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ARTICLE

Robert Y. Wang · Tiantian Zhang · Qiuyang Bao
David M. Rawson

Study on fish embryo responses to the treatment of cryoprotective chemicals using impedance spectroscopy

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Abstract Investigations using electrical impedance spectroscopy to measure the responses of fish embryos to the cryoprotective chemicals, methanol and dimethyl sulphoxide (DMSO), were carried out. Zebrafish (*Danio rerio*) embryos were used as a model to study the newly proposed technique. The normalised permittivity and conductivity changes of the embryos were measured continuously over a 20-min period in a customised embryo-holding chamber. The normalised permittivity and conductivity spectra were obtained during embryo exposure to different concentrations of methanol (1.0, 2.0 and 3.0 M) and DMSO (0.5, 1.0 and 2.0 M) solutions. The results showed significant permittivity and conductivity changes after embryo exposure to methanol and DMSO at the optimum embryo loading level (six embryos). Embryos in different concentrations of methanol and DMSO also resulted in quantitative responses shown in the normalised permittivity and conductivity spectra. The results demonstrated that fish embryo membrane permeability to cryoprotective chemicals could be monitored in real-time. The measurement of permittivity at a lower frequency range (10–10^3 Hz) and conductivity at a higher frequency range (10^4–10^6 Hz) during fish embryo exposure to cryoprotective chemicals using impedance spectroscopy can be used as a new tool for the fast screening of most effective cryoprotective chemicals. The results from the present study also demonstrated the possibility of quantifying the level of cryoprotective chemicals penetrating the fish embryos.

R. Y. Wang · T. Zhang (✉) · Q. Bao · D. M. Rawson
Luton Institute of Research in the Applied Natural Sciences (LIRANS), University of Luton, The Spires, 2 Adelaide Street, Luton, Bedfordshire, LU1 5DU, UK
E-mail: tiantian.zhang@luton.ac.uk
Tel.: +44-1582-743729
Fax: +44-1582-743701

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Introduction

Electrical impedance spectroscopy (EIS) is a widely used technique for measuring electrochemical phenomenon on solid, porous, synthetic and biological materials. EIS measures the electrical properties of biological materials, i.e. the conductivity and permittivity as a function of applied alternative current (AC) frequency. For many years, the technique has been used to determine the impedance properties of normal and damaged biological tissues (Schwan 1957; Ackmann and Seitz 1984; Foster and Schwan 1989; Gabriel et al. 1996). McRae et al. (1999) and Paulsen et al. (1999) demonstrated the application of the technique as a non-invasive method for measuring the damaged tissues in human tumours. Applications of the technique in membrane studies were also reported by Moreno and Valiente (1996) and Wiegand (2000) for non-invasive measurement of biological membranes during ionic transportation processes. Wiegand (2000) studied a supported bilayer focussing on their electrical properties using EIS. In this work, the electrical characteristics of the lipid bilayers were studied with a number of structural and environment variables. The technique was also used for the evaluation of the incorporation kinetics and selective conductivity of ion channels of membranes (Benavente and Ramos-Barrado 1997).

Although considerable progress has been made in cryopreservation of embryos of mammalian species over the last two decades, successful cryopreservation of fish embryos has not been achieved and low fish embryo membrane permeability to water and cryoprotectants has been identified as one of the major obstacles to their successful cryopreservation (Zhang 2004). Information on membrane permeability is vital for the successful

cryopreservation of fish embryos. Obtaining accurate information on cryoprotectant penetration into fish embryos and selecting the most effective cryoprotective chemicals is important in designing optimum cryopreservation protocols.

At present, a conventional volumetric measurement-based technique is used in fish embryo membrane permeability studies (Zhang and Rawson 1996, 1998). This method is inherently difficult with zebrafish embryos because a high percentage of the embryo volume is osmotically inactive (Zhang and Rawson 1998), and volumetric changes during exposure to both permeating and non-permeating solutes are therefore small (Hagedorn et al. 1997). Also the embryos are only approximately spherical, which makes accurate estimations of volume changes difficult and the method is lengthy, reducing the capacity for multi-embryo measurement. Although some new techniques have been applied in fish embryo membrane permeability studies including nuclear magnetic resonance (NMR) spectroscopy (Hagedorn et al. 1996, 1997), these methods do not provide real-time or quantitative information on cryoprotectant penetration into the embryos.

