In Vitro Biocompatibility of Various Polymer-Based Microelectrode Arrays for Retinal Prosthesis

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PURPOSE. The purpose of our study is to evaluate the biocompatibility of various polymers used as microelectrode arrays (MEAs) in retinal prostheses through in vitro cytotoxicity testing following a standardized method.

METHODS. Three types of polymer-based MEAs were examined: silicone-based platinum, polyimide-based gold and liquid crystal polymer (LCP)-based gold MEAs. The silicone/platinum MEAs were fabricated by a Nd:YAG laser, polyimide/gold MEAs by a semiconductor manufacturing technique, and LCP/gold MEAs by laser micromachining and thermal-bonding process. All experimental procedures followed the International Organization for Standardization (ISO) 10993-5. To obtain the extracts of specimens, 4 g of each type of MEA were eluted by culture media, MEM, for 24 hours. Then, several diluents of extracts, including the original extracts, were applied to a cultured-cell monolayer, L929 fibroblasts. The morphologic changes of cells were analyzed by microscope after 24 and 48 hours of incubation. The quantitative evaluations of cell viability were performed by MTT assay after 24 hours of incubation.

RESULTS. The microscopic evaluations revealed that extracts from polymer-based MEAs did not induce morphologic changes or reduction of cells compared with control irrespective of concentrations of extracts. The MTT assay showed high viability values of approximately 80 to 130% regardless of diluted ratio of extracts from polymer-based MEAs. None of the polymers demonstrated a significant reduction of cell viability when compared with control.

CONCLUSIONS. All types of polymer-based MEAs, including silicone/platinum, polyimide/gold, and LCP/gold MEAs, meet the criteria of biocompatibility guided by international standards, ISO 10993-5. (*Invest Ophthalmol Vis Sci.* 2012;53:2653–2657) DOI:10.1167/iovs.11-9341

To our knowledge, there has been no established treatment for blind patients secondary to outer retinal degeneration, such as age-related macular degeneration (AMD) or retinitis pigmentosa (RP). These diseases lead to progressive loss of photoreceptors, but significant numbers of inner retinal cells, including bipolar and ganglion cells, remain even in advanced stages of disease.[1,2] For blind patients with AMD or RP, a retinal prosthesis is a possible device therapy to restore limited forms of vision.

In general, retinal prostheses consist of several components. Images are captured by a digital camera and processed by a microprocessor for conversion to an electrical signal. Then, to integrate into the retinal network, this signal is transmitted to the microelectrode embedded in the patient's eye, which stimulates electrically the remaining retinal cells.

Successful realization of a retinal prosthesis depends on the survival and function of remaining retinal cells. The intraocular tissue, especially the retina, is vulnerable to toxic or foreign body reactions, which might result in fatal deterioration of surviving retinal tissue. Therefore, the biocompatibility of a microelectrode array (MEA), which in a retinal neuroprosthesis, represents the device that interfaces directly with retina, is an essential prerequisite. A variety of biocompatible and nontoxic substrates have been used for microelectrode arrays in retinal prostheses. The potential characteristics of certain polymers, being thin, flexible and biocompatible, make them suitable for retinal prosthesis MEAs. Several polymers have been used for substrate materials, including polyimide, parylene, silicone and liquid crystal polymer (LCP).[3–9] The stability and biocompatibility of the polymer-based MEAs are the determinants of success or failure with intraocular implantation.

To ensure the biocompatibility of medical devices, in vitro cytotoxicity assays are widely accepted as a preliminary step. Numerous methods have been used depending on the nature of the specimens. To achieve reliable results, the assays must be performed according to international standards. The International Organization for Standardization (ISO) gives guidance to assess the in vitro cytotoxicity of biomedical devices.[10]

The fabrication process of several polymer-based MEAs for retinal prostheses has been reported previously.[6–9] The aim of

our study was to evaluate the biocompatibility and cytotoxicity of the polymers used for microelectrode arrays of retinal prostheses through in vitro cytotoxicity tests following a standardized method (ISO 10993-5).[10]

METHODS

Fabrication Process of Microelectrode Arrays

Three types of polymer-based microelectrode arrays were examined as follows: silicone-based platinum, polyimide-based gold, and liquid crystal polymer-based gold MEAs. The fabrication process of these MEAs has been reported previously.[6–9]

