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Fabrication, Characterization and Biological Evaluation of PRGD/PDLLA/β-TCP Scaffold for Nerve Regeneration ⋆

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Abstract

A novel nerve repairing material PRGD/PDLLA/β-TCP was synthesized and characterized with Scanning Electron Microscope (SEM), Fourier Transform Infrared (FTIR) spectroscopy, and mass loss ratio. The effects of PDLLA or PRGD/PDLLA/β-TCP on viability and growth of Schwann Cells (SCs) were investigated by MTT assay and SEM. After implantation of different materials, histological assessment was performed. The results showed that, compared with PDLLA, PRGD/PDLLA/β-TCP materials displayed better biocompatibility, degradation property and less inflammatory reaction. Moreover, PRGD/PDLLA/β-TCP materials promoted the adhesion and proliferation of Schwann cells and exhibited better degradation performance than pure PDLLA. These results indicated that PRGD/PDLLA/β-TCP has a potential application in the fields of nerve regeneration.

Keywords: Schwann Cells; PRGD/PDLLA/β-TCP Composite; Implantation; Inflammatory Factors; Biocompatibility

1 Introduction

Numerous investigations have been accomplished toward scaffold materials for neural tissue engineering over the past 30 years [1–3]. Scaffold materials have a significant effect on peripheral and central nervous system regeneration. A variety of natural or synthetic polymers have been used for fabricating biodegradable nerve scaffold materials. After nerve tissue was repaired, the conduit will gradually degrade without inducing an inflammation [4, 5]. Commonly used natural and synthetic biodegradable polymers including collagen and hyaluronic acid,

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PDLLA [6], poly(L-lactide-co-glycolide) (PLGA), polyester urethane, poly-ε-caprolactone (P-CL) and poly(phosphoester) have been synthesized into conduits for conducting nerve growth [7, 8]. However, none of these polymers might be considered to be ideal nerve scaffold materials.

Currently, nerve scaffold materials is inclined to induce strong tissue reaction and aseptic inflammation, so these biodegradable nerve scaffolds hardly have the same repairing effects on nerve regeneration as autologous nerve transplantation.

Due to good biocompatibility, PDLLA and β-TCP have been approved to be applied in clinics by Food and Drug Administration (FDA). The PDLLA is used in animal experiments on nerve repair and shows good repairing effects [9, 10]. However, PDLLA degradation is acidic and easily causes aseptic inflammation in vivo, affecting its further application as nerve repairing material. β-TCP provides calcium ion which guides the direction of nerve axon growth cone regeneration and neutralize the acidity generated during PDLLA degradation [11]. Some literatures reported its regulation of inflammation by affecting inflammatory cytokines [12]. Furthermore, the PDLLA lacks bioactive signals existing in the Extracellular Matrix (ECM) for cell adhesion and proliferation. Partial sequences of protein polypeptide are known to promote cellular attachment and proliferation by providing anchorage points and thereby triggering signal transduction by activation of integrin receptors [13]. Therefore, the above problems can be overcame by introducing of partial sequences of protein polypeptide, such as ECM adhesion proteins and cell-binding peptides into PDLLA to enhance cell adhesion and proliferation. The Arg-Gly-Asp (RGD) sequence, found within many ECM proteins, has been the most extensively studied motifs and substrates and found widespread use in adhesion research [14].

In this research, we attempted to synthesize PRGD/PDLLA/β-TCP materials to promote the adhesion and proliferation of cell in nerve tissue regenerative process. We also studied their degradability and investigated cytocompatibility in vitro; the experiment of transplantation *in vivo* was conducted to detect histocompatibility and inflammation of materials to provide theoretical basis for the study and application of nerve repairing materials.