The complex nature of fish embryos requires the development of a new membrane permeability assessment method. An alternative approach is to measure the electrical impedance response of fish embryos during their exposure to cryoprotective chemicals that could provide important information on embryo membrane permeability. A direct correlation between the complex dielectric response, which includes the permittivity and conductivity, and cell membrane potential at low frequencies in living cell suspensions has been reported by Prodan et al. (1999, 2004). The work provides a possible approach to study embryo membrane permeability using EIS technique. At an optimal frequency it would also be possible to correlate the changes of permittivity and conductivity values with embryo responses to different concentrations of cryoprotective chemicals, thereby providing quantitative information on cryoprotectant penetration into the embryos. The objectives of the present study were (1) to assess the sensitivity of the EIS technique in studying zebrafish embryo membrane permeability; (2) to investigate permisttivity and conductivity spectra responses to different concentrations of the selected cryoprotective chemicals.

Materials and methods

Instrumentation

Permittivity and conductivity spectra were recorded using Solartron SI1260 Impedance/Gain-Phase Analyser (Novocontrol Technologies, Germany) with the impedance measurement range from 10 mΩ to 100 MΩ (accuracy 0.1% and 0.1°), AC amplitude range 0.01–3.0 V rms, a resolution in loss factor <0.4 mrad and frequency from 10 μHz to 32 MHz. Six parameters including dielectric constant (permittivity), specific conductivity, serial/parallel impedance, capacity and tan (*d*), temperature and actual time are measured by the instrument. In this study, the AC output voltage of the system was set at 1.0 V rms for current measurement across the frequency range $10–10^6$ Hz with 12 logarithmic sweeping points per decade. An embryo-holding chamber was designed and made from a gold-plated brass BDS 1,200 sample cell (Novocontrol Technologies, Germany), which contains an embryo-holding chamber and a cover lid. The holding chamber is placed between the two parallel electrodes in the analyser. The embryo holding chamber was made with a diameter of 5.0 mm and 0.7 mm in depth, which can hold up to ten embryos (Fig. 1). A thin Teflon isolative ring was placed on top of the embryo holding chamber for preventing unwanted contact between the chamber and cover lid. Geometry of the embryo-holding chamber was specially designed for intact zebrafish embryos, which are approximately 1.0 mm in diameter. When the embryos are loaded into the chamber, the outer membrane (chorion) of the embryo should be in contact with both sides of the holding chamber that are linked to the system electrodes.

Zebrafish breeding and embryo collection

Adult zebrafish were held in a closed re-circulating system in 12-l tanks with 8–12 fishes per tank. The temperature of the system water (deionised water containing 250 mg/l ocean salt) was maintained at 28°C and the photoperiod was fixed at 14 h light: 10 h dark. Zebrafish were fed three times daily with TetraMin flake food (Tetra, Germany) and newly hatched brine shrimps.

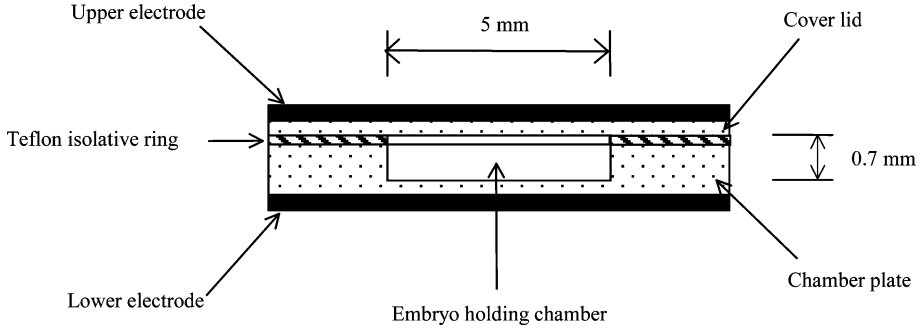

Fig. 1 Schematic diagram of the embryo-holding chamber and connections with the system electrodes

Embryos were collected from breeding trays each morning and maintained in embryo medium (60 μg/ml ocean salt) at 28°C until 50% epiboly stage. Intact embryos at 50% epiboly stage were used in this study. The developmental stage of the embryos was examined using a laboratory microscope (Lecia, UK). All embryos used in the experiments were kept in the embryo medium for at least 1 h before use.

