

Fig. 7. Experimental results of vibration amplitude and piezoelectric output from (a) Ch. 6, (b) Ch. 12 and (c) Ch. 18 at various frequencies in air.

relatively large outputs. The specific frequency is defined as LRF of electrode. LRF decreases as the channel number increases, i.e., as the position  $x$  increases. Extremal amplitudes at other frequencies of LRF may indicate the effect of standing waves. That is, in case of the electrode locates on the antinode of standing wave, the amplitude from the electrode increases. On the other hand, in case of the electrode locates on the node, the amplitude decreases. As reported in Refs. [7,8,17], pretension in the membrane may be a main reason for the standing wave which results in the multiple-peaks as shown in Fig. 7. However, it is difficult to precisely control the pretension in our fabrication process. Therefore, the mechanism of this result remained to be solved in this study. The frequency dependences of vibration and the piezoelectric output are qualitatively similar to each other. The reason of their similarity can be explained as follows. Since ABM is relatively narrow in  $y$  direction compared with that in the  $x$  direction, the vibration is mainly effected by the boundary conditions at  $y = \pm b(x)/2$ . As the result of that, the piezoelectric output is dominated by the ABM's local structural strain in  $y$  direction. Furthermore, the piezoelectric constant in  $y$  direction is larger than that in  $x$  direction. This may make the strong dependence of piezoelectric output on the strain in  $y$  direction.

Fig. 8 shows the relationship between LRF  $f_r$  and position  $x$ . Circles are LRF which are determined by the vibration and the

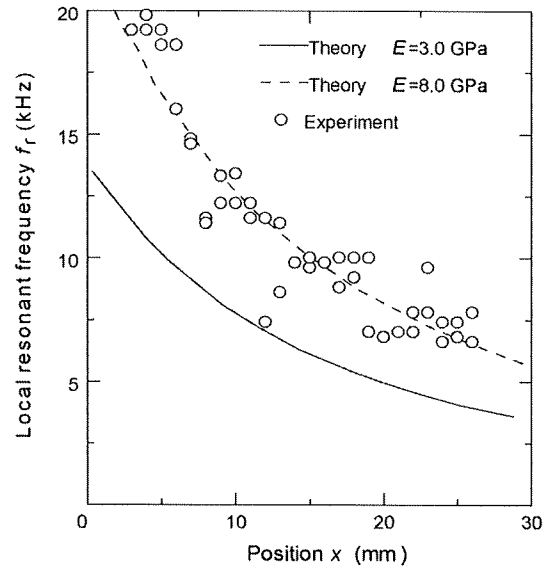


Fig. 8. Comparison of theoretically and experimentally obtained results of local resonant frequency  $f_r$  in air.

piezoelectric output. LRF decreases from 19.8 to 6.6 kHz as  $x$  increases. This experimental LRF is in qualitative agreement with the theoretical prediction of Eq. (16) which is drawn by the solid line. In the quantitative comparison, however, almost all experimental results are slightly higher than the predictions. One of possible reasons is the underestimation of  $E$ . Since  $E$  from literatures widely distributes as 3.0–11.0 GPa [18,19], we used a reference value of 3.0 GPa for the prediction. If we use higher value of 8.0 GPa, the precision is improved as shown by the broken line in Fig. 8.

Fig. 9(a) shows the relationship between the external sound pressure and the amplitude of vibration in ABM. This investigation is conducted at LRF of each electrode. To show the results over the range of 60–90 dB SPL, those are drawn in the log-dB SPL plot. From the gradient of results, it is found that the amplitude of vibration at LRF linearly increases with the sound pressure. The amplitude increases with the channel number, that is, with ABM's width. Fig. 9(b) shows the relationship between the sound pressure and the amplitude of piezoelectric output. The amplitude of piezoelectric output also shows the linear relationship with the sound pressure. These results suggest that the device can detect not only the frequency of acoustic wave but also the magnitude of it.

### 3.2. Performance test in liquid environment

The performance of device in the liquid environment is investigated by filling the fluid channel with the silicone oil with the

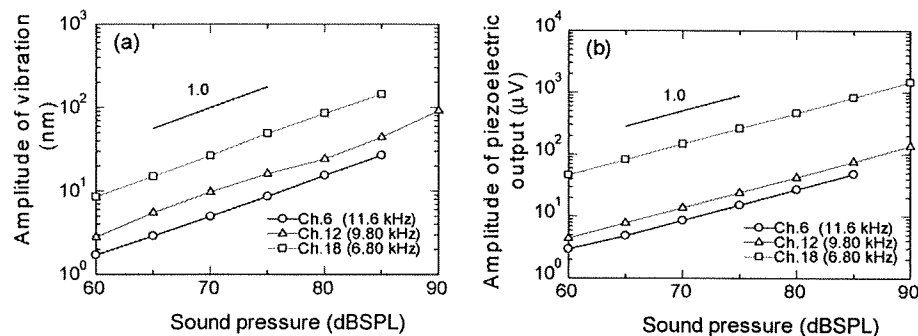


Fig. 9. Experimental results in effect of sound pressure on amplitudes (a) of vibration and (b) of piezoelectric output in air.

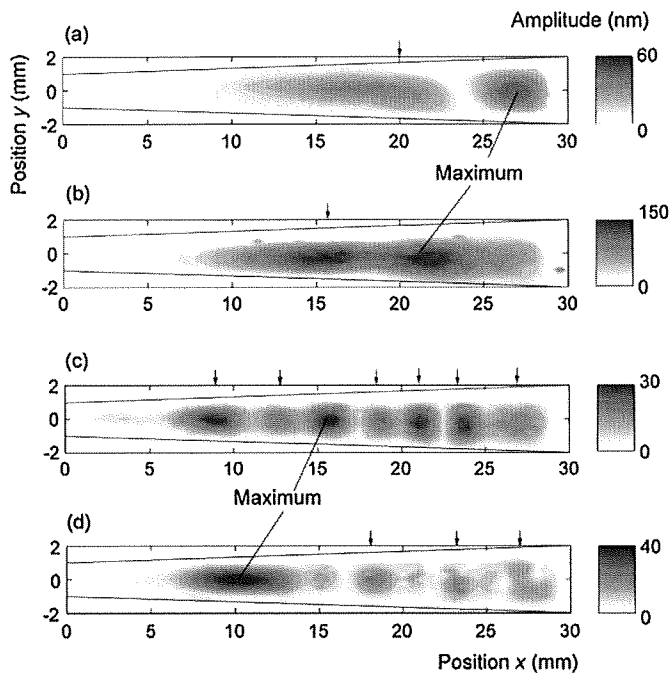


Fig. 10. Experimental results of contour maps of vibration amplitude at (a)  $f=1.5$  kHz, (b) 2.0 kHz, (c) 3.0 kHz and (d) 4.0 kHz in silicone oil of  $1.75 \times 10^{-3}$  Pa s.

viscosity of  $1.75 \times 10^{-3}$  Pa s. This investigation is intended to test the applicability of device for implanting into the cochlea. Since the outputs from ABM are relatively small compared with that in the air environment, the sound pressure is increased to be 85 dB SPL for this experiment. This decrease of outputs in the silicone oil may be caused by energy dissipation in liquid environment due to the viscosity and the stronger fluid-structure interaction of ABM due to the density, however, the detailed mechanism has not been clarified. Fig. 10 shows the contour maps of amplitude distribution at (a)  $f=1.5$  kHz, (b) 2.0 kHz, (c) 3.0 kHz and (d) 4.0 kHz, respectively. The qualitative frequency dependence of vibration is similar to that in the air environment. That is, the location with the maximum amplitude is shifted to the smaller  $x$  as the frequency increases. However, the frequency ranges where ABM shows peak amplitude in the silicone oil are lower than that in the air environment. Comparing Fig. 6(a) and Fig. 10(d), the effect of surrounding fluid can be discussed in detail. In spite of driving the device at the same frequency of 4.0 kHz, these results clearly show the different vibration behavior. It is found that the maximum amplitude is found at the smaller  $x$  in the silicone oil compared with that in the air environment. This difference may be caused by the fluid-structure interaction as discussed in the earlier section. That is, compared with the result in the air environment, the effective mass for the vibration is increased in the silicone oil. As a result, the place with maximum amplitude is shifted to the smaller  $x$  in the silicone oil at the same frequency. Furthermore, the effects of standing wave in the silicone oil which are indicated by arrows seem relatively large compared with that in the air environment. This fact is predicted by the theoretical analysis that the resonance is relaxed due to the surrounding liquid as shown in Fig. 3.

