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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.[1] proposed a piezoelectric power generator with a muscle-tendon unit. Platt et al.[2] proposed a self-powered embedded sensor for orthopedic implants. Schubert et al.[3] and Schrag et al.[4] 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[5]-[7].

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 microelectromechannical 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[8]-[10]. 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[5],[6]. The effects of surrounding fluid of ABM have been studied by the comparison between theoretical results and experimental ones[6]. 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[7]. 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[11]. 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.

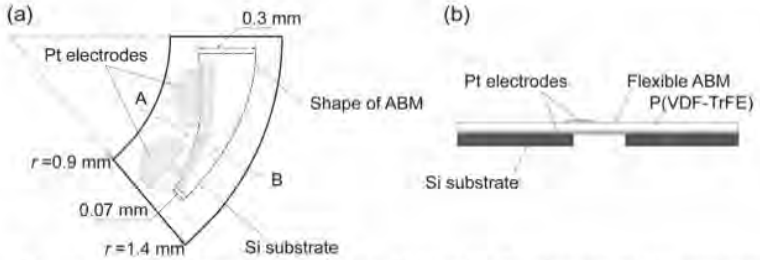

Fig. 1 Schematic of fully self-contained implantable artificial cochlea[7]; (a) top view and (b) cross sectional view at AB of (a).

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 ^{o}C for 12 hours to evaporate DMF and at 145 ^{o}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[12] and the collagen from the calf skin[13], 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 6.7×10^{-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 ^{o}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 6.2×10^{-4} g/ml, and insulin of 3.5×10^{-5} g/ml[14] 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 ^{o}C and 5% CO_2 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^{TM} 500/525 green, Sigma Aldrich Japan)[15] 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,

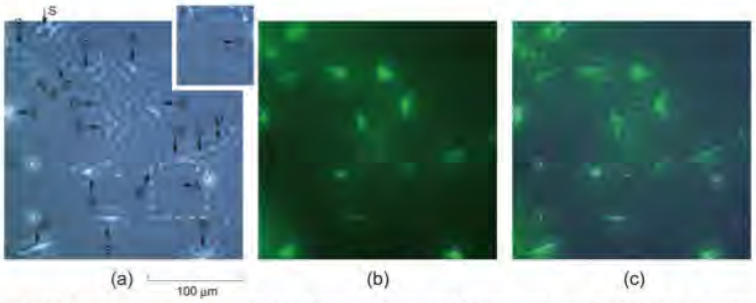

Fig. 2 (a) Phase contrast photograph, (b) fluorescent one, and (c) merged one of (a) and (b) of cultured neurons over P(VDF-TrFE) film modified by fibronectin and fabricated on glass substrate. Symbols A, D, and S indicate axon, dendrite, and soma, respectively. Inset in (a) is enlarged view around axon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

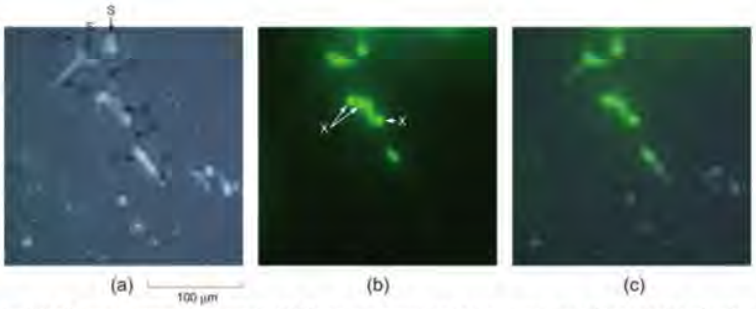

Fig. 3 (a) Phase contrast photograph, (b) fluorescent one, and (c) merged one of (a) and (b) of cultured neurons over P(VDF-TrFE) film modified by collagen and fabricated on glass substrate. Symbols D, S, and X indicate dendrite, soma, and dead cell, respectively.

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

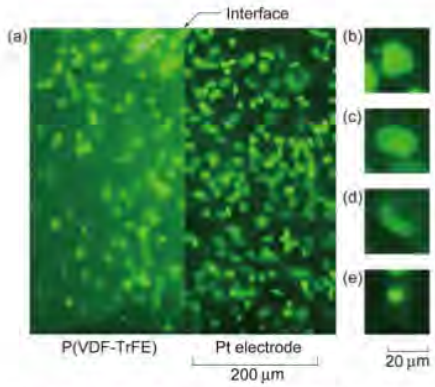

Fig. 4 (a) Fluorescent photograph of cultured neurons over P(VDF-TrFE) film and Pt electrode modified by fibronectin on Si substrate; (b)~(d) distorted stained area corresponding to living neurons and (e) circular area corresponding to dead one.

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[7], 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[16], 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 no cytotoxic effects on cultured neurons. The results presented here would provide the useful suggestions for further development of artificial organs research.

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