Studies of permittivity and conductivity spectra during embryos' exposure to methanol and dimethyl sulphoxide

Embryo medium was prepared by dissolving 60 μg/ml ocean salt (ZM SYSTEMS Ltd, UK) in deionised water shortly before use. Two commonly used cryoprotective chemicals, methanol (Sigma, UK) and dimethyl sulfoxide (DMSO, Sigma, UK), were selected for the experiments. Different concentrations of methanol at 1.0, 2.0 and 3.0 M and DMSO at 0.5, 1.0 and 2.0 M were made up with the embryo medium. For each experiment, six embryos were loaded into the holding chamber with a pipette. Six embryos were used because this loading level was confirmed as the optimum loading level from the previous study (Zhang et al. 2004). The excess solutions were removed using tissue paper. For cryoprotectant-treated embryos, the embryo medium in the holding chamber was carefully removed with tissue paper and replaced with the medium containing cryoprotectant. The holding chamber was then quickly covered with the lid and secured between the two electrodes. A continue sweep was set for 20 min at 1-min intervals. All experiments were run under room temperature at 23 ± 1°C. The holding chamber was carefully cleaned and recalibrated with standard KCl solutions before each experiment. The permittivity data at each frequency were normalised by subtracting $t = 0$ data from the subsequent data points. The absolute values of the subtracted data were used to present the changing trends over the time and between the different concentrations at 5 min after starting the experiment. The data normalisation is essential in order to extract the information on permittivity change, which is caused solely due to cryoprotectant penetration to embryo membrane, and to minimise the impact of the polarisation effect on the electrodes. The presented data of both normalised permittivity and conductivity were an average of ten runs. Where measurements are quoted numerically, then the standard deviations (SD) are indicated by the '±' values. The SD is shown by a bar in graphs.

Results and discussion

Changes of permittivity and conductivity during embryo exposure to methanol and DMSO over time

The normalised permittivity spectra of six embryos loaded in embryo medium, 2.0 M methanol and 2.0 M DMSO over 20 min are presented in Figs. 2, 3 and 4, respectively. In the experiment with embryo medium (Fig. 2), a clear increasing trend in the normalised permittivity spectra was obtained within 10 min in the frequency range of 10–10^2 Hz. At 10 Hz, the values were increased from 772.5 ± 38.47 (SD) to 3349.3 ± 207.99 (SD) and from 616.4 ± 30.7 (SD) to 1687.11 ± 104.77 (SD) for 10^2 Hz. Relatively small increases or unchanged values were obtained between 10^3 Hz and 10^6 Hz. The normalised permittivity values remained relatively steady after 10 min of the experiment. The same changing patterns were also recorded in both methanol and DMSO experiments. In the experiment with methanol and DMSO, the normalised permittivity values were increased from 10.28 ± 1.34 (SD) to 23.72 ± 2.61(SD) at 10 Hz (Fig. 3) and 29.83 ± 2.42 (SD) to 63.9 ± 7.03 (SD) for DMSO (Fig. 4), which shows a higher degree of increase of normalised permittivity then methanol.

For methanol- and DMSO-treated embryos, the increases in the normalised permittivity within the first 10 min could be caused by the water, ion and cryoprotective chemical exchanges from both sides of the membrane, the equilibrium was then reached, which was reflected by the measured steady permittivity between 10 and 20 min. The differences in the normalised permittivity spectra between the untreated embryos (in embryo medium) and treated embryos (in both 2.0 M methanol and 2.0 M DMSO) reflected different levels of ion strength in embryo medium and cryoprotectant solutions, and embryo membrane permeability to the cryoprotective chemicals. The untreated embryos show the largest normalised permittivity increases in the first 10 min in comparison with methanol- and DMSO-treated embryos. This is mainly caused by the presence