For the silicone-based platinum MEA fabrication, medical grade silicone rubber MED-1000 (NuSil, Carpinteria, CA) was used as a substrate and platinum foil (Goodfellow Cambridge Ltd., Huntington, UK) as the electrode material. Platinum foil was placed on the silicone rubber applied by spin coating. Electrode, pads, and connecting wires were patterned by a Nd:YAG laser (Quanta Ray GCR-5A, Spectral Physics, Mountain View, CA),[6,7] and another insulating layer of silicone was spun on. Then, the electrode array was completed by laser micromachining.

The polyimide-based gold microelectrode array was fabricated based on a semiconductor manufacturing technique.[8] Polyimide (PI2525, HD Micro systems, Parlin, NJ) was prepared as the substrate and gold was used for the electrode material. On the lower polyimide layer, three layers of titanium, gold and titanium were evaporated in that order. Patterning of the metal layer was conducted by photolithography and then the upper polyimide layer was added. The electrodes were opened and the whole structure was defined by reactive ion etching.

The LCP-based gold microelectrode array was fabricated through conventional micromachining and a thermal-bonding press.[9] Two types of LCP (Vecstar FA-25N; Kuraray Co., Ltd., Tokyo, Japan) films, low-melting temperature and high-melting temperature LCP, were used for substrate and cover, respectively. The titanium and gold films of 50 and 300 nm were deposited sequentially on the LCP substrate and patterned using photolithography and wet etching. The fabricated substrate was encapsulated by a cover layer through a thermal-bonding process, and the structure then outlined by laser-machining.

Sample Preparation

Preparations of the test specimens, namely three types of MEAs, were performed according to ISO 10993-5 and 10993-12.[10,11] The specimens were packed separately and sterilized by ethylene oxide to prevent bacterial contamination. The standard extraction temperature and times recommended by ISO 10993-12 are as follows:[11] $37 \pm 1°C$ for 24 ± 2 hours, $37 \pm 1°C$ for 72 ± 2 hours, $50 \pm 2°C$ for 72 ± 2 hours, $70 \pm 2°C$ for 24 ± 2 hours, and $121 \pm 2°C$ for 1 ± 0.2 hours. To obtain extracts of specimens, 4 g of each type of MEA were immersed in 20 mL of culture medium (MEM) for a 24-hour incubation in a humidified atmosphere with 5% carbon dioxide at 37°C in a shaking incubator (Jeiotech, Seoul, Korea). These values were chosen as the polymer might be denatured at higher temperatures and shaking for a long time might damage the electrode itself. The original extracts and a dilution series of extracts were used for cytotoxicity tests.

Cell Culture

L929 mouse fibroblasts were used to determine the biocompatibility and cytotoxicity of the MEAs. The cells were cultured in MEM (#41500; Gibco, Langley, OK) supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. All experiments were performed on cultured-cell monolayer in the logarithmic phase of growth.

Qualitative Evaluation of Cytotoxicity

L929 fibroblasts were seeded in 12-well plates in 1 mL culture medium at a concentration of 1×10^5 cells/mL and cultured for 24 hours at 37°C to 80% confluence. Then, the growth medium was removed and 1 mL of test extract was added carefully into each well including the original, 1:2 and 1:4 diluted extracts of MEAs. The extract of high-density polyethylene (HDPE) was added for negative control, and 10% dimethyl sulfoxide (DMSO) for positive control. After 24 and 48 hours of incubation at 37°C, the cytotoxic effect was determined by observation using an inverted phase contrast microscope (IX70, Olympus, Tokyo, Japan).

Qualitative parameters, including general morphology of cells, cell lysis and cell growth, were assessed. The changes from normal morphology were evaluated by numerical grades guided by ISO 10993-5[10] as follows: grade 0 as none (no changes, no cell lysis, no reduction of cell growth), 1 as slight (not more than 20% of the cells are round, loosely attached and show morphological changes), 2 as mild (not more than 50% of the cells are round, no extensive cell lysis), 3 as moderate (not more than 70% of the cells are round or lysed, cell layers not completely destroyed), and 4 as severe (nearly complete destruction of the cell layers). The experiments were repeated three times.