Fig. 11 shows the frequency dependences of vibration and piezoelectric output, where they are indicated by a solid line and the broken one, respectively. As same with those in the air environment, the two amplitudes show the similar tendency to each other. The amplitudes have peaks at each specific frequency which is also described as LRF for convenience. LRF of Ch. 6, Ch. 12, and Ch. 18 are obtained as 3.64, 2.32, and 1.88 kHz, respectively. From Fig. 11, it is confirmed that the ABM's frequency selectivity is success-

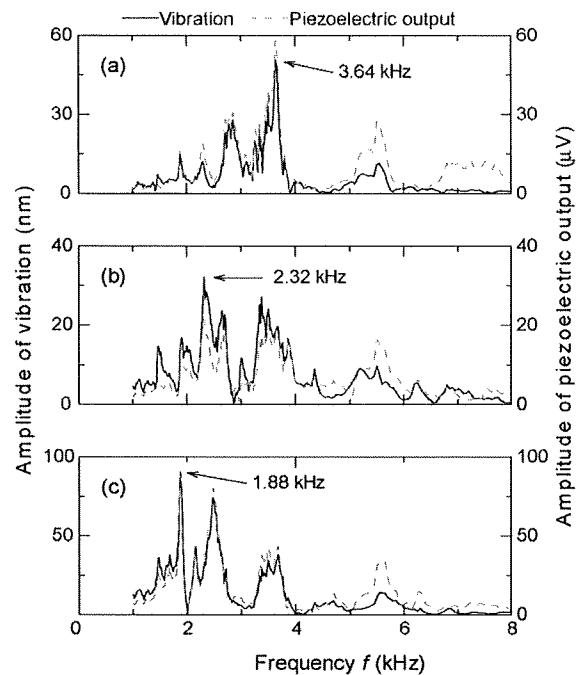


Fig. 11. Experimental results of vibration amplitude and piezoelectric output from (a) Ch. 6, (b) Ch. 12 and (c) Ch. 18 at various frequencies in silicone oil of  $1.75 \times 10^{-3}$  Pa s.

fully realized even in the liquid environment. However, comparing Figs. 7 and 11, the peak height at the LRF becomes low, i.e., the selectivity seems worse than that in the air environment. This result qualitatively agrees with the theoretical result that the peak is relaxed in the liquid environment as shown in Fig. 3. In order to dramatically improve the frequency selectivity, it is expected that the sensor including the active feedback control which mimics the biological cochlea should be developed in future.

Fig. 12 shows the relationship between  $f_r$  and  $x$  in the silicone oil. From Fig. 12, it is found that the LRF decreases from 4.9 to 1.4 kHz as  $x$  increases, where the range is lower than that in the air environment. The experimentally obtained LRF is compared

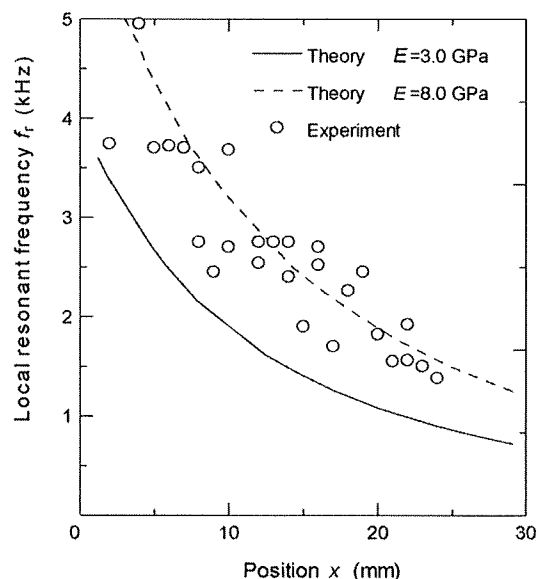


Fig. 12. Comparison of theoretically and experimentally obtained results of local resonant frequency  $f_r$  in silicon oil of  $1.75 \times 10^{-3}$  Pa s.

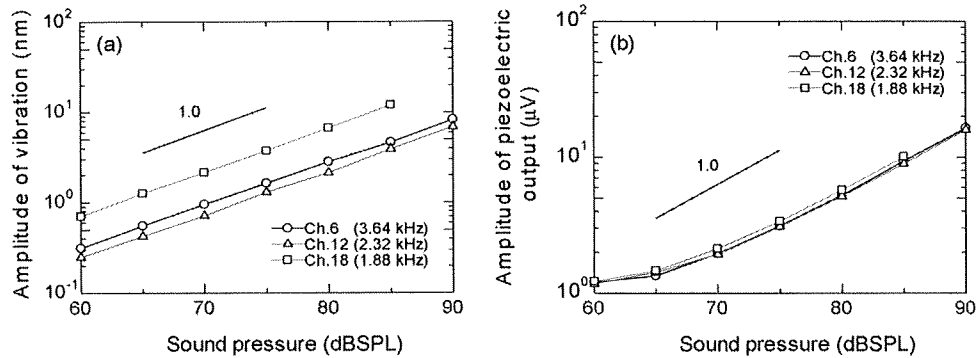


Fig. 13. Experimental results in effect of sound pressure on amplitudes (a) of vibration and (b) of piezoelectric output in silicone oil of  $1.75 \times 10^{-3}$  Pa s.

with the theoretically obtained one in Fig. 12. The predicted LRF of  $E=8.0$  GPa which is drawn by the broken line reasonably agrees with the experimentally obtained ones. From this agreement, it can be said that the theoretical analysis well reproduces the effect of the fluid-structure interaction on LRF. Since the theoretical analysis is carried out with inviscid fluid model, it can also be said that the decrease of LRF in the liquid environment is governed by the increase of effective mass for the vibration under the condition of constant sound pressure.

Fig. 13(a) shows the relationships between the sound pressure and the amplitudes of vibration. The amplitude of vibration linearly increases with the sound pressure. However, the amplitude of piezoelectric output shown in Fig. 13 (b) seems to be nonlinear especially at the low sound pressure level of 60–70 dB SPL. It may be due to the viscosity of liquid, however, it is difficult to confirm the reason for the nonlinear relationship due to the lack of the basic knowledge, where it is in our future work. As shown in Fig. 13(b), the amplitude of piezoelectric output is about  $16 \mu\text{V}$  at 90 dB SPL in the silicone oil. The acoustic wave of 90 dB SPL is relatively loud for the normal hearing. Even applying such a high sound pressure, the developed device can generate several tens of  $\mu\text{V}$  at most. To effectively stimulate nerve cells [20], the electric output should be amplified. One of the methods for the amplification is to use equipment such as a hearing aid, where it amplifies the sound pressure. Another solution is the downsizing of device using a fully micro-machining process, since the thinner membrane can generate the larger voltage. That is, the piezoelectric voltage  $V_p$  is proportional to the stress  $\sigma$  and the square of thickness  $h$  as  $V_p \propto \sigma h^2$ . On the other hand, the stress  $\sigma$  of ABM is inversely proportional to the cube of thickness as  $\sigma \propto h^{-3}$ . Consequently, the voltage is inversely proportional to the thickness as  $V_p \propto h^{-1}$ . The reduction of thickness can be easily realized by means of microfabrication and the thin films technologies. Thus, the implantable device will be developed based on those technologies in our future work.

### 3.3. Effect of viscosity on frequency selectivity

Fig. 14 shows the contour maps of amplitude distribution using the higher viscous silicone oil of  $1.75 \times 10^{-2}$  Pa s at (a)  $f=1.5$  kHz, (b) 2.0 kHz, (c) 3.0 kHz and (d) 4.0 kHz, respectively. The viscosity of the silicone oil is ten times higher than that in the previous section. The positions of maximum amplitude are  $x=28.0, 24.0, 20.5,$  and  $10.5$  mm for  $f=1.5, 2.0, 3.0,$  and  $4.0$  kHz, respectively as shown in Fig. 14. On the other hand, those are  $x=27.0, 21.0, 16.0,$  and  $10.0$  mm in the results of  $1.75 \times 10^{-3}$  Pa s as shown in Fig. 10. From this result, it can be said that the effect of viscosity on the position of the resonating place seems small. It is also found that the local maximum amplitudes due to the standing wave are relatively small in Fig. 14 compared with those in Fig. 10. That is

Table 2

Ratio  $a_1/a_2$  of height between the highest peak and the secondary highest one.