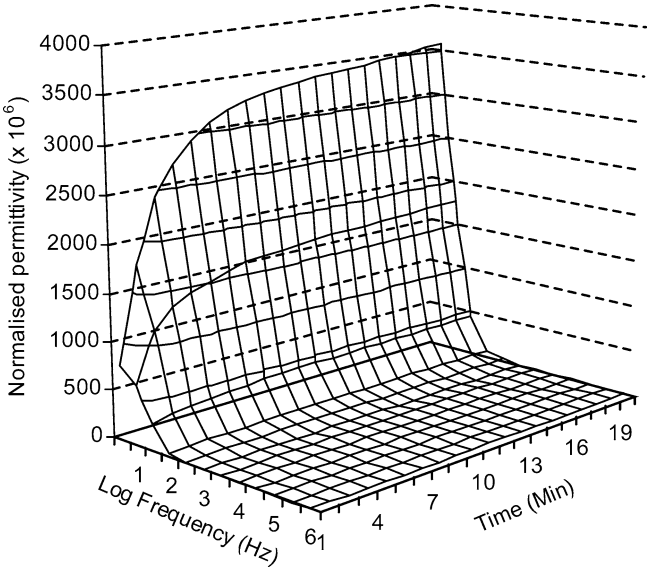

Fig. 2 Three-dimensional presentation of the normalised permittivity on the measured six embryos over 20 min in the given frequency range of 10–10^6 Hz in embryo medium

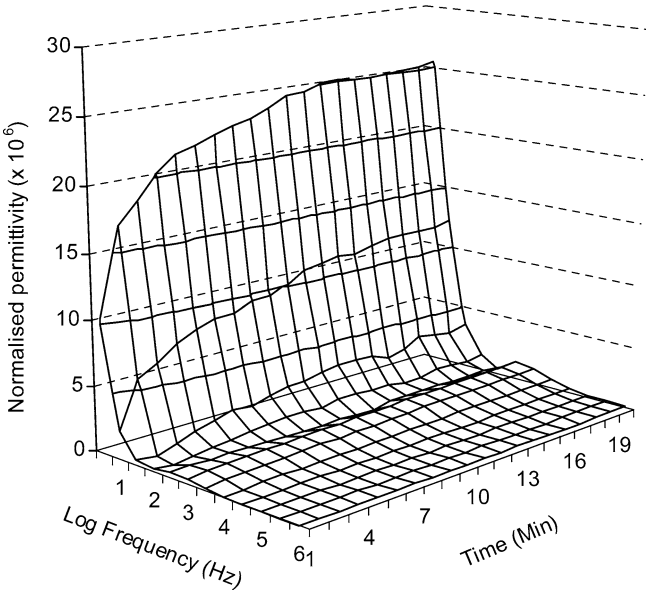

Fig. 3 Three-dimensional presentation of the normalised permittivity on the measured six embryos over 20 min in the given frequency range of 10–10^6 Hz in 2.0 M methanol

of stronger ionic properties in both intra-embryonic fluid and the extra-embryonic media in the untreated embryos than those of the 2.0 M methanol- and DMSO-treated embryos. The normalised permittivity values measured from 2.0 M methanol exposure experiment were lower than those obtained with 2.0 M DMSO, which could be explained by the movements of more methanol molecules than DMSO to the intra-embryonic fluid of the treated embryos. This demonstrated that zebrafish embryo chorion is more permeable to methanol than DMSO. The normalised permittivity spectrum shows that more methanol molecules penetrated into the embryos and changed the ionic properties of the intra-embryonic fluid. These results are in agreement with the results obtained by Zhang and Rawson (1996) using a conventional volumetric measurement-based technique. Despa (1995) also demonstrated that the value of the dielectric permittivity at low frequencies decreased (increased in normalised permittivity) gradually with the increase of membrane permeability for ions, while the electrical permittivity at high frequencies was unchanged. The effect was considered especially important for analysing the dielectric spectrum of a tissue or membrane that has undergone the influence of various chemical agents.

Biological membranes play an important role in establishing the resting and active electric properties through regulation of the movement of ions between the extracellular and intracellular environments (Alcaraz et al. 2001). During cryoprotectant treatment, embryos shrank initially due to water losses of the intra-embryonic fluid, and then re-expanded indicating cryoprotectant penetration (Mazur 2004). The embryo membrane permeability to different cryoprotective chemicals was reflected by different embryo shrink-swell profiles in different cryoprotective chemical solutions (Zhang and Rawson 1996). The results from the present study demonstrate that the processes of osmosis and cryoprotectant penetration can be measured by EIS technique. The permittivity spectra also reflected the differences in embryo membrane permeability to different cryoprotective chemicals. The results are in good agreement with the studies of Asami (1977) and Despa (1995), which demonstrated that the electric permittivity at low frequencies decreases gradually with the increase in membrane permeability to ions.

