JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 429–433

A preliminary assessment of poly(pyrrole) in nerve guide studies

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This study investigates the biocompatibility of polypyrrole, a conducting polymer, and comments on its potential as an effective guidance channel for the regeneration of nervous tissue. The polymer was prepared in our laboratories by an electro-polymerization process. Pyrrole is placed in an electrolyte and when a potential is applied polypyrrole is deposited at the anode. After polymerization the polypyrrole is easily removed from the anode. Extraction in methanol for a period of 1 week was carried out to remove residual electrolyte. The biocompatibility of the material was assessed *in vitro* and *in vivo*. The response of two cell lines growing in contact with the polymer was evaluated. L929 mouse fibroblast and neuro2a neuroblastoma cells contacted the polypyrrole in a specially constructed cell culture chamber which allowed a controlled current to pass through the material. *In vivo*, the material was evaluated following implantation into a rat model.Furthermore, the effect of charge on the cell lines was examined using the same cell culture chamber, but substituting platinum wire for the polypyrrole. Finally, the polypyrrole was deposited directly onto the platinum wire and introduced to the cell culture chamber. The results demonstrate that the polypyrrole is cytocompatible *in vitro* if prepared by appropriate extraction techniques. *In vivo* there was only a minimal tissue response after 4 weeks *in situ*. The cell culture chamber model proved successful and allowed a current up to 1 mA to be applied across the polypyrrole or platinum wire while in contact with both cell lines. Some evidence of toxicity was evident when a current of 1 mA was applied across the polymer for periods up to 96 h. However, it is clear from these experiments that polypyrrole can be an effective medium for carrying current in a biological environment.

1. Introduction

The repair of severed nerves poses a significant clinical problem although it is recognized that the peripheral nervous system is capable of repair. Over recent years it has been established that the attachment of the distal and proximal stumps of the nerve into either end of a tube can greatly increase the degree of regeneration and performance of the repaired nerve.

Two principle techniques are employed experimentally in attempts to influence the repair of severed peripheral nerves. Synthetic materials are used to form guidance channels and bioelectric fields are employed to influence the behaviour of the nerve cell. A number of materials have been investigated for use as a tube or guidance channel to encourage the joining of distal and proximal stumps of damaged nerves. Further studies have shown the effect of electric fields on the growth and orientation of nerve cells. *In vitro* and *in vivo* techniques have demonstrated an increase in the growth of neurones, however, the exact role of the bioelectric field remains unclear.

Many materials have been investigated for use as the tube or guidance channel and probably the most well studied of these is silicone rubber [1]. Tubulization is thought to provide advantages for the repair of nerves by aiding the guidance of the growing fibres by mechanical orientation and confinement and by reducing the infiltration of fibrosis and scaring. It is also thought that it helps to contain growth and tropic factors in the vicinity of the growing axons [1]. It has been suggested [2] that perm-selective materials, which allow exchange of nutrients and growth and trophic factors with waste products, but restrict the migration of cellular material into the area between the two stumps, encourages growth.

In vitro studies [3] have shown that if primary nerve cell cultures are subjected to an electric field there is an increase in the rate of growth of the neurite towards the cathode rather than the anode. An *in vivo* study [4] has shown that the implantation of silver/silver chloride wick electrodes either side of sciatic nerve lesions in rats, and the passage of DC current between the electrodes resulted in a significant improvement in the regeneration of the nerve assessed by the recovery of the motor function. Guidance channels constructed from piezoelectric polymer materials such as polyvinylidene fluoride (PVDF) [5], and vinylidene fluoride–trifluoroethylene copolymer [6] have been shown to enhance neural regeneration in mice. *In vitro*, when mouse neuroblastoma cells were seeded

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directly onto electrically poled PVDF there was enhanced neurite outgrowth [7]. In a similar study [8], fluorinated ethylenepropylene films were treated with a coronal charging process thus generating positively or negatively charged surfaces and it was shown that significantly higher levels of neurite outgrowths were observed on the positive surfaces rather than the negative or uncharged substrates.

Polypyrrole is a conducting polymer which can be produced simply in the laboratory by electro-polymerization onto a stable conducting material from an electrolyte containing the monomer pyrrole [9]. Studies [10, 11] on the electrochemical properties of polypyrrole have demonstrated, using cyclic voltametry, that, although complex, the potential can be repeatedly cycled without degradation indicating a chemical reversibility, if not a strictly electrochemical reversibility.

The conductivity and mechanical properties of the polypyrrole depend on the exact conditions and electrolyte mixture during polymerization [12]. In this way it is possible to design a polymer with specific properties for the application intended.

