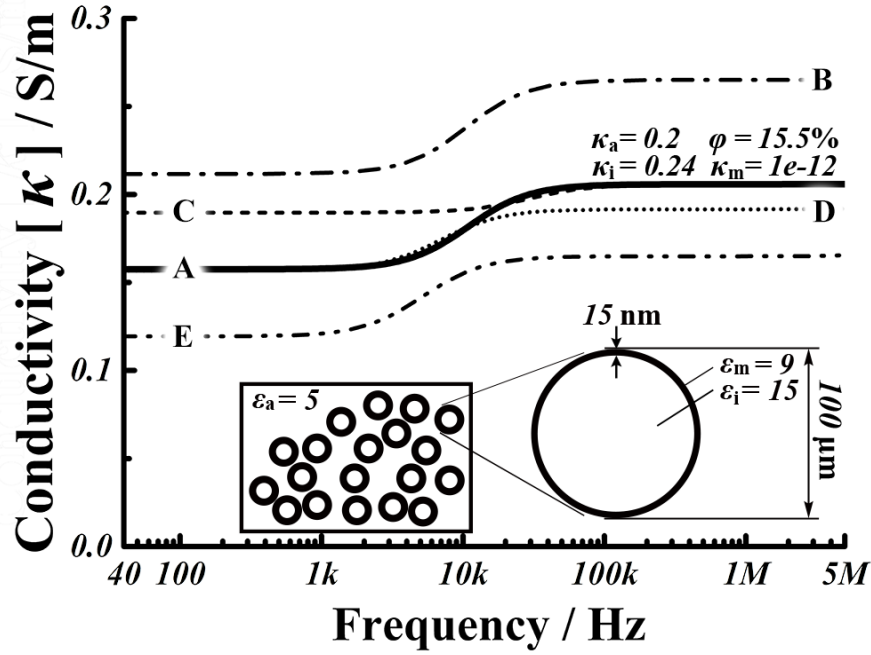


Fig. 4-9. Theoretical simulation of the dielectric behavior of *E. gracilis* cells in suspension with “single-shell” model. The theory is based on an electrical model in which a conducting homogeneous sphere (*ie*, the cytoplasm) is enclosed by a thin less-conductive layer (*ie*, the plasma membrane). As shown in the inset, the model consists of shelled spheres (100 μ m in diameter with a shell thickness of 15 nm), and the phase parameters (relative permittivity ϵ and conductivity κ) are represented with the subscripts (a, extracellular medium; m, plasma membrane, i; inner phase of cell). The solid curve (A) was obtained with a set of phase parameters $\kappa_a = 0.20$ S/m, $\kappa_m = 1 \times 10^{-12}$ S/m, $\kappa_i = 0.24$ S/m, $\epsilon_a = 5$, $\epsilon_m = 9$, $\epsilon_i = 15$, and the volume fraction of the spheres occupied in the medium (ϕ) was 15.5%. For obtaining other curves, different values for κ_a , κ_m , κ_i , or ϕ were substituted for the above set of values, while other parameters remained unchanged. Namely, in B, κ_a was raised to 0.20 S/m, which resulted in the overall raise of conductivity at all frequencies. The increase in κ_m (1×10^{-4} S/m) resulted in an increase of suspension conductivity only in the low frequency range (C), and the decrease in κ_i (0.15 S/m) resulted in a decrease in suspension conductivity only in the high frequency range (D). An overall decrease in suspension conductivity (E) was achieved with a smaller value for κ_a (0.15 S/m), while exactly the same curve was also obtained with a set of parameters ($\kappa_a = 0.10$ S/m, $\phi = 31\%$).

Chapter 5: Concluding remarks

Biomonitoring of water quality is essential for the safe supply of drinking water, which offers many advantages over the classical physico-chemical methods of analyses. They usually involve simple observations, and requires only short time, small money and space. However, biomonitoring has also disadvantages. They are not able to provide an exact figure of water quality parameters, and cannot pinpoint the exact cause of water quality problems. It is principally because biomonitoring techniques usually employ only a single parameter (swimming behavior, electromyographic response, etc.), although chemical nature of toxicant is highly complicated. In the present paper, we found different dielectric responses that are characteristic to three different toxic chemicals, even though they showed similar morphological and behavioral responses to *E. gracilis*. If we can make use of an adequate set of multiple dielectric parameters extracted from a wide frequency-range data, it should provide us with a good starting point to identify the toxicant.