Frequency $f$ (Hz)	Ratio at $1.75 \times 10^{-3}$ Pa s $a_1/a_2$	Ratio at $1.75 \times 10^{-2}$ Pa s $a_1/a_2$
1.5	1.52	1.69
2.0	1.03	1.56
3.0	1.08	1.96
4.0	1.89	2.08

quantitatively confirmed by the ratio  $a_1/a_2$  which is the ratio of amplitudes between the highest peak  $a_1$  and the secondary highest peak  $a_2$  in Table 2. Comparing the results between  $1.75 \times 10^{-3}$  Pa s and  $1.75 \times 10^{-2}$  Pa s, it is found that the highest peak is significant in the higher viscous silicone oil. This may be caused because the wave is damped more rapidly in the higher viscous one. From the viewpoint of application, this result indicates that the effect of viscosity may contribute to improve the frequency selectivity. It is also possible to discuss the effect of viscosity by comparing the result between in the air and in the liquid environments. However, there are two different types of fluid-structure interactions of the increase in the effective mass and the viscous damping between

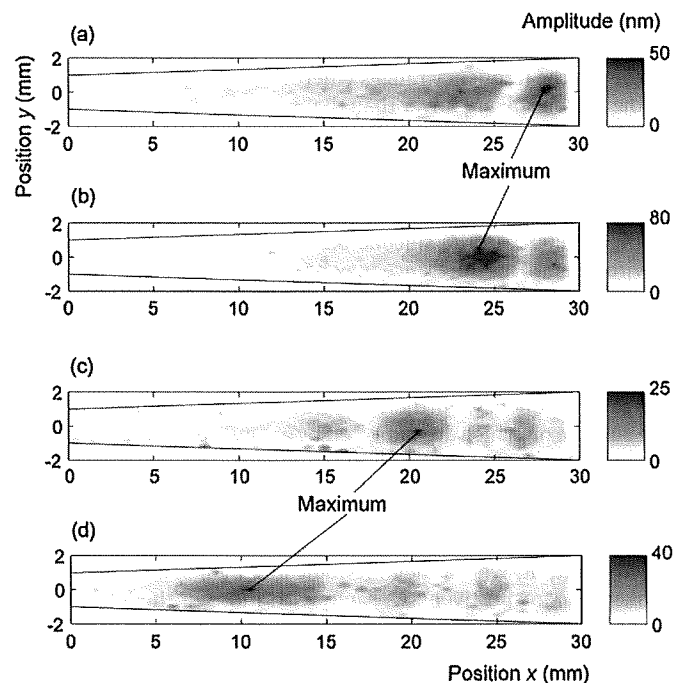


Fig. 14. Experimental results of contour maps of vibration amplitude at (a)  $f=1.5$  kHz, (b) 2.0 kHz, (c) 3.0 kHz and (d) 4.0 kHz in silicone oil of  $1.75 \times 10^{-2}$  Pa s.

those conditions. Since they contribute to the frequency selectivity oppositely, it is difficult to discuss separately. The development of theoretical analysis using full Navier–Stokes equation should be made in future research.

#### 4. Concluding remarks

In this paper, we reported a novel piezoelectric artificial cochlea which worked as a sensor with the acoustic/electric conversion and with the frequency selectivity based on MEMS technology. The basic performances of prototype device both in the air and in the liquid environments were investigated experimentally and theoretically.

The vibrating characteristics of trapezoidal ABM were measured by applying acoustic waves at a certain frequency. The location with the maximum amplitude was shifted toward narrower area of trapezoidal ABM as the frequency increased. This phenomenon indicated that the developed device successfully realized the frequency selectivity.

The frequency dependences of vibration and piezoelectric output were investigated both in the air and in the silicone oil of  $1.75 \times 10^{-3}$  Pa s. The resonant frequencies were determined to be over the ranges of 6.6–19.8 kHz in the air and 1.4–4.9 kHz in the silicone oil, respectively. The decrease in the resonant frequency due to the silicone oil must be the effect of fluid–structure interaction, that is, the interaction between the acoustic wave in the fluid and the membrane vibration. The interaction contributed to increase the effective mass for the vibration. This consideration was confirmed by the reasonable agreement between the experiment and the theory in terms of local resonant frequency.

The viscous effect of surrounding fluid on the vibration was explored using the higher viscous silicone oil of  $1.75 \times 10^{-2}$  Pa s. The effect on the resonating place seemed to be small between  $1.75 \times 10^{-3}$  and  $1.75 \times 10^{-2}$  Pa s. However, it was found that the higher viscous liquid suppressed the standing wave and improved the frequency selectivity.

To develop the fully implantable device in our future work, the amplification of voltage may be required to effectively stimulate nerve cells. Furthermore, the present device is relatively large for the implantation into a cochlea. These problems can be solved by the use of microfabrication and thin films technologies. The miniaturization is accomplished by the technology straightforward. And the larger electric signals can be generated using the thinner ABM, since the voltage is expected to be inversely proportional to the thickness. As a matter of course, these further developments must be conducted considering the frequency dependence. Thus, the theoretical approach which is described here is useful to design it in our future work.

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# Transplantation of Bone Marrow-Derived Neurospheres Into Guinea Pig Cochlea

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**Objectives/Hypothesis.** To investigate the potential of neurally induced bone marrow stromal cells (BMSCs) as transplants for replacement of spiral ganglion neurons.

**Methods.** BMSCs were harvested from the femurs and tibias of adult guinea pigs. BMSCs were cultured with neural induction media and formed spheres. The capacity of BMSC-derived spheres for neural differentiation was examined by immunocytochemistry in vitro. BMSC-derived spheres were injected into the modiolus of the intact cochleae or those locally damaged by ouabain, followed by histological and functional analyses.

**Results.** In vitro analysis revealed a high capacity of BMSC-derived spheres for neural differentiation. After transplantation into the cochlear modiolus, the survival and neural differentiation of BMSC-derived spheres was observed in both the intact and damaged cochleae. In intact cochleae, transplants settled in various portions of the cochlea, including the cochlear modiolus, whereas in damaged cochleae, transplants were predominantly observed in the internal auditory meatus. Transplantation of BMSC-derived spheres resulted in no functional recovery of the cochlea or protection of host spiral ganglion neurons.

**Conclusions.** The present findings indicate that BMSC-derived spheres can be a source for replacement of spiral ganglion neurons, although further manipulations are required for functional recovery.

**Key Words:** Allograft, cell therapy, cochlea, neurosphere, spiral ganglion neuron, regeneration.

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## INTRODUCTION

Treatment options for sensorineural hearing loss are currently limited to cochlear implants and hearing aids. Hence, there is a requirement for alternative means of biological therapy, including cell-based therapy. Indeed, recent studies have indicated that cell-based therapy could be utilized as a therapeutic option for inner ear disorders.<sup>1-3</sup> Spiral ganglion neurons (SGNs), primary auditory neurons, are located in the modiolus of the cochlea and transmit sound stimulation to the central auditory system. The loss of SGNs, therefore, compromises auditory function. In addition, SGN loss also reduces the effectiveness of cochlear implants, which can improve impaired hearing by stimulating SGNs. SGNs are, therefore, a primary target for cell transplantation in the auditory system.

Bone marrow stromal cells (BMSCs) are a heterogeneous population of stem/progenitor cells with pluripotent capacity to differentiate toward a neuronal phenotype,<sup>4,5</sup> and consequently the possible use of BMSCs for the treatment of neurological diseases has acquired enormous importance. BMSCs have great potential as therapeutic agents, because they are easy to isolate and expand. Previously, the potential of BMSC transplantation for the treatment of inner ear disorders has been investigated.<sup>6-9</sup> These previous studies have demonstrated that undifferentiated BMSCs are able to settle in the cochlea and have a high capacity for migration. However, limited numbers of transplants differentiated into neurons after transplantation into the intact or damaged cochlea,<sup>6-8</sup> which indicates that neural induction of BMSCs before transplantation is required for SGN replacement by BMSC transplantation.