2 Experimental Methods

2.1 Acquisition of Cells

Newborn SD rats were sentenced by being soaked into 75% alcohol; sciatic nerve was removed under sterile conditions, cut into explanted tissues at the size of 1 mm^3 on clean bench, and then the explanted tissues were put into flask. The tissues were arranged at the distance of 1 cm. The flask was inverted and put into incubator at 5% CO_2 and 37 °C for 8 h; when the explanted tissues were firmly adhered to the bottom surface, turn over the flask and add a little culture medium. According to cell growth, culture medium was timely replenished. Generally, every 2-3 days, the culture medium was replaced with a new one. One week's culture of separated sciatic nerve explanted tissues, an observation of cell growth state was that the cells isolated from the marginal explanted tissues had covered the bottom surface. After abandoning culture medium, 0.25% trypsin was used for enzymatic digestion and separation, and differential adhesion for purifying Schwann cells.

2.2 Material Fabrication

The polymer RGD peptide modification of poly (lactic acid)-co-[(glycolic acid)-alt-(L-lysine)] (PRGD) was fabricated by the following steps [15]. Firstly, (3S)-3-[4-(benzyloxycarbonylamino) butyl] morpholine-2, 5-dione (BMD) was synthesized by bromoacetyl bromide and Nε-(benzyloxycarbonyl)-L-lysine. Secondly, poly (lactic acid)-co-[(glycolic acid)-alt-(Nε-benzyloxycarbonyl-L-lysine)] (PLGLZ) was obtained by copolymerization of D, L-lactide and BMD. Then, poly (lactic acid)-co-[(glycolic acid)-alt-(L-lysine)] (PLGL) was synthesized by catalytic hydrogenation of PLGL. Finally, PLGL was modified with RGD peptide. PRGD (0.05 g) and PDLLA (0.9 g) was dissolved in ethyl acetate at a concentration of 5 wt%. β-TCP (0.05 g) was added to ethyl acetate solution and mixed thoroughly. PRGD/PDLLA/β-TCP materials were prepared by using solvent volatilization method.

2.3 PRGD/PDLLA/TCP Characterization

The chemical compositions were characterized through the TR-FTIR transmission spectra. FT-IR spectra were recorded on a Nicolet Nexus FTIR Spectrometer (Nexus, Thermo Scientific, USA) equipped with a Nic-Plan TM IR microscope. Data were collected in transmission mode, 128 scans per point at 4 cm^{-1} resolution. The measurement was in the range of 4000-500 cm^{-1} and performed at random positions in the samples.

2.4 Degradation Experiment of Materials

Different materials of PDLLA and PRGD/PDLLA/β-TCP were dried to be constant weight. They weighed for initial mass W1 and were put into glass, and then PBS of the same volume (PH=7.4±0.2) was added, and then thermostatic degradation was conducted at 37 °C. The initial value of the prepared phosphoric acid buffer solution was PH=7.2.

Mass loss rate of composite conduit materials were detected at different time periods (0 W, 4 W, 8 W, 12 W, 16 W, 20 W, 24 W). Materials were taken out from degradation solution and washed with distilled water, water on which was removed with filter paper, and the materials were dried at vacuum to be constant weight. The calculation of mass loss W_2 is:

$$\text{Massloss}(\%) = (W_1 - W_2)/W_1 \times 100\% \quad (1)$$

W_1 : Initial mass W_2 : mass after degradation

2.5 Effect of Materials on Schwann Cells

PRGD/PDLLA/β-TCP materials (PDLLA materials were used as control) were made to be at the size of the well of 96-well plate, irradiated for 30 min front and back and put into 96-well plate. 3 pieces of each material at each time point, there were a total of 4 culture plates. Schwann cells were distributed into RPMI-1640 culture medium containing 10% FBS; 90 μL Schwann cells at the concentration of 4×10^4/mL for each well was inoculated on material surface, and then cultured in incubators at 100% relative humility, 37 °C and 5% CO_2 for different time. One 96-well plate was taken out at each time point and added into 10 μL MTT for another 4 hours of light-proof static culture in incubator. The supernatant was removed, and 150 μL DMSO was added into

each well and mixed together. When purple formazan particles were fully dissolved and mixed and 100 μL supernatant was sucked away, the solution was added into new corresponding 96-well plate; the Optical Density (OD) of each well at 490 nm was detected with ELISA. With 570 nm as reference wavelength, cell growth of different materials was detected.