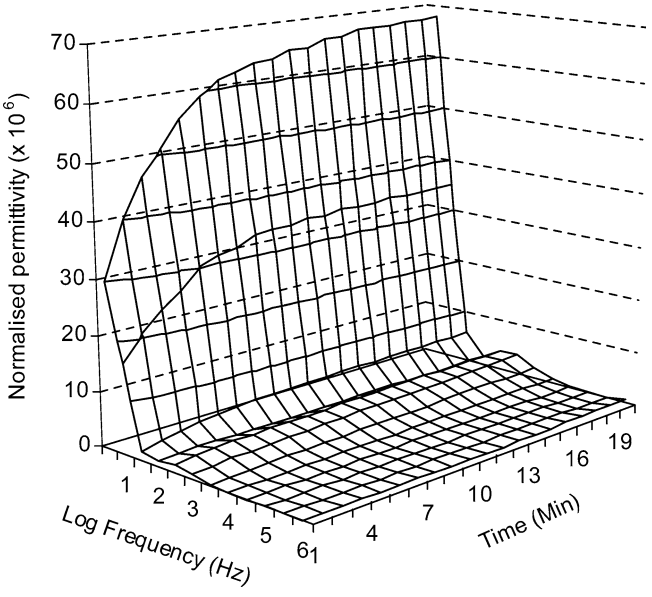

Fig. 4 Three-dimensional presentation of the normalised permittivity on the measured six embryos over 20 min in the given frequency range of 10–10^6 Hz in 2.0 M DMSO

Changes of permittivity and conductivity during embryo exposure to different concentrations of methanol and DMSO

The normalised embryo permittivity responses to the treatment of different concentrations of methanol and DMSO at 5 min of the experiments are presented in Figs. 5 and 6. In general, the permittivity spectra of embryos in methanol were approximately tenfold lower than the permittivity measured from the untreated embryos in the low frequency range (10–10^3 Hz; Fig. 5), demonstrating the direct impact of methanol on permittivity. The spectra measured from the methanol-treated embryos showed that in the frequency range 10–10^3 Hz normalised permittivity values decreased with decreasing concentrations of methanol. Similar trends were also recorded for embryos treated with 0.5, 1.0 and 2.0 M DMSO solutions (Fig. 6). The results showed that the amount of methanol and DMSO penetration into the embryos also depended on the concentrations of the cryoprotectants. Embryos exposed to higher concentrations of both methanol and

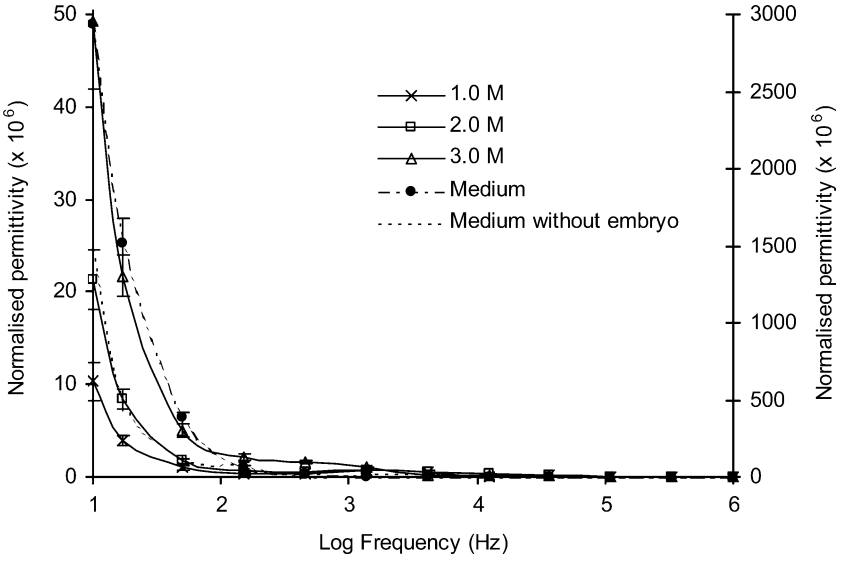

Fig. 5 Effect of different concentrations of methanol on normalised permittivity change after 5 min exposure to concentrations of 1.0, 2.0 and 3.0 M (*solid lines*, left-hand axis versus frequency). Six embryos were exposed to the methanol in the frequency range of 10–10^6 Hz. The reference spectra of the medium with or without embryos are presented with *dotted* and *dashed lines* (right-hand axis versus frequency)

DMSO had larger permittivity value changes in the frequency range of 10–10^3 Hz. The results also showed that methanol-treated embryos had larger permittivity changes than DMSO. The permittivity spectra responses to different concentrations of methanol and DMSO demonstrated that the changes of the intra-embryonic fluid caused by the penetrated methanol or DMSO can be measured by the EIS technique and it is clearly sensitive to commonly used concentration levels of cryoprotectants in cryobiological studies.