The aim of this study was to elucidate the neural expression profile of neurally induced BMSCs of guinea pigs and their ability to retain neural differentiation potential when transplanted into the intact or damaged cochleae of guinea pigs. In addition, we examined the capacity of neurally induced BMSCs for functional and histological replacement of SGNs.

## MATERIALS AND METHODS

### *Experimental Animals*

A total of 18 Hartley-strain guinea pigs were purchased from Japan SLC Inc. (Hamamatsu, Japan). The Animal

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Research Committee of the Graduate School of Medicine, Kyoto University, Kyoto, Japan, approved all of the experimental protocols. Animal care was carried out under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

### BMSCs

Bone marrow was isolated from the femurs and tibias of 6- to 8-week-old guinea pigs ( $n = 4$ , Japan SLC Inc.). Under general anesthesia with midazolam (8 mg/kg, intramuscular injection) and xylazine (8 mg/kg, intramuscular injection), the epiphyses of the femurs and tibias were removed, and the marrow was flushed out into a 100-mm culture dish. The isolated bone marrow, composed of hematopoietic and stromal cells, was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Thermo Trace Ltd., Noble Park, Victoria, Australia) and 1% antibiotic-antimycotic (Invitrogen) at 37°C with 5% CO<sub>2</sub>. The medium was changed twice weekly until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. The BMSCs were passaged three to five times before use. BMSCs at this stage were defined as undifferentiated.

### Neural Induction of BMSCs

For neural induction, cultured BMSCs were enzymatically detached from culture dishes. The BMSCs were plated into 100-mm culture dishes at a density of  $2 \times 10^6$  cells/well, and cultured in serum-free DMEM/F-12 medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/mL of basic fibroblast growth factor (bFGF, Invitrogen), and 20 ng/mL of epidermal growth factor (EGF, Invitrogen). We added the same amounts of bFGF and EGF every 3 days. After 7 days of culture, BMSC-derived spheres were collected and fixed with 4% paraformaldehyde (PFA) for 15 minutes. The characteristics of BMSC-derived spheres were examined by immunohistochemistry for nestin and Musashi-1. Anti-nestin rabbit polyclonal antibody (1:500; BD Biosciences, San Jose, CA) or anti-Musashi rabbit polyclonal antibody (1:500; Chemicon, Billerica, MA) was used as the primary antibody. Alexa Fluor 488-conjugated anti-mouse donkey IgG (1:1000; Molecular Probes, Eugene, OR) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) were used as the secondary antibodies. We counted the numbers of spheres and the number of marker-positive spheres in five randomly selected fields (3.4 mm<sup>2</sup> in area), and then calculated the ratio of nestin or Musashi-1 expressing spheres to the total number of spheres. Four independent cultures were performed.

### In Vitro Neural Differentiation

To investigate the ability of BMSC-derived spheres to neurally differentiate, BMSC-derived spheres were plated onto 8-well chamber slides at a density of 100 spheres/well in serum-free DMEM/F-12 medium supplemented with B27, retinoic acid (1 mM, Sigma, St. Louis, MO), and dibutyl cyclic adenosine monophosphate (AMP) (1 mM, Sigma). After 7 days of culture, the cells were fixed with 4% PFA for 15 minutes and immunostained for beta-III tubulin. Anti-beta-III tubulin rabbit polyclonal antibody (1:500; Sigma) was used as the primary antibody, and Alexa Fluor 488-conjugated anti-mouse donkey IgG (1:1000; Molecular Probes) was used as the secondary antibody. We counted the total numbers of the cells and the number of beta-III tubulin-positive cells in five randomly selected fields (3.4 mm<sup>2</sup> in area), and then calculated the ratio of beta-III

tubulin expressing cells to the total number of cells. Four independent cultures were performed.

### Transplantation Procedure

After labeling with DiI (Invitrogen, 5 μg/mL), the cell suspension of BMSC-derived spheres ( $10^5$  cells in 10 μL DMEM) was injected into the cochlear modiolus of guinea pigs weighing 300 to 330 g as described previously.<sup>10</sup> Briefly, under general anesthesia with midazolam and xylazine, a small hole was made on the left otic bulla to expose the round window niche and the basal turn of the cochlea. After cochleostomy in the basal turn of the cochlea, a glass pipette, which was connected to a microsyringe (Hamilton, Reno, NV), was inserted into the cochlear modiolus of the basal portion of the cochlea. The glass pipette was removed 1 minute after completion of the infusion. Finally, the cochleostomy site was closed with a fat graft and then covered with fibrin glue.

### BMSC-Derived Sphere Transplantation

BMSC-derived spheres were transplanted into intact or damaged cochleae of guinea pigs weighing 300 to 330 g. Four weeks after transplantation, four intact guinea pigs were transcardially perfused with phosphate buffered saline (PBS) at pH 7.4, followed by 4% PFA, and sacrificed under general anesthesia with an overdose of pentobarbital. The temporal bones were immediately dissected out and immersed in the same fixative for 4 hours at 4°C.

Ten guinea pigs received local application of ouabain (5 μL at a concentration of 5 mM in saline; Sigma), which causes SGN degeneration,<sup>9</sup> through the round window membrane under general anesthesia with midazolam and xylazine. One week after application, the electrically evoked auditory brainstem response (eABR), which has been used for functional evaluation of SGNs, was measured before cell transplantation as previously described.<sup>10</sup> Eight animals that showed no eABRs were used in the following experiments. Immediately after the eABR measurements, four animals received transplantation of BMSC-derived spheres similar to intact guinea pigs, and the other four animals received an injection of the culture media and were used as controls. Four weeks later, the cochlear specimens were collected after eABR recording.

Specimens (10-μm thick) were prepared using a cryostat after decalcification with 0.1 M ethylenediaminetetraacetic acid in PBS for 3 weeks at 4°C. Then, immunostaining for beta-III tubulin was performed, followed by nuclear staining with 4',6-diamino-2-phenylindole dihydrochloride (DAPI; 2 μg/mL PBS, Molecular Probes). Specimens were viewed with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems Inc., Wetzlar, Germany). Five midmodiolar sections (each separated by 30 μm) were provided for quantitative analyses from each tissue sample. We defined the cells that were positive for DiI with a distinct nucleus identified by DAPI as surviving transplants. The numbers of transplants were counted in the internal auditory meatus and in five cochlear compartments (the modiolus, the scala vestibuli, the scala media, the scala tympani, and the lateral wall). We also counted the numbers of beta-III tubulin-positive transplants, and calculated the ratio of beta-III tubulin expressing transplants to total surviving transplants. In addition, the densities of SGNs in the Rosenthal canals were quantified in ouabain-treated specimens as described previously.<sup>11</sup> All data are presented as the mean  $\pm$  1 standard deviation.

### Statistical Analyses

We statistically compared the total numbers of surviving transplants and the ratios for beta-III tubulin expression

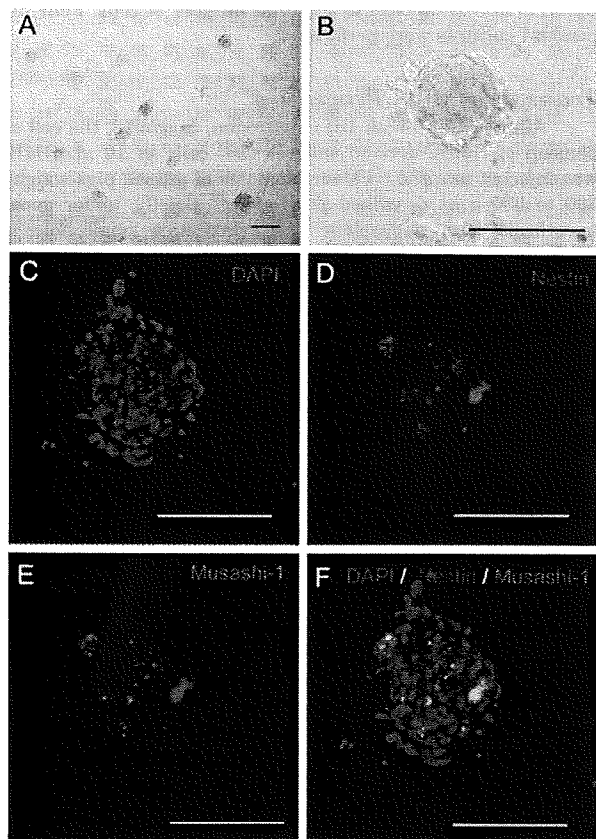


Fig. 1. BMSC-derived spheres. (A, B) Phase contrast images. (C) 4',6-diamino-2-phenylindole dihydrochloride (DAPI) staining. (D) Immunostaining for nestin. (E) Immunostaining for Musashi-1. (F) Merged image. Scale bars = 500  $\mu$ m.

between transplants in damaged cochleae and those in intact cochleae, using unpaired *t* tests. The difference in the locations of surviving transplants between damaged and intact cochleae was examined by two-way analysis of variance. In damaged models, the difference in the density of remaining SGNs between control and transplanted cochleae were compared using unpaired *t* tests. *P* values of < .05 were considered to be statistically significant.