A preliminary study has been carried out to investigate the biocompatibility of polypyrrole and its potential as a guidance channel for the regeneration of nerves. It has been shown that stable, self-supporting sheets of this material which rapidly reach a stable rest potential in phosphate buffered saline can be manufactured. This material was evaluated using conventional biocompatibility assessment techniques using a specially constructed cell culture chamber which allowed two growing cell lines to contact a biomaterial carrying a current.

2. Materials and methods

2.1. Preparation of polypyrrole

Pyrrole (Aldrich Chemicals) was slightly yellow when purchased, and was distilled to collect a clear liquid at 131 °C, then stored at 4 °C until required.

The electrolyte is prepared using 7 g tetra–ethyl–ammonium p-toluene sulphonate, dissolved in 100 mL acetonitrile (Aldrich). To this is added 3 ml of pyrrole. A simple electro-chemical cell was prepared with a titanium sheet as the cathode and initially a gold-coated histology slide as the anode. Subsequently a fine platinum wire (0.2 mm diameter) was used as the anode. A current of 0.08 A was applied between the electrodes and polymer film was allowed to form for approximately one hour. This provided a free-standing film that could easily be stripped away from the glass anode. With the platinum electrode, one hour produced an uneven, thick coating of polymer, and it was found that a period of approximately five minutes was sufficient to produce an effective coating. After polymerization the polypyrrole was maintained in 100% methanol until it was required for cell culture experiments.

2.2. Biocompatibility of polypyrrole

The biocompatibility of the prepared polymer was assessed using *in vitro* and *in vivo* techniques. *In vitro* the material was examined in direct contact test and using an MTT assay. Both tests were carried out using standard operating procedures well established in our laboratories.

Preparing samples

(a) Rinse in a stream of water for 1 h.
(b) Immerse in 5% detergent solution for 3 h.
(c) Place the solution in sonic bath for 30 min.
(d) Transfer to distilled water and place in sonic bath for 30 min.
(e) Rinse in distilled water and dry.
(f) Double pack the samples and sterilise by autoclave.
(g) Dry in hot oven and store in dry conditions.

Preparing aqueous extracts

(a) Prepare samples as detailed above.
(b) Add 10 ml of sterile, distilled water to glass test-tube.
(c) Add test samples to provide total surface area of 200 mm^2.
(d) Seal test-tubes with poly (propylene) lined caps.
(e) Store at 80 °C for 1 week.
(f) Invert tubes every 24 h.

Passaging

(a) Discard the medium from a 25 cm^2 culture flask of confluent cells.
(b) Rinse the cells (L929 mouse fibroblast) with Ca/Mg-free phosphate-buffered saline.
(c) Replace the PBS with 2–3 ml of 0.25% trypsin solution.
(d) Incubate at 37 °C until the cell monolayer is almost free from the surface.
(e) Replace the trypsin with 10 ml full growth medium and make a suspension by gentle pipetting.
(f) Count cells if required using a haemocytometer.
(g) Transfer appropriate aliquot of the cell suspension to fresh culture medium in a sterile 25 cm^2 flask.
(h) Incubate until confluence, replenishing the medium if necessary.

Direct contact test

(a) Using a sterile spatula, place a spot of Silastic adhesive into the centre of a 35 mm Petri dish.
(b) Push a test sample into the adhesive and allow to cure (1–3 h).
(c) Passage cells as described above and prepare an appropriate cell suspension in fresh culture medium (5×10^4 cells ml^{-1}).
(d) Add 3 ml of this suspension to the Petri dish and incubate at 37 °C.
(e) Examine cells by phase contrast microscopy at 24 and 48 h.
(f) Compare cell growth and morphology in the test plates with that in control plates (silicone only).
(g) At 48 h prepare photomicrographs as appropriate.

This type of test is readily evaluated qualitatively using a phase contrast microscope. The plates were examined for any evidence of toxins leaching from the test material and adversely affecting the cells. Observations can be directly compared with controls, and photomicrographs prepared. The silastic adhesive

itself was used as a non-toxic control. Flat sheet samples were cut to 5 mm^2 pieces, washed in a sonic bath for 30 min in distilled water then autoclave-sterilized. Five samples of each material were examined.

The MTT assay is a sensitive, reliable and quantitative measure of cell viability and has proved useful in accurately examining the cytotoxicity of a variety of biomaterials. Extracts of the material under the test are readily examined. The appropriate extracts are directly introduced to the test plate.

Procedure

(a) Cells are prepared in a 96-well microtitre plate in full growth medium (200 µl per well).
(b) When close to confluence the culture medium is removed from all wells of the plate and replaced with dilutions of the extract prepared in growth medium.
(c) Twelve wells are used for each extract dilution and for growth medium controls.
(d) The extract and control medium remain in contact with the cell line for 24 h at 37 °C.
(e) After this time the culture medium/extract is removed and replaced by the MTT salt (100 µl).
(f) The plate is incubated for 4 h at 37 °C.
(g) The MTT is removed and replaced by isopropanol (50 µl) and spectrophotometric readings taken at 570 nm on a multi-well plate reader.

