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Communication

Biocompatibility of polypyrrole particles: an in-vivo study in mice

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Abstract

The objectives of this study were the chemical synthesis of polypyrrole particles, the investigation and estimation of the impact of polypyrrole particle concentration, and the evaluation of the effect of duration of treatment on immune-related haematological parameters and peritoneum cells in mice. The results showed that chemically prepared polypyrrole particles did not have any detectable cytotoxic effect on mouse peritoneum cells. Polypyrrole particles did not induce any allergic response, nor did they affect spleen, kidney or liver indexes. Moreover, no effect of polypyrrole particles on immune-related haematological parameters was observed. No inflammation was detected in the peritoneum of mice after a 6-week period of treatment with polypyrrole particles. In conclusion, chemically synthesized polypyrrole particles showed good biocompatibility in mice and are attractive candidates for biomedical applications in-vivo.

Introduction

Electrically conducting polymer polypyrrole (Ppy) has been investigated for numerous applications. Previous studies have used this polymer and its composites with other materials in analytical and biological sensing devices (Ramanaviciene & Ramanavicius 2002), as a suitable substrate for cell attachment, in the manipulation of mammalian cell growth and function (Aoki et al 1996; Garner et al 1999), as a promising candidate for tissue engineering and wound-healing applications (Collier et al 2000), and to provide bioelectric fields in cultures of nerve cells (Schmidt et al 1997). Among various electrically conducting polymers, Ppy has been the most widely studied material for potential biomedical applications (Tessier et al 2000), mainly because of its relatively high environmental stability, unique electrical properties (Ramanavicius et al 1999), and because it can be easily synthesized chemically as a black powder and electrochemically as thin films on various electrodes from aqueous media (Wang et al 2003; Ramanaviciene & Ramanavicius 2004b). Moreover, electrically deposited Ppy may be doped with various dopants to advance its physical, chemical and electrical properties (Masuda & Asano 2003; Ramanaviciene & Ramanavicius 2004a; Li et al 2005). Ppy-based particles belong to the unique class of materials with potential applications in optical/visual immunodiagnostic assays due to their deep black colour and intense optical absorbance.

The facile preparation of Ppy in aqueous media and its surface modification by various specific functional groups makes this polymer particularly suitable for the covalent attachment of proteins (Benabderrahmane et al 2005). Ppy-coated glucose oxidase nanoparticles and other polymeric nanoparticles are attractive candidates for biomedical applications in-vivo (Ramanavicius et al 2005). Based on the unique properties and versatile applications of this conducting polymer, the biocompatibility of Ppy has been extensively studied in several in-vivo and in-vitro systems. The effect of Ppy on nerve tissue, endothelial cells, osteoblasts, muscle, hypodermic tissues and brain tissue have been evaluated (Garner et al 1999; De Giglio et al 2000; Jiang et al 2002; Wang et al 2003). The extracted solution of chemically prepared Ppy powder possesses neither acute nor subacute toxicity, it does not change body temperature, cause haemolysis of red blood cells, cause allergic responses such as nose scratching, piloerection, dyspnoea and spasm, or cause mutagenesis of cells (Wang

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et al 2003). It is therefore important to test the influence of this polymer on the immune system of model animals, on immune-related haematological parameters and peritoneum cells (Ramanaviciene et al 2004).

Our primary objectives in the present study were: (i) the chemical synthesis of Ppy particles; (ii) investigation and estimation of the impact of the Ppy particle concentration; and (iii) evaluation of the effect of treatment duration on immune-related haematological parameters and peritoneum cells in mice.