2.6 SEM Observation of Cells on Material Surface

PDLLA and PRGD/PDLLA/β-TCP materials of the same size were sterilized by ultraviolet ray, 30 min for each side, front and back; different membranous materials were respectively put into 24-well plate and 3 pieces of each material were used. Cells in flask were digested with 0.25% trypsin and then prepared to be cell suspension of 2×10^4/mL. The cells were seeded onto the composite film in 24-well plate, 1 ml for each well, and then incubated; change the liquid every other day; 4 days later, materials were taken out and fixed with 2.5% glutaraldehyde, and conventionally dehydrated and dried. SEM was used for observation of cell growth in materials.

2.7 Tissue Reaction in Vivo

SD rats (250-400 g) were anaesthetized with 2% sodium pentobarbital. The neck skin was cut open, and different materials were subcutaneously embedded and fixed. PRGD/PDLLA/β-TCP materials were experimental materials and pure PDLLA materials were control. Each material was embedded in three corresponding positions for each rat to observe wound healing and tissue regeneration. At a specific time point, the wounds were open to observe the degradation of materials and local inflammation. In the fourth week, the materials and surrounding tissues were taken out for routine tissue fixation, dehydration, wax embedding and HE staining. An observation of reactions of surrounding tissues was conducted.

2.8 Statistical Analysis

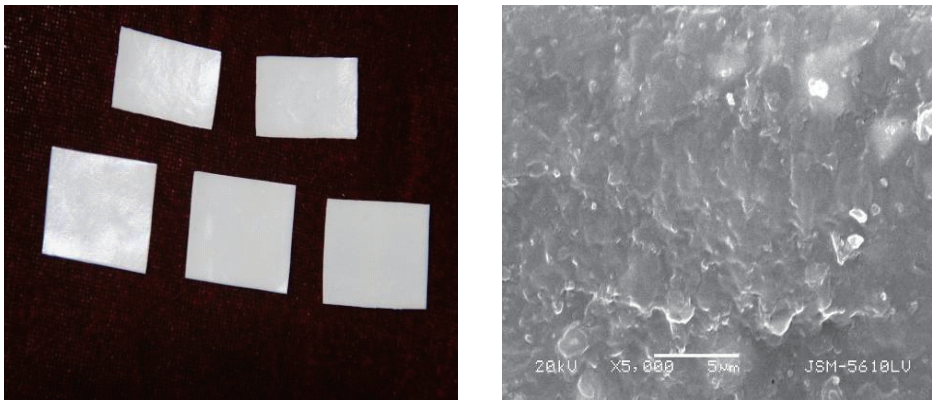
The measured data were expressed as mean $\pm$ standard deviation. All biological experiments were conducted one-factor analysis of variance, and $P < 0.05$ refers to the result is of statistical significance.

3 Experimental Results

3.1 Characterization of PRGD/PDLLA/β-TCP Materials

PRGD/PDLLA/β-TCP materials were fabricated into film with solvent volatilization methods. Fig. 1 (a) displays the optical image of the PRGD/PDLLA/β-TCP materials. It shows the SEM image of the PRGD/PDLLA/β-TCP materials, indicating that different phases of PRGD/PDLLA/β-TCP materials are very homogeneous (Fig. 1 (b)). Few obvious cracks can be found in PRGD/PDLLA/β-TCP materials. These microstructures likely improve the mechanical properties of PRGD/PDLLA/β-TCP materials. Fig. 2 shows the FTIR spectras of the PDLLA, PRGD and PRGD/PDLLA/β-TCP. In the spectrum of the PRGD/PDLLA/β-TCP, the band at 1753 cm^{-1} is a characteristic peak of PDLLA. The two bands at 611 cm^{-1} and 557

cm^{-1} are the characteristic peaks of the β-TCP. The peak at 3436 cm^{-1} is related to NH peak attributed to the characteristic peak of the PRGD. All the above four peaks are presented in the PRGD/PDLLA/β-TCP spectrum, verifying that the PRGD/PDLLA/β-TCP was successfully synthesized.