In this case the extracts were examined as a 50:50 dilution in × 2 strength culture medium. Reduction of tetrazolium to formazan can be visualized by the addition of isopropanol to form a coloured product. Plates are then transferred to a spectrophotometer. The optical density is directly proportional to the number and activity of viable cells present.

The material was further examined in a rat model. Again, the test method followed a well-established standard operating procedure. Two samples were placed in each animal and two animals were used for 2-week and 4-week time periods. At the end of the appropriate time period, animals were sacrificed and a block of muscle containing the implant was excised and fixed. Thin sections were prepared from each block, and examined using conventional histology.

2.3. Cellular response to activated polymer

A simple model was designed to allow monitoring of the response of cells to a biomaterial carrying a current. Two cell lines were employed; L929 and mouse neuro 2a neuroblastoma cells (ICN-Flow, UK). The test cell consisted of a 24-well tissue culture plate modified to allow a 0.2 mm platinum wire to pass through eight of the individual wells. The test model is detailed in fig. 1. The platinum wire (or polypyrrole coated wire) does not make direct contact with the cells, but passes a few millimeters above the substrate to which the cells are adhered. The response of the cells immediately beneath the wire can be determined using an inverted phase contrast microscope. At the end of the experiment, the wire is readily removed and the cells stained and examined. Throughout the period

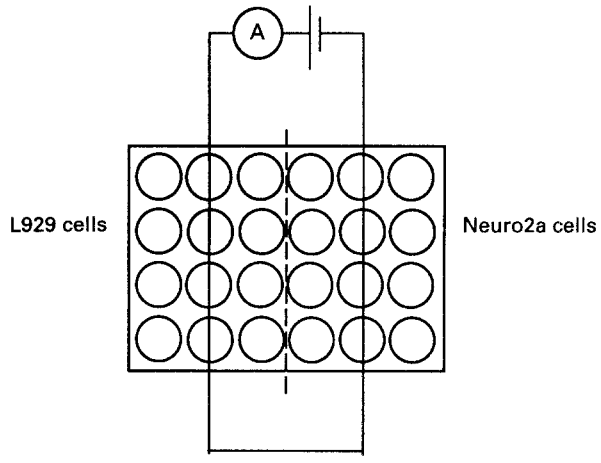

Figure 1 Test model designed to allow a current to be applied to two cell lines via a platinum wire or polypyrrole coated wire. Twelve wells, as illustrated, hold L929 mouse fibroblasts, and twelve hold neuro 2a mouse neuroblastoma cells. A current of 1 mA was applied throughout the course of the experiment and the test model was maintained during that time at 37 °C/5% CO_2. Cells were examined using phase contrast microscopy at various time periods up to 96 h. At this time the culture medium was removed and the cells stained.

of the experimental procedure the test chamber was maintained at 37 °C/5% CO_2. The wells on either side of those carrying the wire act as controls. Initial experiments examined the cell response to the platinum wire carrying a current of 1 mA at time periods up to 96 h. Jaffe and Poo [3] employed current in the range 0.3–3 mA. For this preliminary study a current in the middle of this range was selected. Subsequently, the response to polypyrrole coated wire carrying the same current was examined over the same time period.

These tests were carried out to determine if the cytocompatibility of the material is compromised when it is carrying a small current, and to determine further if the cell lines employed are observed to display any morphological changes in response to the current.

3. Results

3.1. Biocompatibility of polypyrrole

Biocompatibility tests on the non-activated polymer demonstrated that it was not cytotoxic when assessed by direct contact or MTT assay. No toxicity was observed in the direct contact test plated after the material had been extracted in methanol for a period of 24 h. MTT assay produced results similar to the culture medium controls. *In vivo* only a minimal inflammatory response was observed after 4 weeks implantation (Fig. 2). No evidence of tissue or cellular necrosis was noted. The implanted samples were also examined in the non-activated state.

3.2. Cells and charge

These experiments examined the cell response to a current of 1 mA carried on a 0.2 mm platinum wire. The cells were exposed to the current for time period up to 96 h. Occasionally throughout the experiment

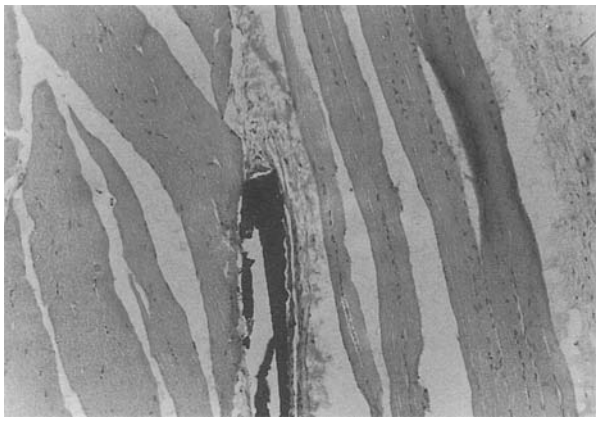

Figure 2 Tissue response to a polypyrrole implant after 4 weeks *in situ*. There is no evidence of cell necrosis or adverse cellular response. The inflammatory response stimulated by the implant is minimal.

