Culture experiments on conductive polymers

Mitsuyoshi Onoda
Graduate School of Engineering, University of Hyogo at Himeji Shosha Campus,
2167 Shosha, Himeji, Hyogo 671-2280 Japan

E-mail: onoda@eng.u-hyogo.ac.jp or ss-syu02@eng.u-hyogo.ac.jp

Abstract. Fibroblast L929 and myoblast C2C12 cells of the mouse connective tissue origin were sown on the surface of conductive polymer films (polypyrrole, PPy and poly(3,4-ethylenedioxythiophene), PEDOT) in the cell culture medium, and the proliferative process of these cells was observed. Without changing the form, fibroblast L929 and myoblast C2C12 cells were observed to proliferate almost similarly to the cell which cultured on a dish on the market and to maintain compatibility. In other word, it has been understood these two kinds of conductive polymers used in this study, the PEDOT films maintain the secretion function of the cell cultured on the surface of these polymers. Therefore, the PPy- and the PEDOT-coated electrode suggested the possibility usable as a nerve stimulation electrode with biocompatibility, because these polymers were effective to culture the cell.

1. Introduction
The conventional common sense to be insulators that polymers don't conduct electricity is overturned, and polymers have alternating carbon-carbon single and double bonds turn into metal high conducting electricity by doping was found. For the development of a new field "Science and Technology of Synthetic Metals", the Nobel Prize in Chemistry at the 20th end of century was awarded jointly to Professor Alan J. Heeger from University of California, Santa Barbara, USA, Professor Alan G. MacDiarmid (death on February 7, 2007) from University of Pensylvanya, USA, and Professor of Emerit Hideki Shirakawa from Tsukuba University, Japan, “for the discovery and development of conductive polymers”.

Conductive polymers have a structure that ranged long alternating carbon-carbon single and double bonds and extended highly conjugated system in their main chain. Because electrons contributing to double bond is easy to move along polymer main chain freely and have extended spatial and widely, the conductive polymers will show the specific properties that does not possess in conventional insulative polymers that polymer main chain consist of single bonds and only σ electrons exist. When thinking about conductive polymers from the viewpoint of electrical and electronic materials, though various kinds of conductive polymers are thought about whether are comprised of only carbon, hydrogen or also include the different kind atoms such as oxygen, nitrogen, sulfur etc., their nature depends on what kind of atoms is bonded how and forms molecules, in other words, being decided by each molecular structure. As one of the most characteristic effect that conductive polymers possess, doping effects are known well. By soaking or putting forcibly electron-accepting (acceptor) or electron-donating (donor) into the conductive polymer by means called the doping, the conductivity can be widely changed from insulator to semiconductor region, and then metal region and

1 To whom any correspondence should be addressed.

Published under licence by IOP Publishing Ltd
accompanying this other properties including the magnetic and optical property change greatly, too. Such a doping effect is occurred by changing the electronic state greatly, because dopant delivers electrons with polymer main chain basically. As for such conductive macromolecule, the use as the ① metals, ② insulators and semiconductors, ③ insulator / metal transition phenomena, ④ reversible doping ability, and so on are possible [1]. By utilizing skillfully the property that conductive polymers possess, for example, practical use devices which application to electronic devices such as condensers, electroluminescent devices, photovoltaic cells, sensors, etc. including rechargeable batteries, and optoelectronic devices, are available began to be realized, and realization of the new electronic devices on organic materials mainly, in other words, the development and progress of the organic electronic technology are doing lively [2, 3].