## RESULTS

### Neural Induction of BMSCs

After 2 to 3 days in vitro, BMSCs began to form spheres. On day 7,  $1.28 \pm 0.71 \times 10^4$  spheres were identified in each culture dish (Fig. 1A, 1B). Immunohistochemistry revealed the expression of nestin and Musashi-1 in the BMSC-derived spheres (Fig. 1C–1E). The expression of nestin was found in  $91.9 \pm 4.7\%$  of total BMSC-derived spheres, and that of Musashi-1 was found in  $93.6 \pm 2.9\%$ , suggesting that neurospheres were generated from guinea pig BMSCs.

### In Vitro Neural Differentiation

We transferred the BMSC-derived spheres into differentiation medium containing retinoic acid and dibutyryl cyclic AMP to examine their capacity for neu-

ral differentiation. Sphere-forming cells attached to culture dishes and the cells migrated from the sphere (Fig. 2A). Then, some of the cells extended processes (Fig. 2B). On day 7,  $89.2 \pm 2.8\%$  of the cells expressed beta-III tubulin (Fig. 2C, 2D), indicating that BMSC-derived spheres have the capacity to differentiate into neurons.

### Transplantation Into Intact Cochleae

DiI-positive transplants were found in all intact cochleae following transplantation of BMSC-derived spheres. In each midmodiolar section,  $74.1 \pm 44.4$  transplants were found. Transplants were located in multiple regions of the cochlea, predominantly in the scala tympani and the modiulus (Fig. 3A, 3C). Transplants were rarely found in the internal auditory meatus. The expression of beta-III tubulin was observed in  $18.6\% \pm 6.4\%$  of transplants (Fig. 3B).

### Transplantation Into Damaged Cochleae

DiI-positive transplants were also identified in all transplanted cochleae that had been damaged by ouabain. The number of surviving transplants in each midmodiolar section was  $72.1 \pm 53.1$ . There was no significant difference in the number of surviving transplants between intact and damaged cochleae, whereas the locations of surviving transplants in the damaged cochleae significantly differed from those in the intact cochleae (*P* = .007). In the damaged cochleae, the settlement of transplants was observed in the modiulus, similar to observations in the intact cochleae; however, the most prominent region for settlement of transplants

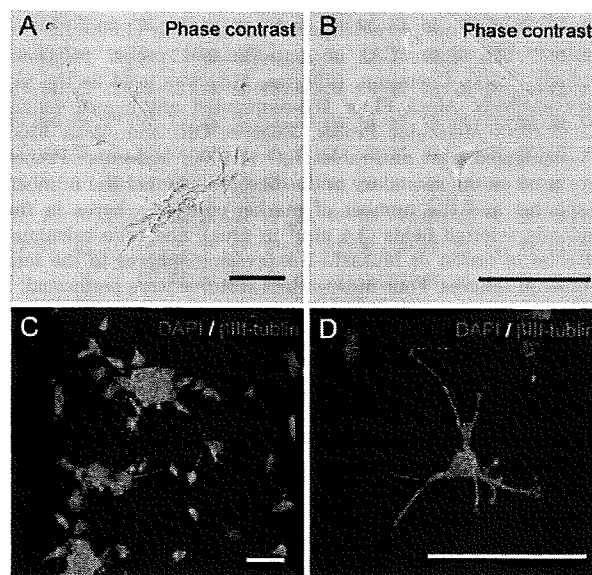


Fig. 2. Neural induction of BMSC-derived spheres in vitro. (A) Phase contrast image on day 3 in vitro. (B) Phase contrast image on day 7 in vitro. (C, D) Immunostaining for beta-III tubulin and nuclear staining with 4',6-diamino-2-phenylindole dihydrochloride (DAPI). Scale bars = 20  $\mu$ m.

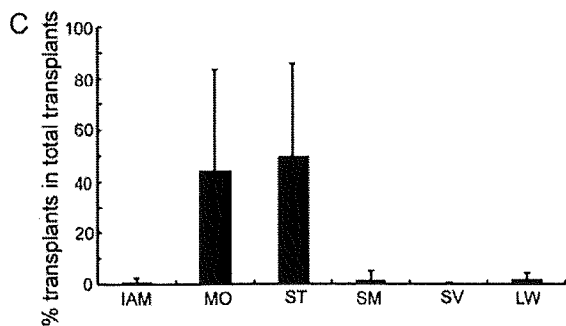
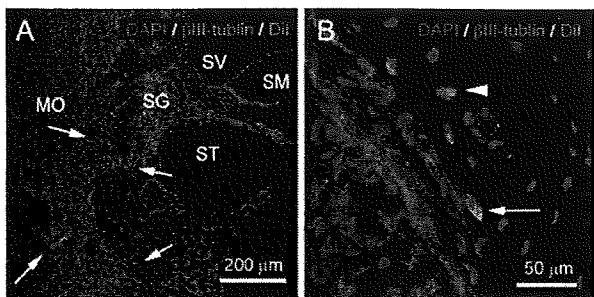


Fig. 3. Transplantation into the intact cochleae. (A) Transplants labeled by Dil (red) are located in the modiolus (MO) of the cochlear basal portion (arrows). (B) Transplant labeled by Dil (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). SV = scala vestibule; SG = spiral ganglion; SM = scala media; ST = scala tympani; LW = lateral wall. Bars represent a standard deviation.

derived spheres on enhancement of the survival of remaining host SGNs. Local ouabain application caused severe degeneration of SGNs, especially in the basal turn of cochleae. No significant differences in the SGN density of the basal, second, or third turn of cochleae were found between transplanted and sham-operated specimens (Fig. 5), indicating that transplantation of BMSC-derived spheres did not promote the survival of remaining host SGNs, which is consistent with eABR results.

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**DISCUSSION**

The present findings demonstrate that guinea pig BMSCs are able to form spheres that have the capacity to differentiate into neurons in vitro. We aimed to replace SGNs, which are located in the cochlear modiolus, with BMSC-derived neurons. We thus directly injected BMSC-derived spheres into the modiolus of intact or damaged cochleae of guinea pigs. For accurate introduction of the cells into the cochlear modiolus, the size of the cochlea is a critical issue. Previously, we tried to introduce transplants into the cochlear modiolus of mice,<sup>12</sup> in which the success rate for the settlement of the transplants was not satisfactory. In addition, functional evaluation following cell transplantation is virtually impossible. On the other hand, guinea pig<sup>10,13</sup> or chinchilla<sup>6</sup> model systems exhibited better settlement

COLOR

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F4 was the internal auditory meatus (Fig. 4A, 4C). The expression of beta-III tubulin was observed in  $24.1 \pm 5.3\%$  of transplants in the damaged cochleae (Fig. 4B). There was no significant difference in the ratio of beta-III tubulin expressing transplants to total number of transplants between intact and damaged cochleae. These findings indicated that SGN degeneration prior to transplantation caused the migration of BMSC-derived spheres into the internal auditory meatus, and had no effects on the survival and neural differentiation of BMSC-derived spheres after transplantation.