Materials and Methods

Synthesis of Ppy particles

Pyrrole (Acros Organics, Geel, Belgium) was purified additionally before polymerization by passing through a 5-cm length Al_2O_3-filled column until it became colourless, and was stored at 4°C in the dark before use. Other chemicals were of analytical grade and were used as received. All solutions were prepared using deionized water purified with a Millipore S.A. water purification system (Molsheim, France). To prepare 100 mL of reaction mixture we used 1.74 mL of pyrrole, 1.7 mL of 30% hydrogen peroxide and 96.56 mL of 0.05 M phosphate buffer pH 2. Thus, the polymerization bulk solution consisted of 0.05 M phosphate buffer pH 2 with 250 mM pyrrole and 150 mM H_2O_2. The mixture was kept at a constant temperature of 23°C for 48 h. The black precipitate formed, based on chemically synthesized Ppy particles, was thoroughly washed three times with saline solution (0.9 % NaCl) and separated by centrifugation at 2000 *g* for 20 min. Afterwards, Ppy particles were dried in a desiccator overnight and weighed. The weight of Ppy particles collected by decantation of supernatant and collection of precipitate after centrifugation at 2000 *g* for 20 min was about 150 mg/100 mL of polymerization solution. The final yield (100×(moles of pyrrole included in polymer particles)/(moles of pyrrole used for polymerization)) after all the polymerization, centrifugation and decantation procedures was 8.5%. We prepared three suspensions with different particle concentrations: 0.24 mg of Ppy particles in 10 mL of saline (1 mL corresponds to 1 mg of Ppy particles kg^{-1}); 1.2 mg of Ppy particles in 10 mL of saline (1 mL corresponds to 5 mg of Ppy particles kg^{-1}) and 2.4 mg of Ppy particles in 10 mL of saline (1 mL corresponds to 10 mg of Ppy particles kg^{-1}). All suspensions were sterilized by autoclavation. An optical microscope (LEICA DM LB; Leica Microsystems Wetzlar GmbH, Germany) and digital camera (GC-X 3E; JVC, Japan) were used for optical imaging of Ppy particles.

Animals and housing

Approval of the Lithuanian Ethic Committee for Laboratory Animal Use (Nr. 0111) was obtained before the start of the experiments. Sixty male BALBc mice, 8 weeks old, 24 ± 1 g, were obtained from the vivarium of the Institute of Immunology of Vilnius University (Vilnius, Lithuania). Mice were randomly divided into groups of six animals each and housed in solid-bottomed cages containing bedding of wood shavings, with food and water freely available. Room temperature was maintained at 21–24 °C and a 12-h light/dark cycle was used.

Treatment with Ppy particles

The mice in all treatment groups were injected intraperitoneally with 1 mL of Ppy particle solution as a single injection dose. The mice in the control group were injected intraperitoneally with 1 mL of sterile saline solution (Group 1). Three groups of mice were injected intraperitoneally with 1 mg kg^{-1}, 5 mg kg^{-1} and 10 mg kg^{-1} Ppy particles, respectively. On the third day after injection, the mice were killed by cervical dislocation and the effect of Ppy particles was examined.

In a second experiment, mice in the Ppy-treated groups were injected intraperitoneally with 5 mg kg^{-1} as described above and the effect of Ppy particles after 1, 3 and 6 weeks was examined.

Study of immune cells in blood and peritoneum. Determination of spleen, kidney and liver indexes

To analyse immune parameters in mice after treatment with Ppy particle suspensions, blood samples were collected from the heart of all animals and 50 μL of K_2-EDTA (6%) was added to each 1-mL blood sample. Blood cell count analysis of neutrophils, lymphocytes, monocytes and eosinophils was performed with a multiparameter automated haematology analyser (Mascot Hemavet; Intelimetric Ltd, Oxford, IN, USA). This method allows the concentrations of selected blood cells to be estimated. Standard limits: neutrophils 0.1–2.4×10^3 μL^{-1}, lymphocytes 0.9–9.3×10^3 μL^{-1}, monocytes 0.0–0.4×10^3 μL^{-1} and eosinophils 0.0–0.2×10^3 μL^{-1}.

The peritoneal cavity of each mouse was lavaged with 4 mL cold saline solution containing heparin (10 IU mL^{-1}), collected in the test tubes and centrifuged at 200 *g* for 5 min. Smears were made after resuspending the cell sediment, fixing it in methanol and staining by Giemsa–Romanovski solution for light microscopic examination. Trypan blue was used for the cell viability test.