(a) (b)

Fig. 1: Observation of conduit morphology of (a) Morphology of PRGD/PDLLA/β-TCP materials; (b) SEM observation of materials surface

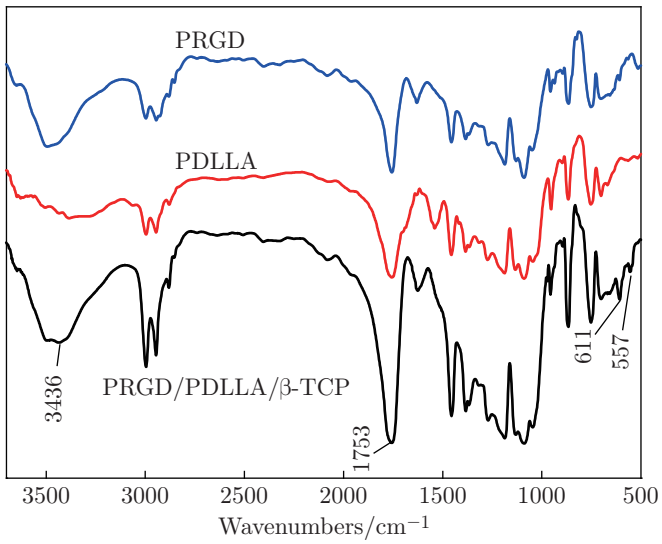


Fig. 2: FTIR spectra of PDLLA, PRGD and PRGD/PDLLA/β-TCP

3.2 Changes of Conduits Mass Loss Rate

It shows that the PRGD/PDLLA/β-TCP and PDLLA materials gradually degrade as time changes (Fig. 3). The degradation velocity of the PDLLA is lower than that of the PRGD/PDLLA /β-TCP materials. After 24 weeksthe mass loss rate of the PDLLA materials was only around 25%. However, the mass loss rate of the PRGD/PDLLA/β-TCP materials was beyond 50% at week 24. It demonstrates that the PRGD/PDLLA/β-TCP materials have more appropriate degradation rate. Our previous study [16] showed that the relative molecular mass of the PRGD smaller than the PDLLA represented a faster degradation rate. The degradation of the

PRGD resulted in rapidly decreasing of pH value of medium and also promoted the degradation of the PDLLA. In addition, the water molecules diffused easily into the interior of the PRGD/PDLLA/β-TCP materials for the interface defects between the β-TCP particles and the PDLLA. This can also promoted the degradation of the PDLLA. Therefore, the degradation of the PRGD/PDLLA/β-TCP composite material is faster than that of pure PDLLA.

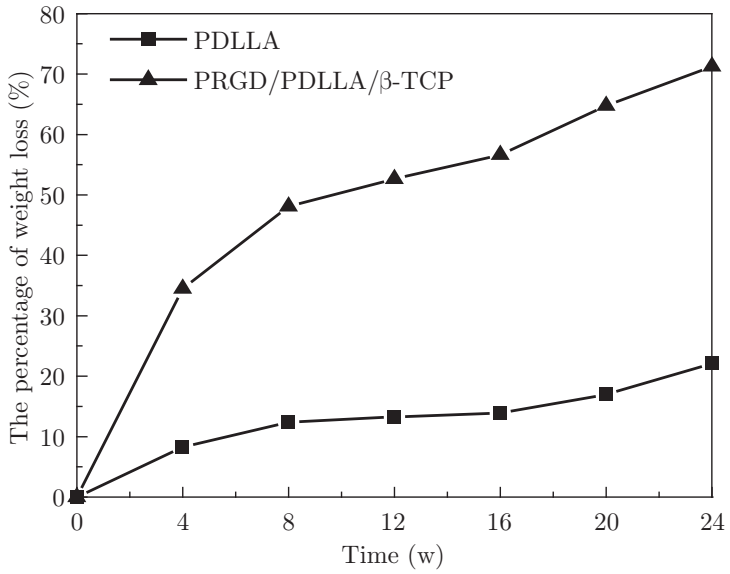


Fig. 3: Mass loss rate curve of different materials

It shows that the change of degradation pH value of PRGD/PDLLA/β-TCP and PDLLA materials as time changes (Fig. 4). PH value of materials sharply declined during the first 4 weeks. Weight loss ratio of β-TCP decreased insignificantly in alkaline medium; therefore PH value of the composite paste was slightly higher than that of PDLLA. After 4 weeks, weight loss ratio of β-TCP increased significantly in acidic medium and neutralized the acidity generated during PRGD/PDLLA degradation.