References

- Ahmed, H., & Häder, D.-P. 2010. Monitoring of waste water samples using the ECOTOX biosystem and the flagellate alga *Euglena gracilis*. *Water, Air, & Soil Pollution*, 216(1-4), 547–560. doi:10.1007/s11270-010-0552-4
- Apostol, S. 1973. A bioassay of toxicity using protozoa in the study of aquatic environment pollution and its prevention. *Environmental Research*, 6(4), 365–372. doi:10.1016/0013-9351(73)90051-0
- Asami, K. 2002. (a). Characterization of biological cells by dielectric spectroscopy. *Journal of Non-Crystalline Solids*, 305(1-3), 268–277. doi:10.1016/S0022-3093(02)01110-9
- Asami, K. 2002. (b). Characterization of heterogeneous systems by dielectric spectroscopy. *Progress in Polymer Science*, 27(8), 1617–1659. doi:10.1016/S0079-6700(02)00015-1
- Asami, K., Hanai, T., & Koizumi, N. 1976. Dielectric properties of yeast cells. *The Journal of Membrane Biology*, 28(1), 169–180. doi:10.1007/BF01869695
- Asami, K., Hanai, T., & Koizumi, N. 1980. (a). Dielectric analysis of *Escherichia coli* suspensions in the light of the theory of interfacial polarization. *Biophysical Journal*, 31(2), 215–28. doi:10.1016/S0006-3495(80)85052-1
- Asami, K., Hanai, T., & Koizumi, N. 1980. (b). Dielectric approach to suspensions of ellipsoidal particles covered with a shell in particular reference to biological cells. *Jpn. J. Appl. Phys.*, 19(2), 359–365. doi:10.1143/JJAP.19.359
- Asami, K., Takahashi, Y., & Takashima, S. 1989. Dielectric properties of mouse lymphocytes and erythrocytes. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1010(1), 49–55. doi:10.1016/0167-4889(89)90183-3
- Asami, K., & Yonezawa, T. 1995. Dielectric behavior of non-spherical cells in culture. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1245(3), 317–324. doi:10.1016/0304-4165(95)00116-6
- Azizullah, A., Jamil, M., Richter, P., & Häder, D.-P. 2013. Fast bioassessment of wastewater and surface water quality using freshwater flagellate *Euglena gracilis*—a case study from Pakistan. *Journal of Applied Phycology*. doi:10.1007/s10811-013-0100-x

- Azizullah, A., Richter, P., Jamil, M., & Häder, D.-P. 2012. Chronic toxicity of a laundry detergent to the freshwater flagellate *Euglena gracilis*. *Ecotoxicology (London, England)*, 21(7), 1957–64. doi:10.1007/s10646-012-0930-3
- Ballatori, N., Shi, C., & Boyer, J. L. 1988. Altered plasma membrane ion permeability in mercury-induced cell injury: studies in hepatocytes of elasmobranch *Raja erinacea*. *Toxicology and Applied Pharmacology*, 95(2), 279–91. PMID:2844008
- Bordi, F., Cametti, C., & Gili, T. 2001. Reduction of the contribution of electrode polarization effects in the radiowave dielectric measurements of highly conductive biological cell suspensions. *Bioelectrochemistry (Amsterdam, Netherlands)*, 54(1), 53–61. PMID:11506975
- Bresadola, M. 1998. Medicine and science in the life of Luigi Galvani (1737-1798). *Brain Research Bulletin*, 46(5), 367–80. doi:10.1016/S0361-9230(98)00023-9
- Broadhurst, M. G., Chiang, C. K., Wahlstrand, K. J., Hill, R. M., Dissado, L. A., & Pugh, J. 1987. The dielectric properties of biological tissue (*Crassula portulacea*) from 10–2 to 109 Hz. *Journal of Molecular Liquids*, 36, 65–73. doi:10.1016/0167-7322(87)80031-4
- Buetow, D. E. 1982. *The Biology of Euglena: Physiology* (pp. 1–27). Academic Press.
- Buetow, D. E. 2011. *Euglena*. *eLS*. doi:10.1002/9780470015902.a0001964.pub3
- Campbell, A. M., & Land, D. V. 1992. Dielectric properties of female human breast tissue measured in vitro at 3.2 GHz. *Physics in Medicine and Biology*, 37(1), 193–210. doi:10.1088/0031-9155/37/1/014
- Cole, K. S. 1928. Electric impedance of suspensions of spheres. *The Journal of General Physiology*, 12(1), 29–36. PMID: 19872446
- Cole, K. S. 1935. Electric impedance of hipponoe eggs. *The Journal of General Physiology*, 18(6), 877–87. PMID: 19872897
- Cole, K. S. 1942. Dispersion and absorption in dielectrics II. direct current characteristics. *The Journal of Chemical Physics*, 10(2), 98. doi:10.1063/1.1723677
- Cole, K. S. 1968. Membranes, ions, and impulses. In *Classical Biophysics* (pp. 0–572). Berkeley: University of California Press.