The spleen, kidney and liver were excised from each mouse and weighed. Spleen, kidney and liver indexes were calculated: (organ weight/bodyweight) × 100.

Statistical analysis

Data were statistically analysed using SYSTAT 11 for Windows software. The results are reported as the mean ± s.e.m. Comparisons of groups for a particular measure were performed using the non-parametric multiple Kruskal–Wallis test. To determine differences between individual treatments and the control, a post-hoc test was performed. Differences were considered statistically significant at $P < 0.05$.

Results

Ppy particles

Figure 1 shows an optical microscope image of the Ppy particles synthesized by oxidative polymerization of pyrrole and obtained after 48 h of polymerization. The particles were susceptible to forming aggregates during the drying process.

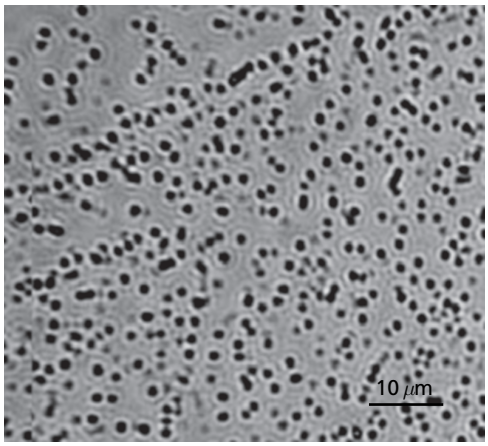

Figure 1 Optical microscope image of chemically synthesized polypyrrole particles.

Dependence of immune cells on different concentrations of Ppy particles

Ppy particles at a concentration of 1 mg kg^{-1}, 5 mg kg^{-1} and 10 mg kg^{-1} had no significant effect on immune cells in mice following measurements of immune-related haematological parameters in blood after a 3-day period; however, the higher concentration (10 mg kg^{-1}) had a tendency to increase neutrophils, monocytes and eosinophils. The relative numbers of peritoneum neutrophils (Kruskal–Wallis test statistic parameter = 11.76, $P < 0.01$) and macrophages (Kruskal–Wallis test statistic parameter = 19.47, $P < 0.01$) in the mice in the Ppy-treated groups were significantly different compared with that of the control group (Table 1). The increase in peritoneum macrophages was evident at 1 mg kg^{-1} Ppy particles (25-times greater), and at 5 mg kg^{-1} and 10 mg kg^{-1} Ppy particles was 26.0- and 57.3-times greater ($P < 0.05$) compared with the control group. In mice treated with 5 and 10 mg kg^{-1} Ppy particles, the relative number of peritoneum neutrophils was 6.2- and 7.6-times greater ($P < 0.05$) compared with the control. The mean number of monocytes was similar in all mice in all groups.

Time dependence of immune cells of mice treated with the same concentration of Ppy particles

Measurements of immune-related haematological parameters in blood after 1, 3 and 6 weeks showed that 5 mg kg^{-1} Ppy particles injected intraperitoneally in mice as a single dose had a significant effect on some immune cells. A statistically significant decrease (by 3.1 times) in the neutrophil number (Kruskal–Wallis test statistic parameter = 8.7, $P < 0.05$) was observed after 6 weeks (Table 2). We detected that the monocyte number (Kruskal–Wallis test statistic parameter = 6.11, $P > 0.05$) decreased by 2.5 times after 1 week, 1.9 times after 3 weeks, and 3.0 times after 6 weeks ($P < 0.05$). The mean number of lymphocytes, basophils and eosinophils was similar in mice in all groups. The relative numbers of peritoneum neutrophils (Kruskal–Wallis test statistic parameter = 4.36, $P > 0.05$), monocytes (Kruskal–Wallis test statistic parameter = 1.82, $P > 0.05$) and macrophages (Kruskal–Wallis test statistic parameter = 4.54, $P > 0.05$) in the mice in the Ppy-treated groups were not significantly different compared with the control mice; however, the number of neutrophils and macrophages had a tendency to increase and the monocyte number had a tendency to decrease (Table 2).