### Quantitative Evaluation of Cytotoxicity

Quantitative evaluation of cytotoxicity was conducted by MTT assay, which is an assay of metabolism of methyltetrazolium salt by mitochondrial dehydrogenase of active cells into formazan crystals. The L929 fibroblasts were seeded in 96-well plates at a density of 2×10^3 cells per well. The cells were incubated for 24 hours at 37°C to 80% confluence. After 24 hours, the culture medium was removed from the wells and equal volumes of the extracts were added into each well including the original, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 diluted extracts. Wells containing culture medium without cells were used as blank value and the cells with an extract of HDPE were used as negative control presenting 100% viability. The positive control was the cells treated with 10% DMSO. After 24 hours of incubation, MTT assay was performed to evaluate the cell viability. A solution of 50 μL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in phosphate-buffered saline was added to each well. After a further incubation period of 4 hours at 37°C, the supernatant was aspirated and formazan crystals were dissolved by 150 μL DMSO. The absorbance was measured at 540 nm using ELISA microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA). Cell viabilities were presented as the percentage of the absorbance of treated cells to the absorbance of control cells cultured with an extract of HDPE. MTT assays were repeated in four separate experiments.

Statistical Analysis

Statistical analysis was performed by SPSS statistical software (version 18.0). To analyze the data, one-way analysis of variance was used with respect to the types of polymer-based MEAs and concentrations of extracts. The analysis was followed by a Tukey post-hoc test for multiple comparisons according to the types of polymers and concentrations. A probability value of < 0.05 was considered significant.

RESULTS

The morphologic changes to L929 fibroblasts after treatment of extracts from the different MEAs was assessed by phase contrast microscopy (Figs. 1–3). After 24 hours of incubation, the cells exposed to the extracts of each MEA maintained normal morphology compared with negative control cells. They did not demonstrate any detrimental changes, such as reduction in cell growth, shrinkage or membrane blebbing of cells. Similarly a dilution of each MEA does not lead to
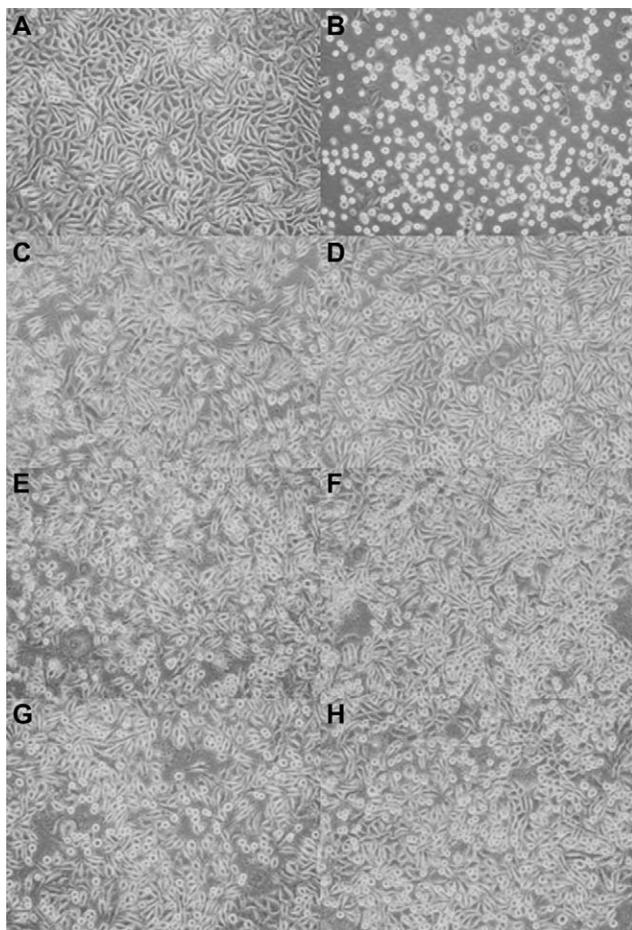
FIGURE 1. Phase-contrast microscopy images of L929 fibroblasts after the treatment with extracts from a silicone-based platinum MEA. The cells were imaged at 200× magnification. (**A**) Negative control cells treated with extract of high-density polyethylene. (**B**) Positive control cells treated with 10% dimethyl sulfoxide. (**C**, **D**) Cells treated with original extracts. (**E**, **F**) Cells treated with 1:2 diluted extracts. (**G**, **H**) Cells treated with 1:4 diluted extracts. (**C**, **E**, **G**) After incubation of 24 hours. (**D**, **F**, **H**) After incubation of 48 hours.
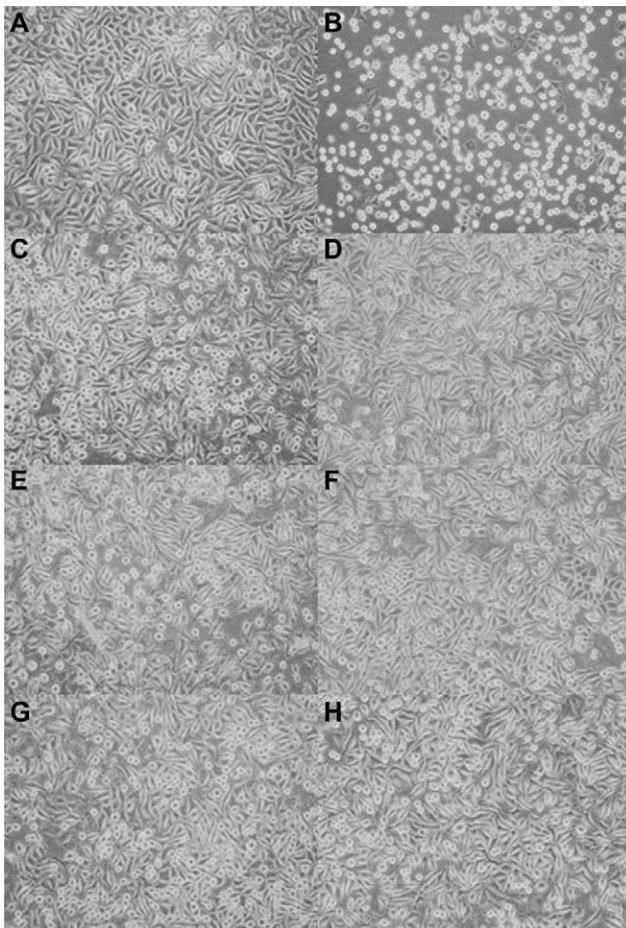