On the other hand, in recent years, the research on the improvement and the reformation of medical equipment are actively examined with the advancement of remarkable medical technology. For instance, the nerve interface technology that directly inputs and outputs some information to man's nervous system is paid attention. If the signal of high accuracy can be inputted with a multichannel to the peripheral nervous system, the achievement of an impossible treatment method is expected in the prior art like a control of the external instrument by the movement instruction signal and an artificial sense by the external sense signal, etc. In order to achieve such a nerve interface technology, the biocompatibility that is appropriate for the burial of a long term can be possessed, meanwhile a steady signal can be inputted, and the development of the nerve stimulation electrode that has a number of channels enough for the control of the aimed device is indispensable. At present, though it is made with the metal electrode such as ITO (indium tin oxide) and platinum as a nerve stimulation electrode, the compatibility of the biological tissue and the metal electrode becomes a problem. As the measures, up to date the surface of the electrode was covered by having used collagen that was one of the matrices outside the cell (existing supermolecule structure besides the cell inorganism) and poly(lysine) that was the cell attachment factor (intercellular bonding) and compatibility was given [4]. However, it caused an increase in impedance by covering the surface of the electrode and decreased the efficiency of the input and output stimulus signals. Therefore, the development of the nerve stimulation electrode in which compatibility with the biological tissue is possessed, and the efficiency of the stimulus signal is not decreased is necessary, and it is extremely important in progressing the nerve interface technology.

In such a background, by thinking about the functional application of conductive polymer, the development to bioelectronics is also expected sufficiently. For example, thinking to use a conductive polymer as metal, namely an electrode, conductive polymers and the compatibility with the biocell become important. If such conductive polymers have a high biocompatibility compared with the metal, the functional application in biomedical engineering as a new artificial internal organmaterial is expected. A new progress and the progress of the nerve interface technology based on the development of the inside of the body burial type nerve stimulation electrode used from being conductive for the artificial sight, the artificial hearing or the artificial retina, etc. are thought. In developing the conductive polymers mentioned above as bioelectronic materials, the compatibility with the most basic and important conductive polymers and biocells have been reported in this article. To date, various conductive polymers have been synthesized, but poly(3,4-ethylenedioxythiophene),

![Polypyrrole : PPy](image1.png)

![Poly(3,4-ethylenedioxythiophene) : PEDOT](image2.png)

**Figure 1.** Examples of conductive polymer with good environmental stability.
PEDOT, the molecular structure of which is shown in Fig. 1, is the most stable after doping and is one of the most attractive materials, together with polyaniline (PAn) and polypyrrole (PPy), because of its good environmental stability and relatively high electrical conductivity. Then, to develop nerve stimulation electrodes of the inside of the body burial type, PPy and/or PEDOT films of biocompatibility were mainly evaluated in the present study. For the biocompatibility of PPy and PEDOT films, the neuronal cell culture experiment was performed and evaluated. Fibroblastic cell (L929) removed from the mouse on the fabricated PPy and/or PEDOT films were cultured, and their survival and growth were observed. In addition, the culture experiment was also performed on myoblast (C2C12) removed from a mouse.

2. Experimental procedure

2.1. Fabrication of conductive polymers

In general, electrochemical polymerization technique is performed with the electrochemical polymerization equipment shown in Fig. 2. Though details of the electrolysis polymerization method are entrusted to technical books, the reaction mechanism of conductive polymer synthesis by electrochemical polymerization has not been clarified, because various factors, such as the composition of the electrolyte and the electrolytic conditions, interact very complexly with the electrode reaction. Therefore, the polymerization reaction condition differs for each individual conductive polymer and the optimum conditions are determined through trial and error. The mechanisms of the electrolysis polymerization reaction are not yet fully clarified under the present conditions, and qualitatively, both the parent electron substitution coupling reaction and the radical coupling reaction are accepted in general. At all events, in the electrolysis polymerization, he dynamic movement of the molecule, which takes part in the monomer, and the electrolytic ion formed by the transfer of an electron from the solvent are caused at the interface between the electrode and the electrolyte.

