PDL 与 PLL 修饰 Aclar 膜应用于 DRG 体外培养观察

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摘 要 外周神经缺损相关疾病给神经外科医生和患者带来严重困扰。自体神经移植被认为是治疗神经缺损的最佳疗法，但常常受到供体神经束长度等因素的限制。另外，人们在神经组织工程原理的基础上，提出组织 - 电极接口的设想，用以克服传统神经接口的缺陷。而该假设也是建立在体外获得足够长的神经束的基础上提出的。因此，我们急需建立体外神经组织培育体系，用来获得足够长的神经束。背根神经节是外周神经系统的发源地，是建立体外外周神经培育体系的理想原材料。文中，将提取 SD 大鼠背根神经节，并使用左旋多聚赖氨酸或者右旋多聚赖氨酸修饰过的 Aclar 膜作为修饰作为背根神经节的生长基底。通过观察背根神经节的生长发育状况，我们发现经右旋多聚赖氨酸修饰的 Aclar 膜更适合用于背根神经节的体外培育。

关键词 修饰；右旋多聚赖氨酸；左旋多聚赖氨酸；背根神经节组织

中图分类号 R 496 **文献标志码** A

An Investigation of PDL and PLL in Coating the Aclar Film for Culturing DRG Explants

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Abstract Peripheral nerve injury and amputation have plagued neurosurgeons and patients. Autologous nerve grafts has been known as the “golden rule” to repair severe lesions but is limited by the length and potentially losing functions originally from the donor nerves. Meanwhile, tissue-electrode interface was proposed to overcome the limitation of the traditional neural interface, but long autologous nerves with original functions are preferred. So finding out a substitute material to replace the autologous nerves as donor nerves becomes critical. Dorsal root ganglia (DRG) proved an ideal original material. In this report, we described a method to isolate the DRG explants from the spinal cord of SD rat, and coated the Aclar films with poly-D-Lysine (PDL) and poly-L-Lysine (PLL) respectively. Then the strategy of culturing the DRG explants on the coated Aclar films was introduced. An experiment was designed to investigate the growth of the DRG explants, specifically the DRG axons cultured on the two separately coated -Aclar films. The results of the experiment show that PDL has an advantage in coating the Aclar film for culturing the DRG explants for nerve tissue growth.

Keywords coating; PDL; PLL; DRG explants

收稿日期：2015-03-02 **修回日期**：2015-03-19

基金项目：国家自然科学基金（61233015）；华中科技大学自主创新基金（2013YGYL004）

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1 Introduction

Peripheral nerve injury (PNI) led by trauma or surgery is a very common phenomenon. However, there are still not sufficient methods for repairing or rehabilitating. Autologous nerve grafts has been known as the “golden rule” to repair several lesions, but the donor nerves would lose function[1,2]. Meanwhile, to the amputees, even they wear the prosthetics, they could not control them at will or feel object vividly with them. The hypothesis, using nerve-electrode to achieve a double neural interface of motor-sensory, was proposed. The proposers[3] have also suggested the best choice to integrate interface with the host nerves was the “autologous nerve” which had enough length, functioned well and could be cultured strongly on the electrode. To sum up, the same requirement of them was a nerve tissue of enough length and normal function. So we need to culture special nerve tissues in vitro, which could grow long with original functions, and be transplantable.

Smith et al.[4] noted that as the body grows, mechanical tension promotes the growth of axons and shows during development as the vertebrae grow in length, the growth of whale spinal axons can reach at least 3 cm of growth per day. With the knowledge that peripheral nerves are mainly developed from the dorsal root ganglia, DRGs promise to be the potential material to ‘stretch’ out long axons.

Smith et al.[4,5] proved DRG explants of both human and rat could grow robust in medium for months, maintaining electrophysiological function. So the DRG explants appear to be an ideal donor for transplantable nervous tissue constructs. On this basis, Cullen et al.[6] proposed an approach to bridge the gap with the DRG axons which were isolated from the spinal cord and stretched with mechanical force on integrated axons in vitro. Pfister et al.[7] supposed a novel method of engineering nervous tissue constructs as a means of interfacing a multi-electrode array (MEA) with regenerating peripheral nerves. Then Cullen et al.[8] conceived to link artificial limbs to the nervous system by tissue-electrode interface on Scientific American.