FIGURE 2. Phase-contrast microscopy images of L929 fibroblasts after the treatment with extracts from a polyimide-based gold MEA. The cells were imaged at 200× magnification. (**A**) Negative control cells treated with extract of high-density polyethylene. (**B**) Positive control cells treated with 10% dimethyl sulfoxide. (**C**, **D**) Cells treated with original extracts. (**E**, **F**) Cells treated with 1:2 diluted extracts. (**G**, **H**) Cells treated with 1:4 diluted extracts. (**C**, **E**, **G**) After incubation of 24 hours. (**D**, **F**, **H**) After incubation of 48 hours.

morphologic changes or reduction of cell growth. The numerical grading was as follows; grade 3 for positive control treated with 10% DMSO, grade 0 for negative control treated with extracts of HDPE and grade 0 for tested cells treated with extracts of each polymer-based MEA regardless of the concentrations of extracts. After 48 hours of incubation, each material did not show cytotoxic effect to the cells exposed to the extracts from the MEA polymers.

The results of MTT assay are shown in Figure 4. After 24 hours of incubation, high viability values of 80–130% were obtained over the whole range of investigated concentrations of extracts as follows: 108.6 ± 10.3 to $132.9 \pm 10.7\%$ for silicone-based platinum MEA, 94.4 ± 4.2 to $116.1 \pm 6.0\%$ for polyimide-based gold MEA and 78.8 ± 7.4 to $100.1 \pm 11.6\%$ for LCP-based gold MEA. The cell viability never dropped below 70% regardless of the types of MEAs and concentrations of extracts. The cells treated with extracts of silicone or polyimide-based MEAs did not show significant reduction of cell viability compared with control cells regardless of concentrations of the extracts. A significant reduction of cell viability compared with control cells occurred only in the cells treated with 1:32 and 1:64 diluted extracts from the LCP-based MEAs ($P = 0.023$,

0.013), but the trend of increasing cytotoxicity was not observed as the concentration of extracts increased further.