- Cole, K. S., & Cole, R. H. 1936. Electric impedance of Arbacia eggs. *The Journal of General Physiology*, 19(4), 625–32. PMID: 19872952
- Cole, K. S., & Cole, R. H. 1941. Dispersion and Absorption in Dielectrics I. Alternating Current Characteristics. *The Journal of Chemical Physics*, 9(4), 341. doi:10.1063/1.1750906
- Daiker, V., Lebert, M., Richter, P., & Häder, D.-P. 2010. Molecular characterization of a calmodulin involved in the signal transduction chain of gravitaxis in *Euglena gracilis*. *Planta*, 231(5), 1229–36. doi:10.1007/s00425-010-1126-9
- Dos Santos Ferreira, V., Rocchetta, I., Conforti, V., Bench, S., Feldman, R., & Levin, M. J. 2007. Gene expression patterns in *Euglena gracilis*: insights into the cellular response to environmental stress. *Gene*, 389(2), 136–45. doi:10.1016/j.gene.2006.10.023
- Fang, J., & Iwasa, K. H. 2007. Effects of chlorpromazine and trinitrophenol on the membrane motor of outer hair cells. *Biophysical Journal*, 93(5), 1809–17. doi:10.1529/biophysj.106.100834
- Finkelstein, G. 2006. Emil du Bois-Reymond vs Ludimar Hermann. *Comptes Rendus Biologies*, 329(5-6), 340–7. doi:10.1016/j.crv.2006.03.005
- Foster, K. R., Schepps, J. L., Stoy, R. D., & Schwan, H. P. 1979. Dielectric properties of brain tissue between 0.01 and 10 GHz. *Physics in Medicine and Biology*, 24(6), 1177–87. PMID: 531093
- Fricke, H. 1933. The electric impedance of suspensions of biological cells. *Cold Spring Harbor Symposia on Quantitative Biology*, 1, 117–124. doi:10.1101/SQB.1933.001.01.015
- Fricke, H., & Curtis, H. J. 1934. (a). Electric impedance of suspensions of yeast cells. *Nature*, 134(3377), 102–103. doi:10.1038/134102b0
- Fricke, H., & Curtis, H. J. 1934. (b). Specific resistance of the interior of the red blood corpuscle. *Nature*, 133(3365), 651–651. doi:10.1038/133651b0
- Fricke, H., & Curtis, H. J. 1935. Electric impedance of suspensions of leucocytes. *Nature*, 135(3411), 436–436. doi:10.1038/135436a0
- Gaïduk, V. I., & McConnell, J. R. 1997. *Dielectric Relaxation and Dynamics of Polar Molecules*. World Scientific.