When embryos were treated with cryoprotectants, the properties of the bathing embryo medium were also changed due to exosmosis of the intra-embryonic water. These changes were revealed by the recorded conductivity spectra in the frequency range of 10^4–10^6 Hz for both methanol- and DMSO-treated embryos (Figs. 7, 8). Conductivity values decreased with increasing concentrations of methanol and DMSO-treated embryos indicating changes of ionic levels of the bathing embryo medium.

The electrical behaviour of biological tissues reveals a high frequency dependence of the dielectric parameters due to the various relaxation phenomena that occur when the current passes through the tissue (Schwan 1988). When the frequency increases, the conductivity rises. The increase in conductivity is associated with a decrease in permittivity (Rigaud et al. 1996). The changing trends and relationship between the measured conductivity and permittivity are clearly shown with both methanol- and DMSO-treated embryos. At low frequencies, the charge time is sufficiently slow to allow the charging and discharging of the membrane to take place in a single period, which induces high permittivity. When the frequencies are increased, the membrane capacitive reactance decreases, which induces an increase in the current flow passing through the intercellular medium and, therefore, an increase in the conductivity. The increase in frequency also prevents the membrane from being completely charged during a

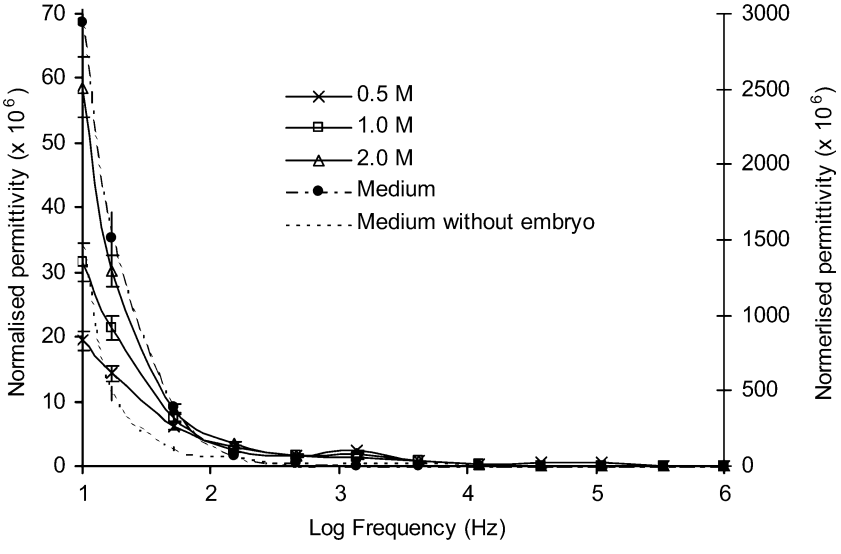

Fig 6 Effect of different concentrations of DMSO on normalised permittivity change after 5 min exposure to concentrations of 0.5, 1.0 and 2.0 M (*solid lines*, left-hand axis versus frequency). Six embryos were exposed to the DMSO in the frequency range of 10–10^6 Hz. The reference spectra of the medium with or without embryos are presented with *dotted* and *dashed lines* (right-hand axis versus frequency)