Table 1 Effect of different concentrations of polypyrrole (Ppy) particles on the peritoneum cell relative number in mice

	NaCl (0.9%) (Group 1, control)	Ppy particles 1 mg kg^{-1} (Group 2)	Ppy particles 5 mg kg^{-1} (Group 3)	Ppy particles 10 mg kg^{-1} (Group 4)
Relative number of neutrophils (%)	0.75 ± 0.31	0.50 ± 0.29	4.67 ± 1.36*	5.67 ± 1.78*
Relative number of monocytes (%)	3.50 ± 0.82	3.25 ± 1.44	4.67 ± 0.80	4.33 ± 1.54
Relative number of macrophages (%)	0.25 ± 0.16	6.25 ± 1.03*	6.50 ± 1.45*	14.33 ± 3.17*

*$P < 0.05$ significantly different compared with the control.

Table 2 Effect of duration of treatment with 5 mg kg^{-1} polypyrrole particles on blood cell number and peritoneum cell relative number in mice

	NaCl (0.9%) (Group 1, control)	After 1 week (Group 2)	After 3 weeks (Group 3)	After 6 weeks (Group 4)
Neutrophil number (10^3 μL^{-1})	1.22 ± 0.33	0.50 ± 0.11	0.78 ± 0.12	0.40 ± 0.04*
Monocyte number (10^3 μL^{-1})	0.57 ± 0.12	0.23 ± 0.05	0.29 ± 0.11	0.19 ± 0.03*
Eosinophil number (10^3 μL^{-1})	0.06 ± 0.02	0.08 ± 0.03	0.07 ± 0.04	0.02 ± 0.01
Relative number of neutrophils (%)	0.50 ± 0.34	1.17 ± 0.31	0.80 ± 0.37	1.33 ± 0.21
Relative number of monocytes (%)	3.17 ± 0.65	3.50 ± 0.92	2.40 ± 0.40	2.17 ± 0.70
Relative number of macrophages (%)	0.50 ± 0.22	1.67 ± 0.33	1.20 ± 0.58	1.17 ± 0.40

*$P < 0.05$ significantly different compared with the control.

Viability of peritoneum cells and spleen, kidney and liver indexes

Peritoneum cell viability was 95–98%. Thus, no cytotoxicity after treatment with Ppy particles was detected. Chemically synthesized Ppy particles adhere to peritoneum organs such as liver, and some are also phagocytosed by the cells (Figure 2). There were no significant differences between spleen, kidney and liver indexes in mice from all groups in the two experiments.

Discussion

Among polymers with potential for biomedical applications, Ppy possesses many attractive properties, such as surface charge, humidity, ease of modification, various methods of preparation, excellent environmental stability and good biocompatibility with rat peripheral nerve tissue in-vitro and in-vivo, and it may be used for bridging the peripheral nerve gap (Wang et al 2003). Functionalized Ppy particles are intensively designed and their physicochemical properties have been investigated (Ramanaviciene et al 2006). These particles have received particular interest because of their promising biomedical applications (Saoudi et al 2004; Benabderrahmane et al 2005; George at al 2005). However, unmodified Ppy is not biodegradable, and substances remaining in the body for a long time may induce chronic inflammation.

Ppy can be readily prepared chemically in acidic medium as a black powder and it is possible to separate and collect particles of approximately the same size by centrifugation. Ppy particles can be synthesized by hydrogen peroxide-induced polymerization, they can be sterilized by autoclavation, and they are susceptible to aggregation during drying (Saoudi et al 2004). Ppy has been the most widely studied material among electrically conducting polymers, but there are no data on the direct effect of Ppy films and particles in mammals. In the present study, we examined the direct effect of chemically synthesized Ppy particles separated by centrifugation on mouse immune-related haematological parameters and peritoneum cells.