With an eye on bridging damaged nervous system with Autologous nerve grafts, or culturing DRG explants on the MEA to make a tissue-electrode interface[9], we firstly need to isolate the DRG explants and culture the isolated DRG explants on the surfaces that could be transferred easily and machined, e.g. being stretched by micro-mechanical force or stimulated by MEA or electrical field[10]. Moreover, the material should be transparent, convenient to observe with a microscope, and adhesive enough between the tissues and the surface to resist the possible external force.

Compared with the traditional glass (slides or cover glass), Aclar film has better flexible, mechanical tolerance. Meanwhile, Aclar film, for all practical purposes, is transparent and chemically inert, contains no volatile components and can accept surface-coating to enhance the adhesion between the cells and the surface. So it is a better material for culturing cells and tissues to be engineered. Kingsley et al.[11] used Aclar film to culture mammalian cells for transmission, proved that the cells adhered to the coating strongly and could be observed with all kinds of microscope. Masurovsky et al.[12] had also taken advantage of Aclar film’s properties to achieve the long-term nerve tissue culture. All in all, Aclar film promises the ideal material to culture the DRG

axons for engineering application.

When it comes to coat the surface to enhance the adhesion strength and tangency between the explants and the underlying materials, some materials, like the rattail collagen and Laminin, which contain highly biologically guidance molecules and adhesion promoters, were proposed[13-15]. However, they are limited to the application in coating the electrical chip or the MEA, for the large molecule could reduce the effective distance from the cell and the underlying surface and the adhesion is not enough to resist the mechanical force[4,5].

The most common approach, for neurons or tissues, is to coat the culturing substrates with some kind of poly, like Poly-L-Lysine (PLL)[16], and Poly-D-Lysine (PDL)[17]. But they both can be taken up through adsorptive endocytosis by cultured cells. The difference between them is that Poly-D-Lysine could not be digested, while Poly-L-Lysine can be digested by Lysosome. Strand et al.[18] found PLL became a powerful toxicant for cells when it exceeded certain threshold. At the level of culturing tissues, the drawback of PLL will be magnified, but the feature of enhancing the adhesion between the tissues with the substrate that both of PLL and PDL have, should remain. So we suppose that Poly-D-Lysine promised to coat substrate for culturing tissues with strongly adhesion between the explant tissues and the underlying material.

In this paper, we described a method to isolate the DRG explants from the spinal cord of SD rat, and coated the Aclar films with PDL and PLL respectively. Then the strategy of culturing the DRG explants on the coated Aclar films was introduced. An experiment was designed to investigate the growth of the DRG explants, specifically the DRG axons, cultured on the two separately coated-Aclar films respectively.

2　Materials and Methods

The experimental protocol was approved by the Ethics Committee for Animal Research, Huazhong University of Science and Technology, China.

2.1　Preparation

The specification of main reagent were shown in table 1.

表 1　主要试剂

Table 1　The main reagent

The Materials	Manufacturers
1 day infant Sprague-Dawley rat	Animal experiment Centre of Wuhan University
Poly-D-Lysine (PDL)	Sigma Inc.
Poly-L-Lysine (PLL)	Sigma Inc.
Dulbecco's modified Eagle's medium(DMEM)	HyClone Inc.
Fetal Bovine Serum (FBS)	HyClone Inc.
Nerve Growth Factor(rat β-NGF)	R&D Inc.
5-Fluoro-2`-Deoxyuridine	Sigma Inc.

Sterilization: Using sterile technique, place autoclaved the Petri dishes, centrifuge tubes, Pipette-tips etc. in exposing to UV light 30 minutes prior to use. Soak all instruments in 75% ethanol for at least 30 minutes. Aclar strips was washed with detergents, which were cut into 25 mm×25 mm to suit the 6 cm-Petri dishes. Then they were socked in 75% ethanol for more than 30 minutes. Expose them to UV light 30 minutes prior to be coated.