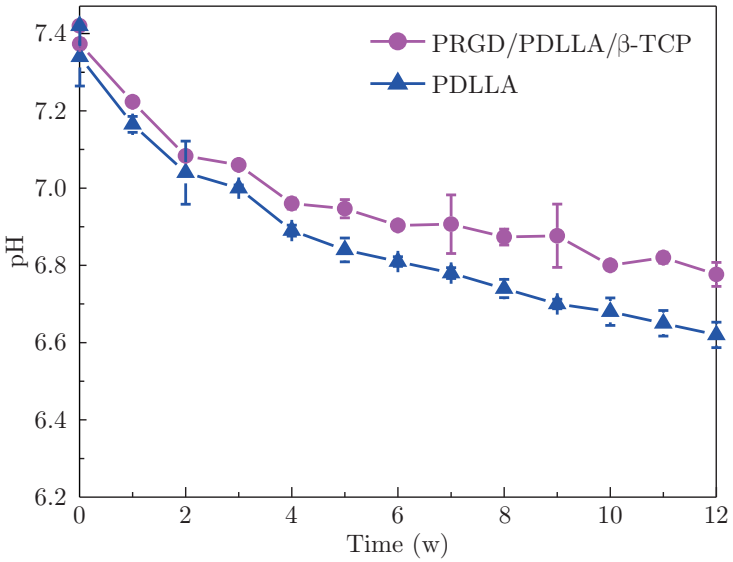


Fig. 4: Change of pH of different materials

3.3 Results of MTT Cell Activity Test

Cell activity was detected with MTT method, as succinodehydrogenase reacting with MTT results in blue- purple crystals, and when DMSO dissolves the blue- purple crystals, the activity is related to its solubility. Mitochondria succinodehydrogenase activity has been proved to be positively correlated with cell activity, so MTT method was adopted to detect cell activity of different material groups. Activity tests on day 2, 4, 6 and 8 after different materials culturing Schwann cells led to the results (Fig. 5) that the cell activity on the PRGD composite materials is higher than that of the control PDLLA materials; on day 4 and 6, the cell activity on the PRGD material surface was significantly higher than that of control; on day 8, it was close to the activity of cell proliferation in plateau.

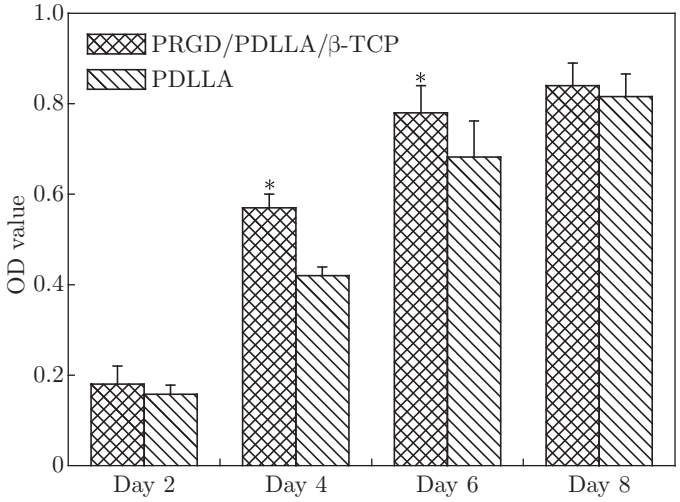
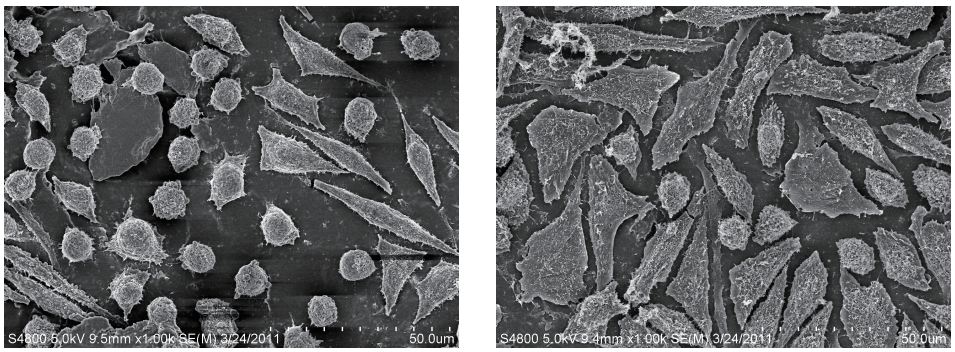