the power was switched off for a period up to 1 h and the cells examined by phase contrast microscopy. Controls were examined at the same time periods. No abnormal cellular responses were observed at any of the time periods examined. Cells grew to confluence in the wells containing the charged wire in an identical manner to those in the control wells. There was no evidence of cell death, even at the closest point between the cells and the wire. No significant morphological changes were apparent with either cell line.

3.3. Cells and activated polymer

For these experiments, the polypyrrole was polymerized directly onto the platinum wire. This procedure proved successful, and the platinum became completely coated with a robust layer of the polymer, which was measured as 0.025 mm thick. The polymer-coated wires were then extracted in methanol to remove any residual electrolyte. The polypyrrole successfully carried the applied current over the course of the experiment. The result was broadly similar to that obtained with the platinum wire alone. In most cases the cells continued to grow to confluence, as did the controls, with the cells growing right up into contact with the wire (Fig. 3). However, in a few of the wells which held the activated polymer, there was some evidence of inhibition of the cell growth although this was patchy. There was no evidence to the naked eye or by microscope, that the polymer was degrading or breaking up in the culture medium.

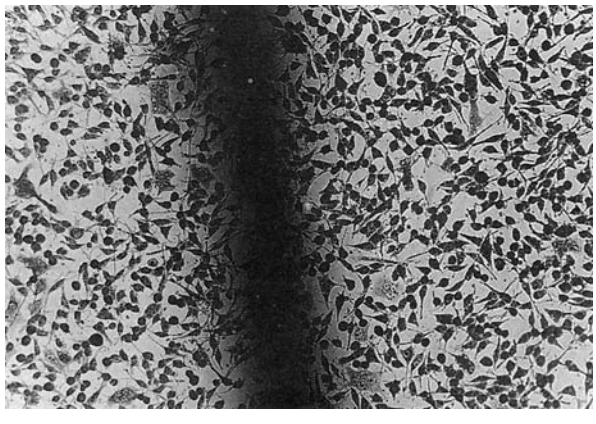

Figure 3 Cellular response to the activated polypyrrole after 96 h. The cells (L929) have been stained by crystal violet.

4. Discussion

This study was designed to evaluate the potential application of polypyrrole, a conducting polymer, for the construction of nerve guides. Many studies have demonstrated the advantages of encasing the proximal and distal ends of a severed nerve within a connecting tube [1]. A few studies have also demonstrated that the application of a current between the ends can enhance the repair of the nerve [4]. The philosophy behind this study was that polypyrrole may be able to perform both these functions as a polymer nerve guide which could carry a current and thus enhance the repair of the nerve.

It was necessary, in the first instance, to establish the biocompatibility of the polypyrrole and ensure that it did not breakdown *in vivo* or *in vitro* with the release of toxic substances. The second part of the study investigated if the biocompatibility was compromized by the passage of a small current through the polypyrrole. In addition, investigations were made to determine if the presence of the polymer carrying the current had any effect on the morphology of the cells *in vitro* since previous studies [7, 8] have shown that charged surfaces can induce neurite outgrowths on mouse neuroblastoma cells. The same cell line was used in this study.

It has been demonstrated in this study that a stable polymer can easily be produced in the laboratory in a variety of forms. The actual mechanical and electrochemical properties of polypyrrole can be altered very simply by changing the electrolyte and conditions of polymerization. These were kept consistent in this preliminary study but further work will optimize these to develop a material more suitable for specific applications.

The initial material evaluated here, exhibited good biocompatibility, both *in vitro* and *in vivo* under non-activated conditions. It was found that when a small current was passed through the polypyrrole *in vitro*, the cells continued to grow until contiguous with the polypyrrole in most cases, however, the cellular response was affected in a few places with evidence of slight growth inhibition. No influence of the current on the morphology of either the L929 or neuroblastoma cell lines was observed. Further work is now in progress to change the electrochemical conditions within the test cell chamber, particularly to place the material in direct contact with the cells and to examine a range of current values and time periods.

In conclusion, the polypyrrole does exhibit good biocompatibility if appropriately prepared. The cell culture test model proved successful and can readily be adapted to allow a number of variables to be examined in further studies. The results obtained show that polypyrrole can be an effective medium for carrying current in a biological environment.

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