![Figure 2. Schematic diagram of electrochemical polymerization equipment.](image)

2.1.1. Fabrication of polypyrrole, PPy films[5]

The electrochemical polymerization was performed using an ITO (indium tin oxide) -coated conductive glass substrate with a working electrode, a nickel (Ni) plate as a counter electrode and a silver (Ag) wire as a reference electrode of the electrochemical cell as shown in Fig. 2. The composition of the polymerization liquid used for the electrolysis polymerization is 0.3 mol/l pyrrole (Tokyo Chemical Industry Co. Ltd.) as the monomer, and tetra-n-butylammonium tetrafluoroborate (TBABF₄, Tokyo Chemical Industry Co., Ltd. (TCI)), sodium p-toluenesulfonate (p-TSNa, TCI), tetra-n-butylammonium hydrogen sulphate (TBAHSO₄, TCI), tetra-methylammonium p-toluenesulphonate (TMA-p-TS, TCI), tetra-n-butylammonium bromide (TBABr, TCI), sodium n-dodecylbenzenesulphonate, LAS, TCI), tetra-n-butylammonium chloride (TBACl, TCI) etc. as the supporting electrolyte. The concentration of supporting electrolyte is changed within the range of 0.3–1.0 mol/l and then distilled water used for the solvent. The electrolysis polymerization was performed
using a potentiostat (Hokuto Denko Inc., HSV-100), and the amount of passing electric charge at the polymerization potential 1.5 V (vs. Ag/Ag⁺) was measured. Moreover, the thickness of the obtained PPy film was measured by the multiple interference method using a multiple interference microscope (Olympus, USPM-RU). The electrical conductivity of the obtained PPy film was about 30–50 S/cm.

2.1.2. Fabrication of poly(3,4-ethylenedioxythiophene), PEDOT films [5]
Jonas and Schrader [3] found that poly(3,4-ethylenedioxythiophene), PEDOT, with the molecular structure shown in Fig. 1, is extremely transparent and its stability is excellent under a typical environment. When the conductive polymer is actually applied, the environmental stability becomes extremely important. It is considered that doped PPy and polythiophene have excellent stability compared with that of positively charged polyacetylene, because of the stabilization effect of the positive charge with the nitrogen atom and the sulfur atom. On the other hand, because of the β position in PEDOT is oxidized easily and blocked by the ethylenedioxy groups, this material has high thermal stability and is excellent in high temperature and stable for a long time compared with PPy.

The electrolysis polymerization was performed by using ITO conductive glass substrate as a working electrode, Ni plate as the counter electrode and Ag wire as the reference electrode of the electrochemical polymerization device shown in Fig. 2. The composition of the polymerization liquid used for the electrolisis polymerization is 0.1 mol/l 3,4-ethylenedioxythiophene (Tokyo Kasei Inc., purity 98%) as the monomer and tetra-n-butylammonium tetrafluoroborate (TBABF₄, Tokyo Chemical Industry Co., Ltd.) as the supporting electrolyte. The concentration of supporting electrolyte is changed within the range of 0.3–1.0 mol/l. Then acetonitrile (Wako Pure Chemical Industries, Ltd., purity 99.5%) was used for the solvent. The electrolysis polymerization was performed using a potentiostat (Hokuto Denko Inc., HSV-100), and the amount of passing electric charge at the polymerization potential of 2.0 V vs. Ag/Ag⁺ was measured and the PEDOT film of about 400 nm in thickness was mainly obtained. The electrical conductivity of the obtained PEDOT film was about 120–200 S/cm.

2.2. Electrochemical properties of PEDOT films

![Oxidized state](image1)  \(\rightleftharpoons\) Reduced state

**Figure 3.** Color change of PEDOT film based on redox reaction.

![Absorbance vs. Energy](image2)

**Figure 4.** Optical absorption spectral change of PEDOT film taken during electrochemical doping.
In general, it is well known electrical, optical or electrochemical properties of conductive polymers changed suddenly owing to the reversible doping. Fig. 3 shows the color change based on the redox reaction of PEDOT films. The PEDOT film is light blue or almost transparent in the doped state, and deep blue in the undoped state. And then, the color change is reversible in the PEDOT films.

On the other hand, Fig. 4 shows the change in the optical absorption spectrum of the PEDOT film with the doping potential. The films in the neutral and reduced states show strong absorption in the visible region and are deep blue in color. With increasing doping potential, the optical absorption in the visible region decreases and the film becomes light blue or transparent in the oxidized state from deep blue.