Fig. 5: Schwann cell activity on surfaces of different materials

3.4 SEM Results of Material Surface

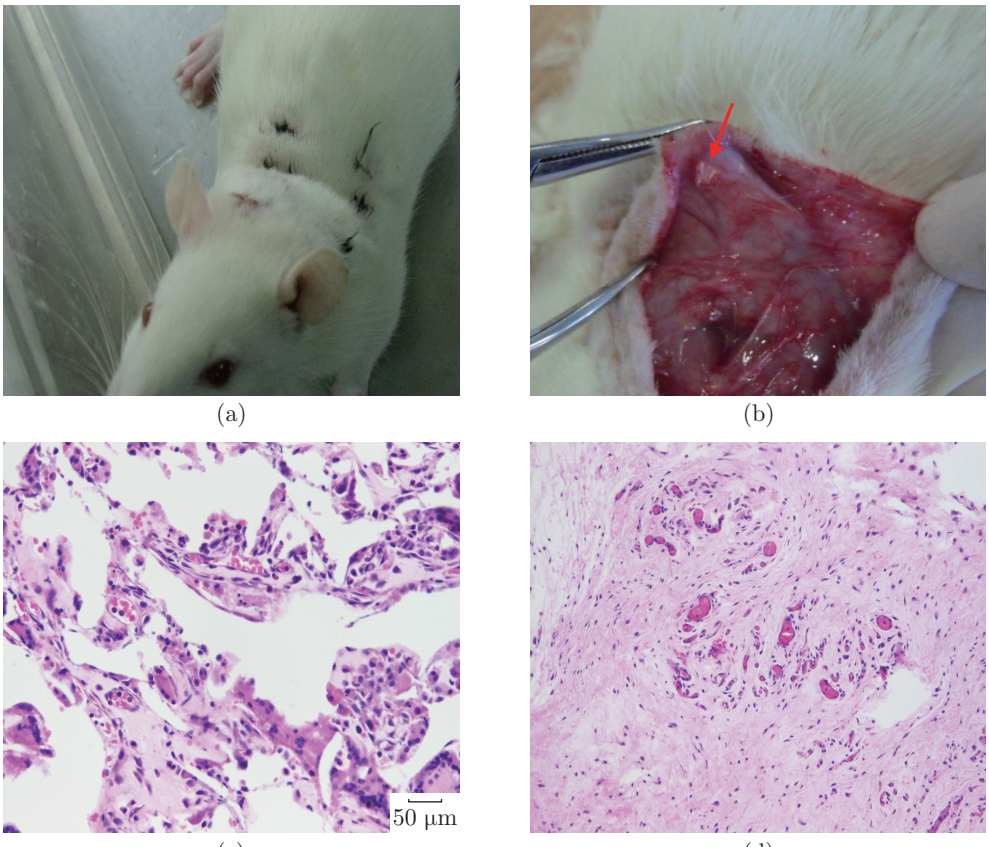
Proliferation of Schwann cells on surfaces of different materials was investigated using SEM (Fig. 6). Most cells on the PDLLA material surface presented to be circular and spreaded unevenly, while most cells on the PRGD/PDLLA/β-TCP materials were in fusiform with full-filled cell body, extensively connected cell process and the morphology close to primary cells. Schwann cells seeded on PRGD/PDLLA/β-TCP materials demonstrated slightly higher metabolic activity compared with those seeded on PDLLA material. These results demonstrate good compatibility of PRGD/PDLLA/β-TCP and Schwann cells. It has been demonstrated that RGD peptide facilitates Schwann cells proliferation and neurite outgrowth at low doses but will disrupt endogenous fibronectin signaling and regeneration at higher doses [17]. Schwann cells grown on the PRGD/PDLLA/β-TCP materials showed a differentiated morphology in comparison to those grown on the PDLLA (Fig. 6). Lack of appropriate cell binding sites on PDLLA can be the main reason of spherical shape of these Schwann cells spreaded unevenly on the surface of materials [18]. Thus, it suggests that PRGD peptide distributed in the PDLLA/β-TCP materials enhance initial attachment of Schwann cells to materials, by presenting more binding sites to the cells. The spreading and process elongation of Schwann cells was significantly better on the surface of the PRGD/PDLLA/β-TCP than on the PDLLA, where a number of spherical shape