- Galvani, L. 1791. De viribus electricitatis in motu musculari commentarius. In *De Bononiensi Scientiarum et Artium Instituto atque Academia Commentarii* (VII., pp. 363–418). Bononiae: Ex Typographia Instituti Scientiarum.
- Green, U., Kremer, J. H., Zillmer, M., & Moldaenke, C. 2003. Detection of chemical threat agents in drinking water by an early warning real-time biomonitor. *Environmental Toxicology*, 18(6), 368–74. doi:10.1002/tox.10138
- Grell, K. G. 1973. *Protozoology* (1st ed., pp. 372–375). Springer.
- Hayden, R. I., Moyse, C. A., Calder, F. W., Crawford, D. P., & Fensom, D. S. 1969. Electrical impedance studies on potato and alfalfa tissue. *Journal of Experimental Botany*, 20(2), 177–200. doi:10.1093/jxb/20.2.177
- Höber, R. 1910. Eine Methode, die elektrische Leitfähigkeit im Innern von Zellen zu messen. *Pflüger's Archiv Für Die Gesamte Physiologie Des Menschen Und Der Tiere*, 133(4-6), 237–253. doi:10.1007/BF01680330
- Höber, R. 1912. Ein zweites Verfahren, die Leitfähigkeit im Innern von Zellen zu messen. *Pflüger's Archiv Für Die Gesamte Physiologie Des Menschen Und Der Tiere*, 148(4-5), 189–221. doi:10.1007/BF01680784
- Hölzel, R. 2009. Dielectric and dielectrophoretic properties of DNA. *IET Nanobiotechnology / IET*, 3(2), 28–45. doi:10.1049/iet-nbt.2008.0014
- Ilic, B., Czaplewski, D., Neuzil, P., Stanczyk, T., Blough, J., & Maclay, G. J. 2000. Preparation and characterization of platinum black electrodes. *Journal of Materials Science*, 35(14), 3447–3457. doi:10.1023/A:1004884723515
- Inaba, A., Manabe, T., Tsuji, H., & Iwamoto, T. 1995. Electrical impedance analysis of tissue properties associated with ethylene induction by electric currents in cucumber (*Cucumis sativus* L.) Fruit. *Plant Physiology*, 107(1), 199–205. PMID: 12228354
- Irimajiri, A., Ando, M., Matsuoka, R., Ichinowatari, T., & Takeuchi, S. 1996. Dielectric monitoring of rouleaux formation in human whole blood: a feasibility study. *Biochimica et Biophysica Acta*, 1290(3), 207–9. PMID:8765121

- Kang, I. J., Moroishi, J., Nakamura, A., & Nagafuchi, K. 2009. Biological monitoring for detection of toxic chemicals in water by the swimming behavior of small freshwater fish. *Biological Monitoring of Water Quality*, 54(1), 209–214.
- Koley, D., & Bard, A. J. 2010. Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). *Proceedings of the National Academy of Sciences of the United States of America*, 107(39), 16783–7. doi:10.1073/pnas.1011614107
- Kyle, U. G., Bosaeus, I., De Lorenzo, A. D., Deurenberg, P., Elia, M., Gómez, J. M., ... Pichard, C. 2004. Bioelectrical impedance analysis--part I: review of principles and methods. *Clinical Nutrition (Edinburgh, Scotland)*, 23(5), 1226–43. doi:10.1016/j.clnu.2004.06.004
- Lee, J. J., Leedale, G. F., & Bradbury, P. (Eds.). 2000. *The illustrated Guide to the Protozoa* (2nd ed., p. 1137). Lawrence Kansas U.S.A: Wiley-Blackwell.
- Lonergan, T. A. 1985. Regulation of cell shape in *Euglena gracilis*. IV. Localization of actin, myosin and calmodulin. *Journal of Cell Science*, 77, 197–208. PMID:3936854
- Lu, Y., Li, B., Xu, J., & Yu, J. 1992. Dielectric properties of human glioma and surrounding tissue. *International Journal of Hyperthermia*, 8(6), 755–760. doi:10.3109/02656739209005023
- Ma, Q., Watanabe, M., & Suzuki, T. 2003. Dielectric spectroscopy (100Hz~100MHz) of frog skeletal muscles: a theoretical analysis with an ellipsoidal-shell model. *Acta biophysica sinica*, 19(3), 309–316.
- Matanguihan, R. M., Konstantinov, K. B., & Yoshida, T. 1994. Dielectric measurement to monitor the growth and the physiological states of biological cells. *Bioprocess Engineering*, 11(6), 213–222. doi:10.1007/BF00387695
- MEP of P.R.China. 2002. Environmental quality standards for surface water, Pub. L. No. GB3838-2002 (2002). P.R.China.
- MEP of P.R.China. 2005. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2006. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.