2.3. Subculture of cells

The subculture is to transfer the cells to other culture vessels. In this experiment, the subculture of fibroblast L929 and myoblast C2C12 was carried out respectively according to the following procedures. The dish used for the cell culture is a dish covered without collagen in fibroblast L929 culture and with collagen in myoblast C2C12. Fig. 5 shows the appearance of the cell culture.

![Figure 5. Appearance of cell culture.](image)

2.3.1. The subculture of fibroblast L929 of mouse uniting organization origin

- The old nutrient medium is pulled out from the dish.
- The cell is washed adding PBS 5 ml (phosphate buffered saline).
- PBS is removed by sucking and because of peeling off of the cell that sticks at the bottom of the dish, 3 ml of trypsin solution is added and then the dish is left for about 5 min every time the CO2 incubator reaches 37°C.
- Nutrient medium Dulbecco's Modified Eagle's Medium (DMEM) containing serum 10% Fetal Calf Serum (FCS) content for amplification was added to the dish covered without collagen and the fibroblast L929 was cultured.

2.3.2. The subculture of fibroblast L929 of mouse uniting organization origin

- The old nutrient medium is pulled out from the dish.
- The cell is washed adding PBS 5 ml (phosphate buffered saline).
- PBS is removed by sucking and because of peeling off of the cell that sticks at the bottom of the dish, 3 ml of trypsin solution is added and then the dish is left for about 5 min every time the CO2 incubator reaches 37°C.
- This solution was putted in conical tube (BD Falcon) and the cells were precipitated by the centrifugal separator, and then trypsin solution was pulled out from the conical tube.
- Nutrient medium Dulbecco's Modified Eagle's Medium (DMEM) containing serum 10% Fetal Calf Serum (FCS) content for amplification was added to the dish covered with collagen and the myoblast C2C12 was cultured.

In the culture experiment on the conductive polymer film, PPy and PEDOT were washed enough without peeling off from the ITO conductive glass substrate with distilled water, acetonitrile, and...
ethanol, in order to remove impurities adhered to the surface of the conductive polymer film and included impurities (oligomer etc. generated by unreactive monomer and the reactive process) during the electrolysis polymerization, and were dried. Conductive polymer coating ITO conductive glass substrate was put in the dish, and the subculture of the abovementioned was executed.

3. Experimental results and discussion

3.1. Culture experiment of fibroblastic cell L929

The fibroblastic cell is one of the cells that compose the mouse uniting organization, and its cytoplasm shows that nucleoli has clear oval nucleus and shows a base good nature. In this experiment, fibroblast L929 cells of the mouse uniting organization origin were used. Cultures were carried out in a humidified incubator with 5% CO2/95% air at 37 °C for several days. Fig. 6 shows the photograph of the cell observed with a headstand microscope (Nikon, TE2000-U) after 24 h sowing the fibroblast L929 cell to the dish. Fibroblast L929 cell bonding on the dish surface and progress to fusiform were observed.

Fig. 7 shows the appearance of fibroblastic cell L929 culture. (a) on dish without collagen, (b) on PPy doped with TBABr and (c) on PEDOT doped with TBABF4. From Fig. 7 (a), the cell proliferates on the dish surface and to all aspects of the dish in 96 h. On the other hand, from Fig. 7 (b) and (c), fibroblast L929 exhibited good adhesion on PPy doped with TBABr- and PEDOT doped with TBABF4-coated ITO surfaces as well as the culture experiment that uses the dish. After 24 h, it is understood that the cell is bonded on conductive polymer films and proliferates. Ninety-six hours later, the cell proliferated to all aspects of the conductive polymer films, PPy and PEDOT, respectively. Though dopants that conductive polymer films possess differ from TBABr in the PPy film and TBABF4 in the PEDOT film, it seems that the kind of dopants don't influence cell proliferation.

Figure 6. A macro photograph of fibroblast L929.

Figure 7. Appearance of fibroblastic cell L929 culture.
In microbiology, colony-forming unit (CFU) is an index of viable cell numbers and generally is given as CFU/ml (colony-forming units per milliliter) for liquids and CFU/g (colony-forming units per gram) for solids. In this study, the number of cells for each unit area was evaluated from this observation photographs in Fig. 7.