**Effects of Transplantation on Cochlear Function**

We used eABR recording to monitor SGN function. All the animals receiving eABR evaluation showed no responses before an injection of a cell suspension or a culture medium. Four weeks postoperation, positive eABRs were identified in two of four animals in each group. Thresholds of eABRs in the two animals that showed positive responses in the transplanted group were 300 and 400  $\mu$ A, and those in the sham-operated group were 250 and 650  $\mu$ A. These findings indicated that transplantation of BMSC-derived spheres into the cochlear modiolus induced no significantly functional recovery of the cochlea.

We quantified SGN densities after cell transplantation or sham operation to evaluate the effects of BMSC-

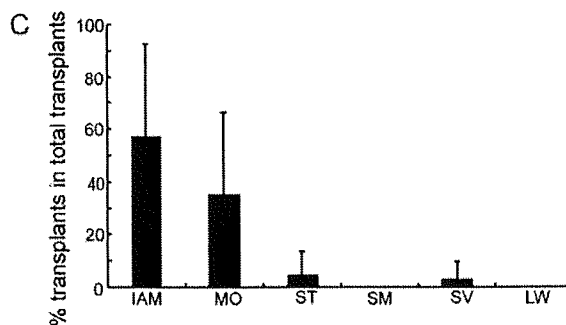
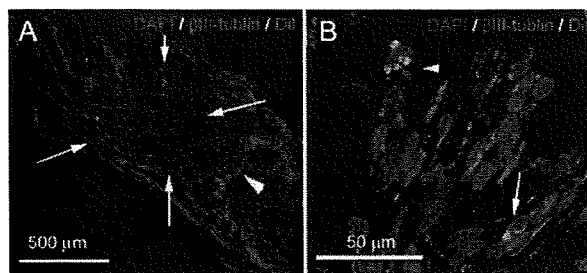


Fig. 4. Transplantation into the damaged cochleae. (A) In the internal auditory meatus (IAM), transplants labeled by Dil (red) are observed (arrows). An arrowhead indicates the location of the glial-schwann junction. (B) A transplant labeled by Dil (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). MO = modiolus; ST = scala tympani; SM = scala media; SG = spiral ganglion; SV = scala vestibule; LW = lateral wall. Bars represent a standard deviation.

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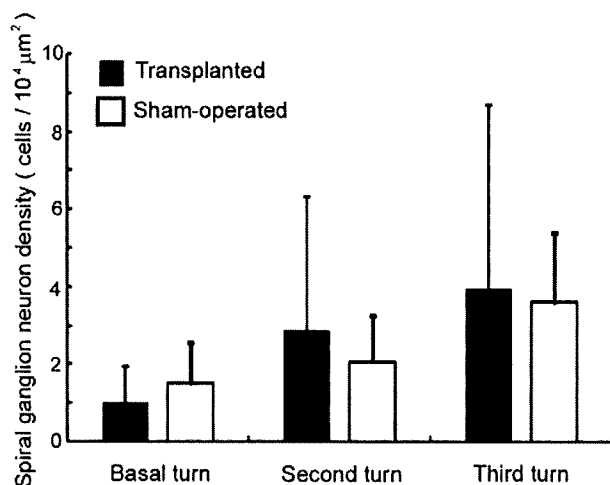


Fig. 5. Densities of remaining spiral ganglion neurons in the basal, second, or third turn of transplanted or sham-operated cochleae. There are no significant differences in the density of spiral ganglion neurons between transplanted and sham-operated cochleae.

of transplants in the cochlear modiolus and enabled functional evaluation using eABRs.<sup>10,13</sup> Moreover, our refined technique for cell introduction into the cochlear modiolus of guinea pigs caused no significant elevation of eABR thresholds.<sup>10</sup> Based on these previous findings, we used guinea pigs as experimental animals in the present study.

After transplantation of BMSC-derived spheres into the intact or damaged cochleae, BMSC-derived neurons were found in various portions of cochleae, including the cochlear modiolus. These findings indicate that BMSCs can be an alternative source of transplants for replacing SGNs. However, measurements of eABRs in the present study revealed no significant improvements of eABR thresholds after transplantation of BMSC-derived spheres. Previously, we demonstrated the recovery of eABR thresholds after transplantation of embryonic stem (ES) cell-derived neural progenitors in a different injury model.<sup>13</sup> There are several possible explanations for this lack of functional recovery following transplantation of BMSC-derived spheres. One is insufficient neurite elongation from BMSC-derived neurons to the central nervous system. Another possibility relates to different subtypes of neurons that are generated from BMSC-derived spheres. Previous studies have demonstrated that glutamatergic neurons are generated from both ES cells<sup>14</sup> and BMSCs,<sup>15</sup> meaning that both cell types have the capacity for differentiation into glutamatergic neurons. To achieve functional SGN restoration by transplantation of BMSC-derived spheres, additional treatments are required to enhance elongation of neurites from BMSC-derived neurons or to induce differentiation of BMSC-derived spheres into glutamatergic neurons.

Interestingly, the localization of transplants was different between the intact and damaged cochleae. In the intact cochleae, a number of transplants were found in the scala tympani. We injected BMSC-derived spheres

through the scala tympani.<sup>10</sup> Therefore, transplants that were found in the scala tympani may have originated from the leakage of injected cell suspensions. In the intact cochlea, there are limited spaces in the cochlear modiolus, because host SGNs and auditory nerves are present, which may cause the leakage of injected cell suspensions into the scala tympani. On the other hand, the loss of host SGNs may result in an increase of spaces for transplants in the cochlear modiolus. Hence, in the damaged cochleae limited numbers of transplants were observed in the perilymphatic spaces including the scala tympani. In the damaged cochleae a number of transplants were found not only in the cochlear modiolus but also in the internal auditory meatus. Transplants in the internal auditory meatus may migrate from the cochlear modiolus, which is an injected site. The degeneration of SGNs could make a path from the cochlear modiolus of the basal portion to the internal auditory meatus, or stimulate production of chemotactic factors that promote the migration activity of BMSC-derived spheres. Future studies should be performed to determine the mechanisms of migration of BMSC-derived spheres into the internal auditory meatus.

## CONCLUSION

The present findings demonstrate that BMSCs are a preferable source of neurospheres and that BMSC-derived spheres retain the ability for neural differentiation after transplantation into the cochlea. Functional restoration of damaged cochleae was not achieved by transplantation of BMSC-derived spheres, although a number of transplant-derived neurons settled in the cochlea and in the internal auditory meatus. To achieve functional restoration of SGNs by transplantation of BMSC-derived spheres, additional treatments including local application of neurotrophic or growth factors may be required.

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## **Culturing Neurons on MEMS Fabricated P(VDF-TrFE) Films for Implantable Artificial Cochlea\***

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### **Abstract**

In this paper, we report an *in vitro* study on the biocompatibility of poly(vinylidene fluoride-trifluoroethylene) (P(VDF-TrFE)) films for the implantable artificial cochlea. The implantable artificial cochlea comprises a piezoelectric membrane made of P(VDF-TrFE), platinum (Pt) thin film electrodes, and a silicon substrate which are designed to stimulate neurons in a cochlea and fabricated by microelectromechanical systems (MEMS) and thin film technologies. The biocompatibility of P(VDF-TrFE) film is evaluated by culturing cerebral cortical neurons from rats on it. The fibronectin from human plasma and the collagen from the calf skin are used as the cell adhesion factors. Since neurons extend dendrites and axons from the somata, it is found that the neurons are successfully cultured on the surface of P(VDF-TrFE) films modified both by the fibronectin and by the collagen. Furthermore, it is also found that the neurons are also successfully cultured over the Pt electrode on the P(VDF-TrFE) of the implantable artificial cochlea modified by the fibronectin. Consequently, the biocompatibility and the applicability of the MEMS fabricated P(VDF-TrFE) films and the implantable artificial cochlea are confirmed.

**Key words:** Biocompatibility, MEMS, Cerebral Cortical Neuron, Medical Equipment, Biomechanical Engineering, Piezoelectric Device

### **1. Introduction**

Piezoelectric materials are promising ones in the field of implantable artificial organs, since they can be used for electric power generators and sensors using the direct piezoelectric effect and for actuators using the inverse piezoelectric effect. For instance, Lewandowski et al.<sup>(1)</sup> proposed a piezoelectric power generator with a muscle-tendon unit. Platt et al.<sup>(2)</sup> proposed a self-powered embedded sensor for orthopedic implants. Schubert et al.<sup>(3)</sup> and Schrag et al.<sup>(4)</sup> proposed micropumps using piezoelectric actuators for an implantable artificial pancreas and artificial bowel sphincter, respectively. Furthermore, authors have proposed a piezoelectric artificial cochlea which realizes the acoustic/electric conversion and the frequency selectivity without an externally supplying energy<sup>(5)-(7)</sup>.