The comparisons of cell viability were performed according to the different polymer-based MEAs. In general, the viability of cells treated with extracts from the silicone-based platinum MEAs was higher than those with other polymers. The cells treated with extracts from the silicone-based platinum MEAs showed greater viability than polyimide or LCP-based gold MEAs ($P < 0.05$), except those with 1:32 and 1:64 diluted extracts of polyimide-based gold MEAs ($P = 0.247, 0.424$). The cells exposed to the polyimide-based gold MEAs demonstrated better viability than those exposed to the LCP-based gold MEAs ($P < 0.05$), except for those treated with 1:2, 1:8, and 1:128 diluted extracts ($P = 0.479, 0.065$ and 0.18, respectively).

DISCUSSION

The problems related to successful realization of a biomedical implant can be categorized into manufacturing process, surgical technique of implantation, biocompatibility of implant and long-term functionality of device. Intraocular implantation of a biomedical device, such as a retinal prosthesis, requires a

FIGURE 3. Phase-contrast microscopy images of L929 fibroblasts after the treatment with extracts from a liquid crystal polymer-based gold MEA. The cells were imaged at 200× magnification. (**A**) Negative control cells treated with extract of high-density polyethylene. (**B**) Positive control cells treated with 10% dimethyl sulfoxide. (**C, D**) Cells treated with original extracts. (**E, F**) Cells treated with 1:2 diluted extracts. (**G, H**) Cells treated with 1:4 diluted extracts. (**C, E, G**) After incubation of 24 hours. (**D, F, H**) After incubation of 48 hours.

higher level of biocompatibility than those implanted in other organs, because a series of inflammatory responses and a fibrous encapsulation of a foreign structure might result in irreversible destruction of the retina with permanent deterio-

ration of vision. A variety of factors might influence the biocompatibility of a retinal prosthesis, including surgical technique, characteristics of implanted material and reactions induced by electrical stimulation. In our study, we tried to evaluate the biocompatibility related to the material properties of each MEA according to international standards. We used end products manufactured by way of prototype fabrication processes rather than raw materials, and in vitro cytotoxicity assays were conducted using the extracts of MEAs following ISO 10993-5.[10]

A variety of polymers have been tried as substrates for an MEA in a retinal prosthesis. We compared three types of polymer-based MEAs, including silicone-based platinum, poly-imide-based gold and liquid crystal polymer-based gold MEAs, of which the fabrication processes have been reported previously.[6-9] These polymers have been cited as suitable candidates for retinal prostheses.[9,12-20] However, each polymer has several disadvantages when considered to be used for such an implant. Silicone might be too brittle to make thin enough. Polyimide is known to absorb moisture and result in delamination of the metal at the electrode site or interface of polyimide layers.[21,22] Recently, LCP has been used as a new substrate material for retinal prosthesis MEAs,[9] having properties of extremely low permeability of moisture and ions.[14,23] However, little is known about the efficacy and safety of LCP-encapsulated MEAs for retinal prostheses.

In our study, in vitro cytotoxicity assays were done based on indirect evaluation using the extracts of each specimen, including the evaluations by phase contrast microscopy and MTT assays. Under microscopic examinations, all types of polymer-based MEAs showed no significant morphologic changes or growth reduction of fibroblasts compared with control groups irrespective of diluted ratios of extracts.