![Graph showing cell number versus time for fibroblast L929 and culture days.](image)

**Figure 8.** Relation between number of cells for each unit area of fibroblast L929 and culture days.

Fig. 8 shows the relation between number of cells for each unit area of fibroblast L929 and culture days. As shown in this figure, in the case of the culture on dish, though the content of the cell for each unit area is slightly a lot of, a remarkable difference between TBABr dope PPy and TBABF4 dope PEDOT cultured cells was not observed in four day culture. The doubling time of the number of cells evaluated from the inclination of this curve was about 23–28 h in the case of the culture on dish. It is reported that the doubling time becomes about 23 h on a collagen coating dish on the market [7]. On the other hand, the doubling time is evaluated for about 25 h on both PPy and PEDOT films obtained by electrolysis polymerization method, and it is considered that these conductive polymers are excellent culture substrates as well as the marketed culture dish.

3.2. Influence of concentration of supporting electrolyte on cell proliferation

![Graph showing cell number versus concentration.](image)

**Figure 9.** Relation between number of cells for each unit area and concentrations of supporting electrolyte.
Fig. 9 shows the relation between number of cells for each unit area of fibrolast L929 culture and the concentration of electrolyte, which evaluated from the observation result after 4 days culture on PPy doped with Cl\(^-\). The composition of the polymerization liquid used for the electrochemical polymerization is 0.3 mol/l as the pyrrole as the monomer and TBACl as the supporting electrolyte and then acetonitrile (Wako Pure Chemical Industries, Ltd.) was used for the solvent. Though the number of culture cells is in the range of about 6~10\(\times\)10\(^{-4}\) pieces/cm\(^2\) and has decreased in the low concentration, thinking about the influence of turbulence like the adhesion of the PPy film and conductive ITO glass substrate etc., the proliferation of the cultured cell doesn't depend on the concentration of the supporting electrolyte.

3.3. Influence of the kind of supporting electrolyte on cell proliferation

In Section 3.1, it was described that the influence of the dopant that the conductive polymers possess seems to be not in the cell culture. Fig. 10 shows the culture experimental results that used the PPy film to understand the influence by the kind of the dopant on the proliferation of the L929 fibroblastic cell.

![Figure 10. Relation between number of cells for each unit area and kinds of supporting electrolyte.](image)

The supporting electrolyte used for the electrolysis polymerization is five kinds of LAS, TBAHSO\(_4\), TBABF\(_4\), TBACL, and \(p\)-TSNa. The concentration of supporting electrolyte is constant with 0.3 mol/l. The number of cells for each unit are after four days culture increases in the PPy film doped with TBAHSO\(_4\), and the cell proliferation is apparently active. However, almost the same number of cells is obtained in the PPy film including other four kinds of dopants, and there is no difference between kinds of supporting electrolyte on the cell proliferation. However, the culture of the cell is greatly influenced also by adhesion of the ITO conductive glass used as a substrate and the PPy film. It is not possible to conclude from an actual experiment result alone. Many kinds of supporting electrolytes are used considering the problem of adhesion and a more detailed examination is necessary.

3.4. Myoblast C2C12 culture experiment

The myoblastic cell is the cell of a single nucleus that becomes an origin of the mouse myotube fiber, and when a lot of these cells fuse and form the syncytium, the myotube fibrocyte is formed. The cytoplasm of the myotube fiber is called sarcoplasm and is occupied in myofibril [8]. Myoblast C2C12 of the mouse skeletal muscle origin starts differentiation to a myotube fibrous cell by lowering serum density in culture fluid. Therefore, to differentiate the myoblast into myotube cells, culture fluid was changed for DMEM where a myoblast contained serum 7%HS (House Serum) for differentiation instructions in a subconfluent state (the state that multiplied to around 80% of the dish base) [9]. The culture fluid is changed every two days, and cell cultures were carried out in incubator under the environment of 100% of humidity with 5% CO\(_2\)/95% air at 37\(^{\circ}\)C for several days. The morphological change of cell according to cell culture period was observed by using a headstand microscope (Nikon, TE2000-U).
Fig. 11 shows the appearance of the C2C12 myoblastic differentiation culture observed by using the PPy film polymerized from the electrolyte with the concentration 1 mol/l p-TSNa and TBACl as the supporting electrolytes. As is obvious from this figure, a differentiation instruction occurs after the subconfluent, and the myoblastic culture on the PPy film forms a myotube fibrous cell. In addition, for C2C12 myoblastic culture and a differentiation instruction, the influence of p-TS and the Cl dopant is not recognized.