Media Formulations: Cultures are maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μg/mL nerve growth factor, and 1% penicillin/streptomycin. Cultures are treated with the mitotic inhibitors formulated with 10 mm 5-fluoro-2`-deoxyuridine (FdU), and 10 mm Uridine to encourage non-neuronal cell elimination.

Coating: coating Aclar strips with 10 μg/mL PDL and 10 μg/mL PLL respectively. The sterile Aclar strips were put into the Petri dishes. Then 1 mL of 10 μg/mL PDL in the culture media on Aclar strip was pooled in. Gently shake the Petri dish to make the PDL solution uniform cover the entire surface of Aclar strip. They were coated in 37℃ CO_2 incubator for at least one hour. After coated, the Aclar strips were rinsed and drained gently with 3×deionized water and cultured media as a final rinse before plating. The above coating steps are suitable for PLL coating.

2.2 Dissection and Isolation of DRGs

The specification of main equipment and instruments were shown in table 2.

表 2 主要设备和仪器

Table 2 The main equipment and instruments

The Devices	Manufacturers
CO_2 incubator(MCO-15AC)	SANYO Inc.
Olympus CKX41 inverted microscope	Olympus Inc.
Canon 600D SLR	Canon Inc.
12cmMicro forceps straight	Tumed Inc.
14cm Micro-scissors	Tumed Inc.
Small size-dissecting scissors	JZ Classic Inc.
Micro-dissecting scissors	JZ Classic Inc.
Aclar 33C film 198 μm	Electron Microscopy Sciences Inc.

Before dissection, soak all instruments in 75% ethanol. The SD rats were anesthetic by intraperitoneal injection with 1% sodium pentobarbital (0.5 mL/100 g body weight). When the rat was successfully anesthetic, the rat would be placed in prone position under a dissecting microscope thoroughly sprayed with 75% ethanol. With pick-up forceps, lift up skin and subcutaneous, then make transverse incision with small dissecting scissors. Cut skin with small-size scissors in an “I” pattern to expose the spinal cord. Then the tissues along the two sides of spinal cord were cut off with micro-dissecting scissors, taking care not to puncture or cut viscera. The spine cord was cut off, from cervical vertebra to coccygeal vertebra carefully, and any remaining viscera from the posterior wall were carefully removed. After the entire spine cord and attached DRGs were peeled off underlying tissues, it was stored into clean a 6 cm-Petri dish, containing almost 4 mL Hanks’ balanced saline solution to retain the tissues active.

After isolating spinal cords, we gently attempted to hold the spine cord with dissecting forceps, very carefully cut through the vertebral column proceeding caudally with micro-scissors (curved). Both halves of vertebral column were teased apart to expose the nerve roots where contain the DRGs carefully, without cutting off the foramen intervertebrale totally. The spinal cord was lifted by grasping cord at rostral end with 12 cm Micro forceps straight. Then carefully pick up the DRGs from the foramens. All the DRGs were harvested by micro-surgical tweezers and transferred to a clean 6 cm dish, containing 3 mL culture media.

2.3 Generation of DRG Explants Cultures

The culture media, containing isolated DRG explants, was transferred into 15 mL-centrifuge tubes with sterile transfer pipet, and was gently centrifuged for 2 minutes at 1200 r to gather the DRG explants, and weak the “wall” around the DRGs to make it easier for the DRG neurons to migrate out after being planted on different surfaces. The supernatant was gently discarded and the DRG explants were re-suspended into 1 mL culture media and mixed immediately by pipetting. Finally, we got the generation of DRG explants cultures for the following experiment.