- MEP of P.R.China. 2007. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2008. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2009. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2010. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2011. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2012. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2013. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2014. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- MEP of P.R.China. 2015. Report on the State of the Environment in China. Ministry of Environmental Protection of the People's Republic of China.
- Millán de Kuhn, R., Streb, C., Breiter, R., Richter, P., Neesse, T., & Häder, D.-P. 2006. Screening for unicellular algae as possible bioassay organisms for monitoring marine water samples. *Water Research*, 40(14), 2695–703. doi:10.1016/j.watres.2006.04.045
- Nopp, P., Harris, N. D., Zhao, T.-X., & Brown, B. H. 1997. Model for the dielectric properties of human lung tissue against frequency and air content. *Medical & Biological Engineering & Computing*, 35(6), 695–702. doi:10.1007/BF02510980
- Oda, Y., Nakano, Y., & Kitaoka, S. 1982. Utilization and toxicity of exogenous amino acids in *Euglena gracilis*. *Microbiology*, 128(4), 853–858. doi:10.1099/00221287-128-4-853

- Patel, P., & Markx, G. H. 2008. Dielectric measurement of cell death. *Enzyme and Microbial Technology*, 43(7), 463–470. doi:10.1016/j.enzmictec.2008.09.005
- Peyman, A. 2012. Comments on “In vivo measurements of electrical conductivity of porcine organs at low frequency: new method of measurement” by J. Spottorno, M. Multigner, G. Rivero, L. Álvarez, J. de la Venta, and M. Santos. *Bioelectromagnetics*, 33(7), 620; discussion 620–1. doi:10.1002/bem.21746
- Peyman, A., & Gabriel, C. 2012. (a). Dielectric properties of porcine glands, gonads and body fluids. *Physics in Medicine and Biology*, 57(19), N339–44. doi:10.1088/0031-9155/57/19/N339
- Peyman, A., & Gabriel, C. 2012. (b). Dielectric properties of rat embryo and foetus as a function of gestation. *Physics in Medicine and Biology*, 57(8), 2103–16. doi:10.1088/0031-9155/57/8/2103
- Peyman, A., Gabriel, C., Benedickter, H. R., & Fröhlich, J. 2011. Dielectric properties of human placenta, umbilical cord and amniotic fluid. *Physics in Medicine and Biology*, 56(7), N93–8. doi:10.1088/0031-9155/56/7/N01
- Peyman, A., Holden, S. J., Watts, S., Perrott, R., & Gabriel, C. 2007. Dielectric properties of porcine cerebrospinal tissues at microwave frequencies: in vivo, in vitro and systematic variation with age. *Physics in Medicine and Biology*, 52(8), 2229–45. doi:10.1088/0031-9155/52/8/013
- Ruiz, L. B., Rocchetta, I., dos Santos Ferreira, V., & Conforti, V. 2004. Isolation, culture and characterization of a new strain of *Euglena gracilis*. *Phycological Research*, 52(2), 168–173. doi:10.1111/j.1440-183.2004.00338.x
- Schmid, G., Neubauer, G., & Mazal, P. R. 2003. Dielectric properties of human brain tissue measured less than 10 h postmortem at frequencies from 800 to 2450 MHz. *Bioelectromagnetics*, 24(6), 423–30. doi:10.1002/bem.10123
- Schwan, H. P. 1968. Electrode polarization impedance and measurements in biological materials. *Annals of the New York Academy of Sciences*, 148(1), 191–209. doi:10.1111/j.1749-6632.1968.tb20349.x
- Seeman, P., Sha’afi, R. I., Galey, W. R., & Solomon, A. K. 1970. The effect of anesthetics (chlorpromazine, ethanol) on erythrocyte permeability to water. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 211(2), 365–368. doi:10.1016/0005-2736(70)90111-2
- Smith, D. G. 2001. *Pennak’s Freshwater Invertebrates of the United States: Porifera to Crustacea*. (4, Ed.). John Wiley & Sons.