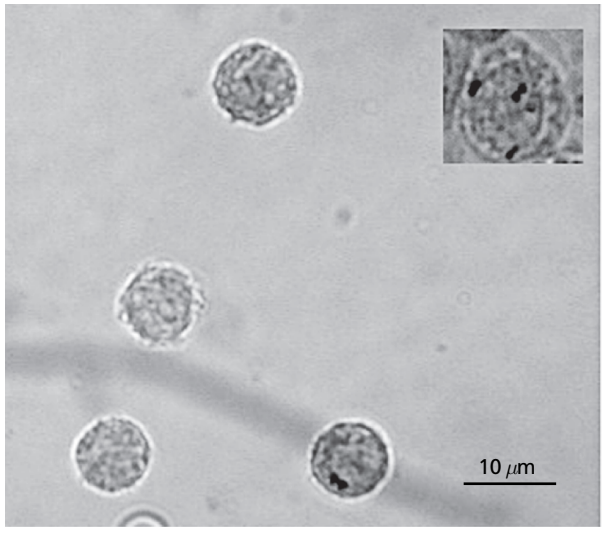

Figure 2 Mice peritoneum macrophages with phagocytosed polypyrrole particles.

Although Ppy is determinable as a polymer with good biocompatibility, especially with enzymes (Ramanavicius et al 1999), DNA (Ramanaviciene & Ramanavicius 2004b), antibodies (Li et al 2005), single cells (Campbell et al 1999) and even with tissues such as nerve tissue (Wang et al 2003), our data show that Ppy particles injected intraperitoneally have an effect on peritoneum cells depending on the concentration and duration of treatment. Microscopic analysis of peritoneum cells shows that after a short period of treatment (3 days) the amount of peritoneum neutrophils and macrophages is already significantly increased (Table 1). Although we observed significant differences between the control group of mice and mice treated with different concentrations of Ppy particles in terms of inflammatory cells (neutrophils) in the peritoneal cavity, the number of leukocytes in the blood did not exceed standard limits. This result indicates that Ppy particles do not induce acute inflammation. After a 6-week period of investigation with one dose ($5\,mg\,kg^{-1}$) of Ppy particles, we observed a statistically insignificant increase in peritoneum macrophages after 1 week, while after 3 weeks and later the amount of macrophages was decreased. George et al (2005) observed similar results after implantation of Ppy implants into the rat cerebral cortex. Staining of macrophages showed the expected increase of this cell type around the implant site after a 3-week period. After a 6-week period, all of the implants induced low but still detectable macrophage activity and, in most cases, the implant site was clear of macrophage activity if the period of treatment exceeded 6 weeks (George et al 2005). Haematological analysis of Ppy-treated and control groups of mice showed no significant differences after a short treatment period (3 days): all determined parameters were within standard limits. However, after 6 weeks we observed a statistically significant (but still within standard limits) decrease in the number of neutrophils. Also, the number of neutrophils in the blood correlated with that in the peritoneum. The decrease in the number of neutrophils in the blood was accompanied by an increase in the number of neutrophils in the peritoneum (Table 2), and these cells in the peritoneum successfully phagocytosed Ppy particles. The decrease in the number of monocytes in blood can be explained in the same way, but the correlation of this cell type was not so clearly manifested. Theoretically, the decrease in the number of neutrophils can also be related to infection. It can be observed during some viral, bacterial and protozoan infections (Jacobson et al 1997). However, in this case, the number of cells counted in the blood should exceed standard limits. Thus, Ppy particles did not induce any symptoms of allergic reaction, since the number of eosinophils in the blood was the same for all mice in all groups. In addition, no cytotoxicity was detected as a result of Ppy particles on peritoneum cells.

Conclusion

Chemically prepared Ppy particles did not cause any cytotoxic effects on mouse peritoneum cells. In addition, the induction of an allergic response or significant changes in

spleen, kidney and liver indexes were not detected. Moreover, standard limits of immune-related haematological parameters or inflammation symptoms were not exceeded in the peritoneum after a 6-week period. Thus, chemically synthesized Ppy particles have good biocompatibility in mice and are an attractive candidate for biomedical applications in-vivo.

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