表 3　实验条件设计表

Table 3　Various test conditions performed for this study

Cases	Surface material	Coated material	DRGs cultures	Temperature (℃)	Concentrations of CO_2 (v/v)	Recording frequency
Blank cases	Petri dishes	none	1 mL	37	5%	every 24h
Experiment cases -PDL	Aclar film	10 μg/mL PDL	1 mL	37	5%	every 24h
Experiment cases -PLL	Aclar film	10 μg/mL PLL	1 mL	37	5%	every 24h

2.4　Experiment Procedure

Various test conditions performed for this study were show in table 3, in the experiment-PDL cases and the experiment-PLL cases: With a 2 mL pipet, the DRG explants culture was transferred to the center of each coated Aclar strip. To each piece of Aclar strip, 1 mL of culture medium is sufficient for the DRG explants to grow and prevent evaporation for at least one hour, while limiting the movement of the DRG explants. Then the DRG explants on the coated Aclar strips were incubated in the 37℃ incubator with 5% CO_2 for 1 hour, which was enough for enhancing the adhesion between the DRG explants and the strips' film to the coated Aclar strips.

When the DRG explants were adhered strongly to the Aclar film, with the sterile transfer pipet, the other 2 mL culture media was pooled into the Petri dish gently, avoiding dripping the liquid straightly to the DRG explants. Then they would be observed on the Olympus CKX41 inverted microscope to find out the adhesive result before being transferred back to the incubator. The culture media was changed every two days. The DRG explants were observed on the Olympus CKX41 inverted microscope, and recorded with a Canon 600D SLR, every 24 hours. In the blank cases, the only difference from the experiment cases was that the DRG explants were planted directly to the Petri dishes without coated Aclar films.

3　Results

Most of the DGR explants in the experiment-PDL and experiment-PLL cases are adhesive to the substrate strongly. The few floating ones would not adhere to the underlying substrate for more liquid filled in the Petri dishes would make a larger resistance. So the first hour for adhesion is very important. If the DRG explants could not adhere to the bottom, hardly the DRG neurons inside them would stretch out the axons or would other supporting cells migrate.

DRG explants cultured in the Petri dish of the blank cases are shown in Fig. 1, where a lot of axons and glial cells migrate from the DRG explants. Both

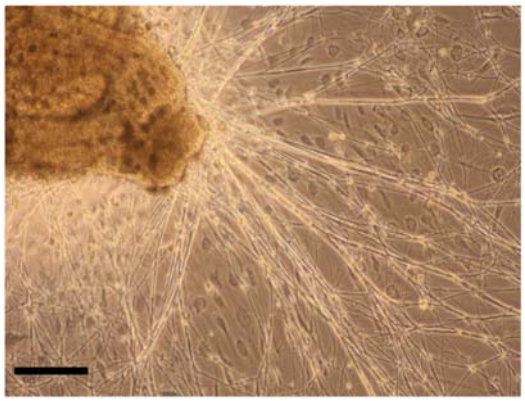

图 1　空白组 DRG 培育 7 天的照片（×100），标尺＝200 μm

Fig. 1　DRG explants cultured in blank cases

The pictures were taken in inverted microscope, on the 7th day after the DRG explants were planted in the Petri dishes with the objective 10X (Scale bar＝200 μm).

the axons and supporting cells appear to be very rough and healthy. And they could be cultured for more than one month.

DRG explants cultured on Aclar film, coated with 10 μg/mL PLL in the Petri dishes of Experiment cases–PLL cases are shown in Fig. 2, in which lots of supporting cells migrate out, and few axons grow out. What's worse, both of them appear sick and could not survive as long as the ones in blank cases and PDL cases. It proves that only PLL is insufficient for coating the substrate to culture the DRG explants.

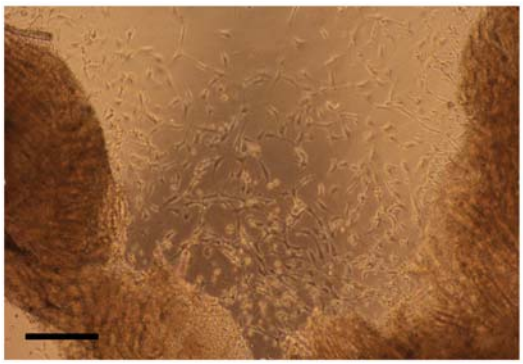

图 2 PLL 组 DRG 培育 7 天的照片（×100），标尺＝200 μm

Fig. 2 DRG explants cultured on the Aclar film, coated with 10 μg/Ml PLL（Experiment cases -PLL）

The picture was taken in inverted microscope on the 7th day after the DRG explants were planted on the PLL coated-Aclar film, with the objective 10X（Scale bar＝200 μm）.