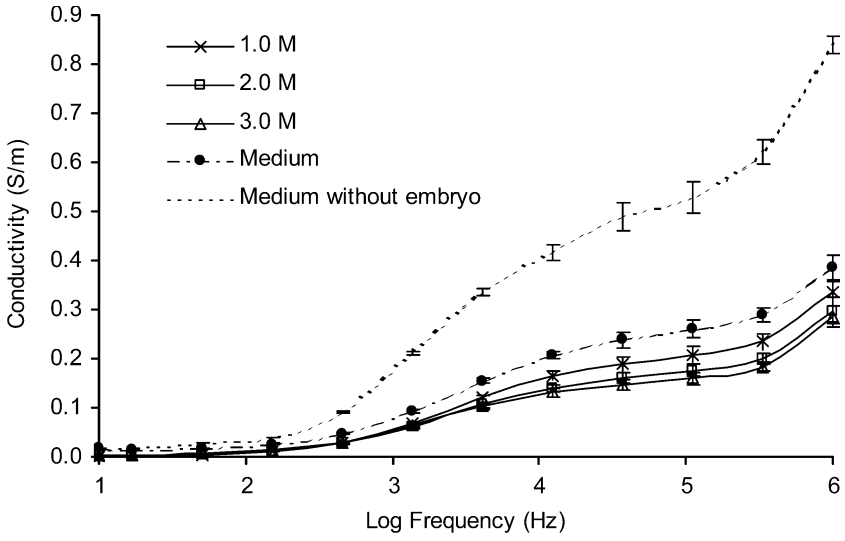

Fig. 7 Effect of different concentrations of methanol on conductivity change after 5 min exposure to concentrations of 1.0, 2.0 and 3.0 M. Six embryos were exposed to the methanol in the frequency range of 10–10^6 Hz

complete cycle, thus causing a decrease in permittivity (Rigaud et al. 1996).

Conclusions

To our knowledge, the EIS technique has never been explored and reported for fish embryo membrane permeability studies. The results presented in this study indicate that the EIS technique is clearly sensitive to methanol and DMSO penetration during the treatment of cryoprotective chemicals. The permittivity and conductivity changes appeared to be frequency-dependent. In the lower frequency range (10–10^4 Hz), permittivity values increases with the increasing concentrations of cryoprotectants, which can be explained as the increasing of cryoprotective chemical compounds in the intra-embryonic fluid. However, the conductivity spectrum has shown a clear decreasing trend with increasing concentrations of the cryoprotective chemicals at the higher frequencies of 10^4–10^6 Hz, resulting from the reduced cryoprotective chemical concentration and the fractional increase of intra-embryonic water in the bathing embryo medium.

The changing trends of normalised permittivity over time in the frequency range of 10–10^4 Hz agree well with the conventional volumetric measurement-based (Zhang and Rawson 1996), which indicated that the significant penetration of the cryoprotective chemicals into the embryos occurs after 3–10 min of exposure to methanol and DMSO. However, EIS method is faster and can monitor changes in both the intra- and the extra-embryonic bathing medium at the same time.

The results obtained from the present study on permittivity and conductivity changes are encouraging for the development of an EIS technique for use in embryo membrane permeability studies. The technique is especially useful for the selection of the suitable cryoprotective chemicals and may also allow quantitative measurements in embryo membrane permeability studies.

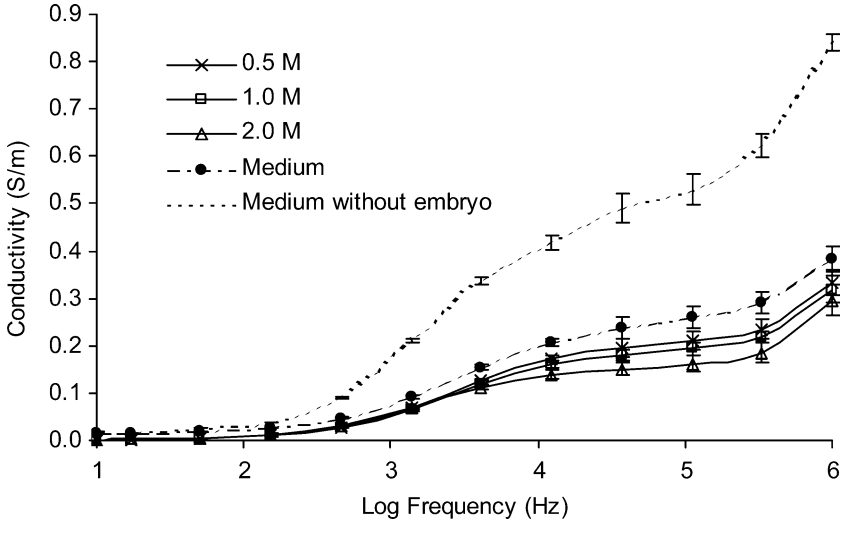

Fig. 8 Effect of different concentrations of DMSO on conductivity change after 5 min exposure to concentrations of 1.0, 2.0 and 3.0 M. Six embryos were exposed to the DMSO in the frequency range of 10–10^6 Hz

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