Fig. 12 shows the appearance of the differentiation culture of the C2C12 myoblast observed by using PEDOT film fabricated from supporting electrolyte TBABF$_4$ of 0.3 mol/l in the concentration. As for the myoblastic culture on the PEDOT film, a differentiation instruction is not clear by the dirt of the lens from the appearance after for four and six days progress after subconfluent. However, from the appearance after 8 days progress, the occurrence of differentiation instruction and the formation of myotube fibrous cell are clear and BF$_4$ dopant does not influence the culture of the C2C12 myoblast and a differentiation instruction as well as PPy film. It is considered that these conductive polymers such as PPy and/or PEDOT films obtained by electrolysis polymerization method are excellent culture substrates and superior in biocompatibility as well as the marketed culture dish.

4. Summary
Fibroblast L929 and myoblast C2C12 cells of the mouse connective tissue origin were sown on the surface of conductive polymer films (PPy and/or PEDOT) in the cell culture medium, and the proliferative process of these cells was observed. Without changing the form, fibroblast L929 and myoblast C2C12 cells were observed to proliferate almost similarly to the cell which cultured on a
dish on the market and to maintain compatibility. In addition, it was able to confirm that the myoblast C2C12 caused an induced differentiation on these conductive polymers films after the subconfluent and formed a myotube fibrous cell. In other words, two kinds of conductive polymers used in this study, PPy and PEDOT films maintained the secretion function of the cell cultured at the surface of these polymers. Therefore, the PPy- and the PEDOT-coated electrode suggested the possibility usable as a nerve stimulation electrode with biocompatibility because these polymers were effective to culture the cell.

References
[1] Yoshino K and Onoda M 1996 Polymer Electronics 266-367 (Japan-Corona) (in Japanese)
[2] Onoda M “Plastics through the electricity and electronics application” 2010 Polyfile 47, No.559 22-27 (in Japanese)
[3] Onoda M “Expectation and problem to application as molecular electronics” 2011 Industrial Materials 59 No.8 42-46 (in Japanese)
[4] James C D Davis R Meyer M Turner A Turner S Withers G Kam L Banker G Craighead H, Isaacson M Turner J and Shain W “Aligned Microcontact Printing of Micrometer-Scale Poly-L-lysine Structures for Controlled Growth of Cultured Neurons on Planar Microelectrode Arrays” 2000 IEEE Transactions on Biomedical Engineering, 47 No. 1 17-21
[5] Abe Y Mathur P C Bhatnagar P Tada K and Onoda M “New fabrication technique of conductive polymer/insulating polymer composite films” 2008 IEE Journal of Transactions on Fundamentals and Materials 128 No.12 703-709 (in Japanese)
[6] Jonas F and Schrader L “Conductive Modification of Polymers with Polypyrrole and Polythiophene” 1991 Synthetic Metals 41-43 831-836
[7] Yamauchi K, Maniwa M Mori T “Cultivation of fibroblast cells on keratin-coated substrata” 1998 Journal of Biomaterials Science, Polymer Edition 9 No.3 259-270
[8] Gerard J Tortora Bryan Derrickson 2010 “Principles of Anatomy and Physiology (12th Editions). Translated by Kuwaki T Kurosawa M Takahashi K and Hosotani Y 3rd Editions 302-308 (Japan-Maruzen) (in Japanese)
[9] Kubo Y “Comparison of initial stages of muscle differentiation in rat and mouse myoblastic and mouse mesodermal stem cell lines” 1991 Journal of Physiology, 442 No.1 743-759