- Smith, S. R., Foster, K. R., & Wolf, G. L. 1986. Dielectric Properties of VX-2 Carcinoma Versus Normal Liver Tissue. *Biomedical Engineering, IEEE Transactions on, BME-33*(5), 522–524. doi:10.1109/TBME.1986.325740
- Stoneman, M. R., Kosempa, M., Gregory, W. D., Gregory, C. W., Marx, J. J., Mikkelsen, W., ... Raicu, V. 2007. Correction of electrode polarization contributions to the dielectric properties of normal and cancerous breast tissues at audio/radiofrequencies. *Physics in Medicine and Biology*, 52(22), 6589–604. doi:10.1088/0031-9155/52/22/003
- Suzaki, T., & Williamson, R. E. 1986. Cell surface displacement during euglenoid movement and its computer simulation. *Cell Motility and the Cytoskeleton*, 6(2), 186–192. doi:10.1002/cm.970060217
- Tahedl, H., & Häder, D. P. 2001. Automated biomonitoring using real time movement analysis of *Euglena gracilis*. *Ecotoxicology and Environmental Safety*, 48(2), 161–9. doi:10.1006/eesa.2000.2004
- Tahedl, H., & Häder, D.-P. 1999. Fast examination of water quality using the automatic biotest ECOTOX based on the movement behavior of a freshwater flagellate. *Water Research*, 33(2), 426–432. doi:10.1016/S0043-1354(98)00224-3
- Thomas, K. V., Thain, J. E., & Waldock, M. J. 1999. Identification of toxic substances in United Kingdom estuaries. *Environmental Toxicology and Chemistry*, 18(3), 401–411. doi:10.1002/etc.5620180306
- Trenfield, M. A., Ng, J. C., Noller, B., Markich, S. J., & van Dam, R. A. 2012. Dissolved organic carbon reduces uranium toxicity to the unicellular eukaryote *Euglena gracilis*. *Ecotoxicology (London, England)*, 21(4), 1013–23. doi:10.1007/s10646-012-0855-x
- Wang, L., Chen, L., Feng, M., & Ma, Q. 2009. Measurement of the Permittivity and Conductivity for 30 normal Blood Samples at Radio Frequency Range. *Beijing Biomedical Engineering*, 28(2), 143–143. doi:10.3969/j.issn.1002-3208.2009.02.08
- Wang, L., CHEN, L., & MA, Q. 2009. Impedance frequency properties of rabbit whole blood cells. *Journal of Zhejiang University(Medical Sciences)*, 38(4), 383–386.
- Watanabe, M., Suzaki, T., & Irimajiri, a. 1991. Dielectric behavior of the frog lens in the 100 Hz to 500 MHz range. Simulation with an allocated ellipsoidal-shells model. *Biophysical Journal*, 59(1), 139–49. doi:10.1016/S0006-3495(91)82206-8

- Watanabe, M., & Suzuki, T. 2001. Cadmium-induced abnormality in strains of *Euglena gracilis*: morphological alteration and its prevention by zinc and cyanocobalamin. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 130(1), 29–39. doi:10.1016/S1532-0456(01)00225-3
- Wilson, C. C., Delamere, N. A., & Paterson, C. A. 1983. Chlorpromazine effects upon rabbit lens water and electrolyte balance. *Experimental Eye Research*, 36(4), 559–65. PMID:6303824
- Woutersen, M., Belkin, S., Brouwer, B., van Wezel, A. P., & Heringa, M. B. 2011. Are luminescent bacteria suitable for online detection and monitoring of toxic compounds in drinking water and its sources? *Analytical and Bioanalytical Chemistry*, 400(4), 915–29. doi:10.1007/s00216-010-4372-6
- Yong-peng, Z., Lin, W., & Qing, M. 2006. Measuring the dielectric spectra of rat skeletal muscle cells. *Journal of ningbo university(natural science & engineering edition)*, 19(3), 418–420. doi:10.3969/j.issn.1001-5132.2006.03.030
- Zhang, M. I. N., Repo, T., Willison, J. H. M., & Sutinen, S. 1995. Electrical impedance analysis in plant tissues: on the biological meaning of Cole-Cole α in Scots pine needles. *European Biophysics Journal*, 24(2), 99–106. doi:10.1007/BF00211405
- 吴舜泽, 夏青, & 刘鸡亮. 2000. 中国流域水污染分析. 环境科学与技术, 89(2), 1–6.

Appendix

A part of this dissertation was published as a paper as shown below:

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