Figure 1 shows a schematic of implantable artificial cochlea we have proposed. The

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device comprises a piezoelectric membrane (ABM) made of poly(vinylidene fluoride-trifluoroethylene) (P(VDF-TrFE)) and discrete electrodes made of platinum (Pt) thin films, which are fabricated on a silicon (Si) substrate by microelectromechanical systems (MEMS) and thin film technologies. When the curved ABM is extended straight, it can be seen that ABM has a trapezoidal shape. The shape which is designed to mimic the biological system, i.e. the basilar membrane in cochlea, enables to analyze the frequency of acoustic wave. To cure the sensorineural hearing loss in the future, the implantable artificial cochlea is inserted into a cochlea which is filled with lymph fluid. ABM in the cochlea is vibrated by externally applying acoustic waves which is transmitted through the outer ear and the middle one. The mechanical deformation of ABM due to the vibration is converted to electric signals by the piezoelectric effect of P(VDF-TrFE) and the electric signals stimulate neurons in the cochlea. Since the proposed device is basically developed by microfabrication technologies, the electrodes can be easily integrated and their number can be increased, whereas the conventional system is limited by the relatively small number of electrodes as 12-22<sup>(8)-(10)</sup>. Furthermore, since the frequency of acoustic wave is analyzed by a biomimetic system, the device may realize more "natural hearing" compared with the conventional system.

The basic mechanisms of frequency analysis and acoustic/electric conversion have been studied using a prototype device fabricated by bulk processes<sup>(5),(6)</sup>. The effects of surrounding fluid of ABM have been studied by the comparison between theoretical results and experimental ones<sup>(6)</sup>. Furthermore, for the miniaturization of device and the amplification of electric signals, a fabrication process based on MEMS and thin film technologies have been developed<sup>(7)</sup>. Although the ultimate goal of our studies is to develop the fully self-contained implantable artificial cochlea, the biocompatibility of device has not been discussed. Since Si and Pt are relatively popular materials in the MEMS field, there are literatures that discuss their biocompatibility<sup>(11)</sup>. However, as far as authors are aware, there are few papers that evaluate the biocompatibility of P(VDF-TrFE) films fabricated by MEMS processes.

Thus, in this paper, we investigate the biocompatibility of P(VDF-TrFE) films for the proposed artificial cochlea in terms of cytotoxicity. To transfer the electric signal from Pt electrodes to auditory neurons in the cochlea over the P(VDF-TrFE) thin film, the neurons should be cultured or at least they should extend neurites on P(VDF-TrFE) films. Therefore, the *in vitro* experiment is carried out by culturing cerebral cortical neurons from rats on the P(VDF-TrFE) films. The cultured neurons are labeled by the fluorescent Nissl stain and observed by a fluorescent microscope. To observe the detailed configurations of neurons by the phase contrast microscope, P(VDF-TrFE) films are also fabricated on glass substrates, instead of Si substrates. Since MEMS fabricated P(VDF-TrFE) films can be applied to develop other biomedical devices, the results obtained here provide not only the fundamental knowledge on the biocompatibility of proposed artificial cochlea but also on that of P(VDF-TrFE) films for other artificial organs.

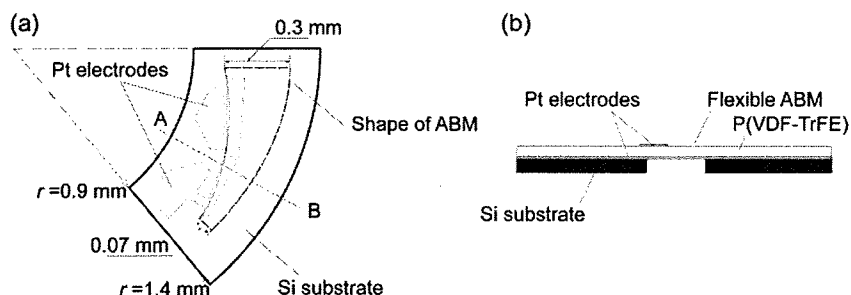


Fig. 1 Schematic of fully self-contained implantable artificial cochlea<sup>(7)</sup>; (a) top view and (b) cross sectional view at AB of (a).

## 2. Materials and methods

The fabrication process of implantable artificial cochlea is described as follows. The surface of Si substrate (100) is pretreated by hexamethyldisilazane (OAP, Tokyo Ohka Kogyo) to enhance the adhesion of P(VDF-TrFE) film. Then, a N,N-dimethylformamide (DMF) solution including P(VDF-TrFE) (KF-W#2200 P(VDF-TrFE), KUREHA) at the concentration of 8.0 wt% is spun on the substrate. The substrate is heated on a hotplate at 50 °C for 12 hours to evaporate DMF and at 145 °C for 2 hours to crystallize P(VDF-TrFE). The Pt electrodes are fabricated on the P(VDF-TrFE) film using a lift off process. The etching process of Si which makes the P(VDF-TrFE) film to be a flexible ABM is omitted in this study, since that is not necessary to discuss the biocompatibility. For the purpose of optical observation using a phase contrast microscope, glass substrates (Micro slide glass, Matsunami) are also used to fabricate P(VDF-TrFE) films instead of Si substrates.

A piece of processed substrate is sterilized by spraying with 70 % ethanol and is put into tissue culture dishes made of polystyrene. Then, the surfaces of substrates are modified by cell adhesion factors. Since it is obvious that the adherent cells have difficulties in growing on the hydrophobic surfaces such as P(VDF-TrFE), two types of popular cell adhesion factors, the fibronectin from the human plasma<sup>(12)</sup> and the collagen from the calf skin<sup>(13)</sup>, are used to focus the discussion on the cytotoxicity of the material. The process of surface modification is briefly described as follows. For the modification by fibronectin, the substrate is immersed in phosphate buffered saline (PBS) containing fibronectin (F0895, Sigma Aldrich Japan) at the concentration of  $6.7 \times 10^{-3}$  g/ml and is incubated at room temperature for more than 45 min. After that, the residual solution is removed. On the other hand, for the modification by the collagen, the substrate is immersed in 0.1 M acetic acid containing 0.1 wt% collagen (C8919, Sigma Aldrich Japan) and is incubated at 4 °C for 12 hours. After removing the residual solution, the substrate is kept at room temperature to be dried for 12 hours.

Dulbecco's modified Eagle's medium (DMEM, Gibco) which contains fetal bovine serum of 5.0 vol%, horse serum of 5.0 vol%, penicillin of  $6.2 \times 10^{-4}$  g/ml, and insulin of  $3.5 \times 10^{-5}$  g/ml<sup>(14)</sup> is poured into the tissue culture dish which contains the surface modified substrate. Then, the DMEM containing dissociated cerebral cortical neurons from rats at postnatal day 1 is introduced to the dish. Since it is quite difficult to obtain enough amounts of neurons from a cochlea, cerebral cortical neurons are used. However, the results are applicable to discuss the biocompatibility as a first step. The dish is placed in an incubator which is maintained at 37 °C and 5% CO<sub>2</sub> to culture neurons on the substrates for 3 days. After culturing 3 days, the neuron is fixed by 4% paraformaldehyde and labeled by fluorescent Nissl stains (NeuroTrace™ 500/525 green, Sigma Aldrich Japan)<sup>(15)</sup> for the subsequent optical observation. Since the Nissl substance labeled by the stain is abundant in neurons, cells with high fluorescent intensity indicate neurons. The viability is evaluated based on the morphology of cells observed by the fluorescent microscope and the phase contrast one. Although most of cells used here are neurons, cells include some glia ones. Thus, the neurons are distinguished based on the observations using both fluorescent photographs and phase contrast ones.