cells were observed. It is therefore speculated that recognition of the PRGD peptide distributed in the PDLLA/β-TCP materials not only favors better cell attachment but also enhances further morphological maturation and spreading of glial cells by providing more focal adhesion sites and increased intracellular signaling [18].



(a) (b)

Fig. 6: SEM observation of Schwann cells on surfaces of different materials. (a) PDLLA; (b) Cells on PRGD composite material surface



(a) (b)

(c) (d)

Fig. 7: Subcutaneous transplantation of different materials. (a) The next day of subcutaneous transplantation of rats; (b) 4 weeks after operation, subcutaneous materials (red arrow) presented to be degraded to some degree, and the surrounding tissues were close to be normal; (c) 4 weeks after PDLLA transplantation, surrounding tissues were HE stained; (d) 4 weeks after subcutaneous embedding of PRGD/PDLLA/β-TCP composite materials, surrounding tissues were HE stained

3.5 Tissue Reaction in Vivo

Tissues reaction of transplanted materials can assess the biocompatibility of the PRGD/PDLLA/β-TCP materials. Different materials were transplanted into rats, and observations of tissue reactions were conducted at different lengths of intervals. The surgical wound healed normally. On the second day after operation, no red or swollen manifestation was observed in the wound (Fig. 7 (a)); 2 weeks later, wounds almost healed; 4 weeks later, wounds almost recovered; tissues surrounding materials were HE stained for observation (Fig. 7 (c), (d)), and macrophages and multinucleated giant cells were occasionally occurred. A few inflammatory cells were found by HE staining of surrounding tissues of the PRGD/PDLLA/β-TCP material. In the PDLLA material group, polykaryocytes representing inflammatory reaction were abundant. Degradation was found in all PRGD/PDLLA/β-TCP materials (Fig. 7 (b)). These results manifest that PRGD/PDLLA/β-TCP materials has good biocompatibility [19–22].

4 Conclusion

The PRGD/PDLLA/β-TCP material was synthesized and characterized. And the biocompatibility of the PRGD/PDLLA/β-TCP materials was also assessed. Compared with the PDLLA, the PRGD/PDLLA/β-TCP materials displayed better degradation property. Proliferation was found in Schwann cells on PRGD/PDLLA/β-TCP materials surface, and the growth state was close to that of primary Schwann cells. The PRGD/PDLLA/β-TCP implantation also demonstrated its small inflammatory reaction and good histocompatibility. These results indicated that the PRGD/PDLLA/β-TCP materials promoted the adhesion and proliferation of Schwann cells and exhibited better degradation performance than pure PDLLA. Therefore, the PRGD/PDLLA/β-TCP materials is a potential material for nerve repair.

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