The MTT assay is considered a good quantitative method to evaluate the cell viability based on the intracellular reduction of methyltetrazolium salt by the viable cells.[24,25] In an MTT assay, the materials that revealed a reduction of cell viability by more than 30% are regarded as cytotoxic according to ISO 10993-5.[10] After incubation for 24 hours, all types of polymer-based MEAs represented high cell viability above approximately 80% over the whole investigated dilution ratios of extracts. Almost all comparisons between cells treated with specimen extracts in comparison to the control group revealed no significant reduction of cell viability. While cells treated with 1:32 and 1:64 diluted extracts of LCP-encapsulated MEAs showed a significant reduction of cell viability compared with control, this outcome was not found in the other six steps of diluted ratios, most of which had higher concentrations than the 1:32 dilution. This anomalous result is difficult to explain,
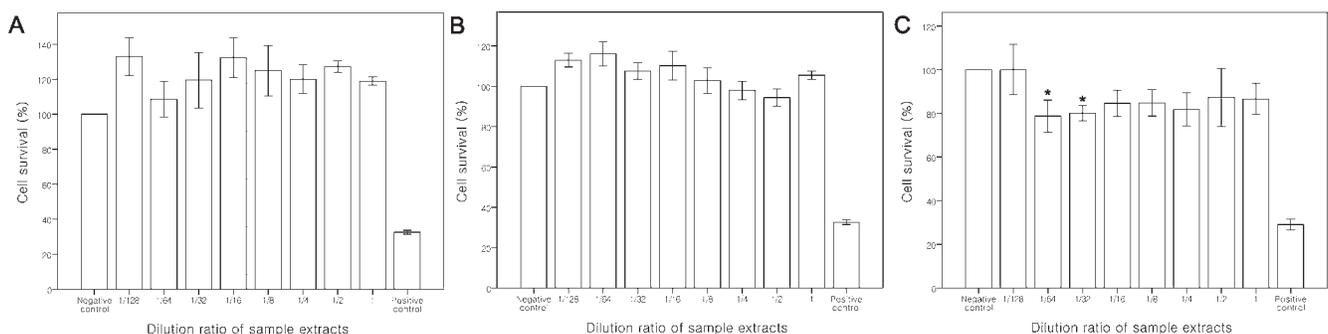
FIGURE 4. Cell viability of L929 fibroblasts according to the concentrations of extracts obtained from polymer-based MEAs assessed by MTT assay. Three types of polymer-based MEAs were evaluated, including silicone-based platinum (**A**), polyimide-based gold (**B**) and liquid crystal polymer-based gold MEAs (**C**). Negative control means the cells treated with an extract from high-density polyethylene and a positive control treated with 10% dimethyl sulfoxide. The bar indicates mean value of cell viability and error bars standard deviation ($n = 4$). *Significant difference of cell viability compared with negative control ($P < 0.05$).

but one possibility is that unknown experimental errors have influenced the cell viability at the concentrations of 1:32 and 1:64 dilutions. This conclusion is based on the facts that the majority comparisons did not show reduction of cell viability and there was no dose-response relationship according to diluted ratios. Therefore, we concluded that all types of MEAs reveal acceptable biocompatibility at any concentrations of extracts on the basis of ISO 10993-5.[10]

The comparisons between the cells treated with different polymer-based MEAs varied according to the concentrations of extracts. Although several comparisons showed higher cell viability of one type of MEA compared with others, the differences of cell viability were not maintained consistently as the concentrations of extracts increased. In some cases, the cell viability even exceeded 100%, which may influence the statistical analysis. Based on these data, the differences of cell viability according to the types of MEAs do not indicate the superiority of one particular MEA in respect of biocompatibility.

In this study, we used mouse L929 fibroblasts to identify the biocompatibility of MEAs for retinal prostheses. There are a few reports about in vitro biocompatibility tests for retinal prostheses using retinal neurons or retinal pigment epithelium that might be more suitable for biocompatibility studies with retinal implants.[26,27] However, L929 fibroblasts have been shown extensively to be a useful tool in standard testing for material biocompatibility and cytotoxicity. The contact between L929 fibroblasts and specimens was limited to an incubation period of 24 or 48 hours in this study. When a retinal prosthesis is applied in a clinical situation, the MEA naturally will be in contact with intraocular tissue for a longer period, likely the lifetime of the patients. These data using a limited incubation period cannot guarantee the long-term biocompatibility of polymer based MEAs for retinal prostheses. However, the controlled process of this study following international standards can provide more confidence in reliability of biocompatibility tests for retinal prostheses.

By in vitro cytotoxicity assays, we reached the following conclusions: all types of polymer-based MEAs, including silicone, polyimide and LCP, meet the criteria of biocompatibility guided by the ISO 10993-5, which means they are biocompatible enough to be a candidate for retinal prosthesis MEAs.

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