## 3. Results and discussion

Figures 2 (a), (b), and (c) show a phase contrast photograph, a fluorescent one, and a merged one of Figs. 2(a) and 2(b), respectively, observed at a same place of P(VDF-TrFE) fabricated on the glass substrate and modified by the fibronectin. Since the P(VDF-TrFE) and the glass substrate are transparent, it is possible to observe neurons, dendrites, and axons using a phase contrast microscope as shown in Fig. 2(a). Symbols of Ss and Ds in Fig. 2(a) are considered as somata and dendrites which adhere on the substrate, respectively,

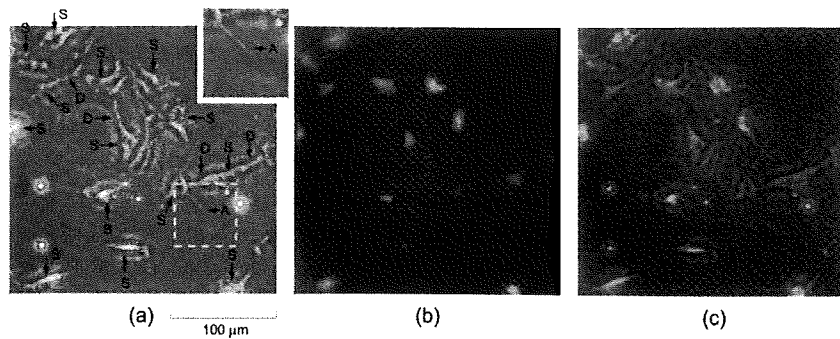


Fig. 2 (a) Phase contrast photograph, (b) fluorescent one, and (c) merged one of (a) and (b) of cultured neurons over P(VDF-TrFE) film modified by fibronectin and fabricated on glass substrate. Symbols A, D, and S indicate axon, dendrite, and soma, respectively. Inset in (a) is enlarged view around axon of A.

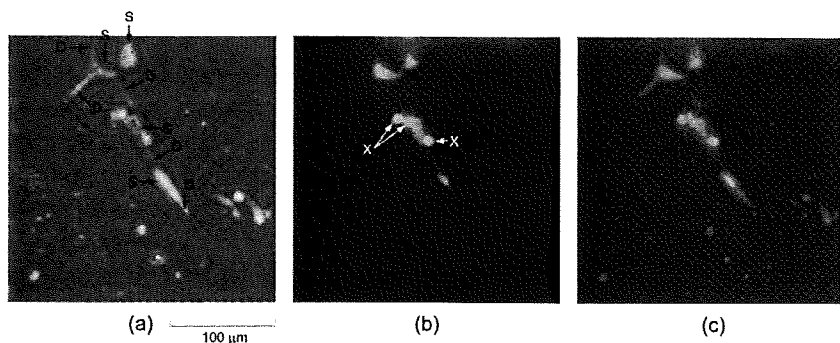


Fig. 3 (a) Phase contrast photograph, (b) fluorescent one, and (c) merged one of (a) and (b) of cultured neurons over P(VDF-TrFE) film modified by collagen and fabricated on glass substrate. Symbols D, S, and X indicate dendrite, soma, and dead cell, respectively.

since the stained areas in Fig. 2(b) correspond to the place of somata and dendrites as shown in Fig. 2(c). Furthermore, symbol A in the inset of Fig. 2(a) must be an axon. From these observation, it can be said that the neurons are successfully cultured on the P(VDF-TrFE) film modified by the fibronectin.

Figures 3 (a), (b), and (c) show a phase contrast photograph, a fluorescent one, and a merged one of Figs. 3(a) and 3(b), respectively, observed at a same place of P(VDF-TrFE) fabricated on the glass substrate and modified by the collagen. Symbols of Ss and Ds in Fig. 3(a) indicate somata and dendrites, respectively, where it is confirmed by the fluorescent photograph of Fig. 3(b) and by the merged one of Fig. 3(c). From the fluorescent photograph of Fig. 3(b), it is possible to roughly evaluate the viability of neurons based on the shapes of stained areas, where living and dead neurons seem to be distorted shapes and circular shapes, respectively. Xs in Fig.3 (b) must be dead neurons which are approximately circular shape. However, since most neurons are living and extend dendrites, it can be said that neurons are successfully cultured on the P(VDF-TrFE) film modified by the collagen. As shown in Figs. 2 and 3, the qualitative difference in terms of viability of neurons is not found between the cell adhesion factors of the fibronectin and the collagen. Consequently, it is found that P(VDF-TrFE) is applicable to a biocompatible material in terms of culturing neurons on it.

Figure 4 shows fluorescent photographs of cultured neurons over the P(VDF-TrFE) film fabricated on a Si substrate and modified by the fibronectin, that is, our proposed implantable artificial cochlea. The relatively dark background at the right half of Fig. 4 (a) indicates the Pt electrode on the P(VDF-TrFE) film. Since the Si substrate is not transparent, the evaluation on the cell viability is carried out based on this fluorescent

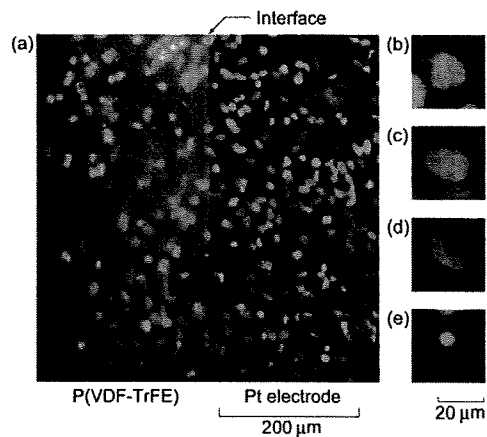


Fig. 4 (a) Fluorescent photograph of cultured neurons over P(VDF-TrFE) film and Pt electrode modified by fibronectin on Si substrate; (b)~(d) distorted stained area corresponding to living neurons and (e) circular area corresponding to dead one.

photograph. Although it is relatively difficult to strictly distinguish between the neuron and the glia cell only from Fig.4 (a), it is found that there are many stained areas with distorted shape. These distorted stained areas correspond to the living neurons or glia cells which adhere on the surface of substrate, where typical distorted and stained areas are shown in Fig.4 (b)~(d). In contrast, the dead cells should have a circular shape as shown in Fig.4 (e), because they do not adhere on the surface of materials. It is found that there are few dead cells in Fig. 4(a). In terms of cell viability, there is no qualitative difference between the area of the P(VDF-TrFE) and the Pt electrode. It may be because the surfaces are uniformly modified by the fibronectin. Furthermore, it is confirmed that there are living neurons or glia cells on the interface between P(VDF-TrFE) and the electrode. From these result, it can be concluded that the Pt thin film electrode is biocompatible and the electrodes must work well for stimulating neurons on it. Consequently, the biocompatibility of proposed artificial cochlea is also confirmed in cytotoxicity. In addition, it is important to discuss the *in vivo* biocompatibility in the context of sensitization, irritation, chronic toxicity, genotoxicity, and fibrous encapsulation.

The present study provides the fundamental knowledge on the biocompatibility of MEMS fabricated P(VDF-TrFE) films and proposed artificial cochlea. However, from the viewpoint of ABM's vibrating characteristics, the neurons should not be cultured on the flexible ABM as shown in Fig. 1(b), since the eigen frequency of vibration is designed before the implantation into a cochlea<sup>(7)</sup>, where the effect of neurons on the vibration is not considered. On the contrary, the neurons should be cultured on the electrodes to be stimulated effectively. Thus, the area where neurons are cultured should be controlled by patterning the cell adhesion factor using MEMS technologies<sup>(16)</sup>, where it is our future work.

#### 4. Concluding remarks

In the present paper, the biocompatibility of fully self-contained implantable artificial cochlea which is made of P(VDF-TrFE) film, the Pt electrodes, and the Si substrate by MEMS and thin films technologies was studied by culturing cerebral cortical neurons from rats. The neurons were successfully cultured on the P(VDF-TrFE) film modified by the fibronectin and the collagen. From the phase contrast photographs, it was found that dendrites and an axon were extended from a soma of a neuron. No qualitative difference in terms of viability of neurons was found between the fibronectin and the collagen.



Furthermore, the neurons were successfully cultured over the P(VDF-TrFE) film and the Pt thin film electrode for the implantable artificial cochlea. Consequently, it was confirmed that the MEMS fabricated P(VDF-TrFE) films and implantable artificial cochlea were not cytotoxic to cultured neurons. The results presented here would provide useful suggestions for further development of artificial organs research.

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