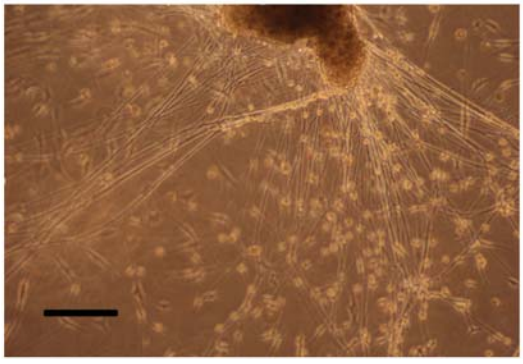

图 3 PDL 组 DRG 培育 7 天的照片（×100），标尺＝200 μm

Fig. 3 DRG explants cultured on the surface of the Aclar film, coated with 10 μg/ml PDL（Experiment cases -PDL）

The picture was taken in inverted microscope on the 7th day after the DRG explants were planted on the PDL coated-Aclar film, with the objective 10X（Scale bar = 200 μm）.

DRG explants cultured on the surface of the Aclar film, coated with 10 μg/mL PDL in the Petri dishes of experiment cases–PDL cases are shown in Fig. 3 in which the axons of the DRG explants appear to be thin but the population is large.

Comparing the result of experiment cases–PDL and experiment cases–PLL, it is not difficult to find that there are more axons on the Aclar strips coated by PDL than PLL. In addition, the axons look healthier. PDL shows a superiority of coating the Aclar films to culture the DRG explants, while enhancing the adhesion between DRG explants and the Aclar film.

4 Discussion

Our results have demonstrated that PDL is superior to PLL in modifying the Aclar film for culturing DRG explants of SD rat. What's more, we found that there are fewer DRG explants floating in the two

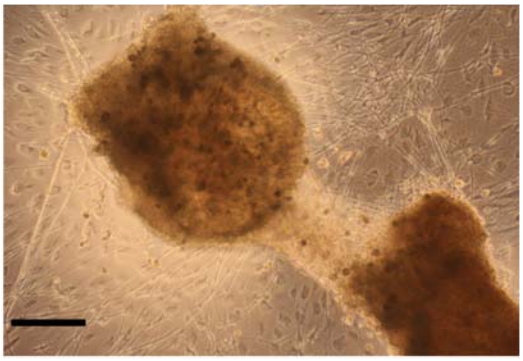

图 4 空白组 DRG 培育 10 天的照片（×100），标尺＝200 μm

Fig. 4 DRG explants cultured in blank cases

The pictures were taken in inverted microscope, on the 10th day after the DRG explants were planted in the Petri dishes with the objective 10X（Scale bar＝200 μm）.

experiments cases than that in the blank cases. It proves that PDL and PLL have enhanced the adhesion between the DRG explants and the coated Aclar film indirectly. The experiment of mechanical force measurement is needed to find a more direct evidence.

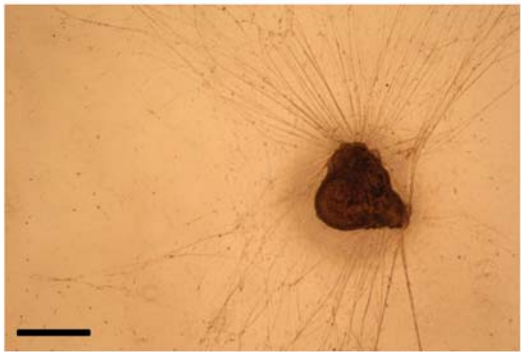

图 5　空白组 DRG 培育 10 天的照片（×40），标尺＝500 μm

Fig. 5　An DRG explants cultured in the Petri dishes（Blank cases）The pictures were taken in inverted microscope, on the 10th day from the DRG explants were planted in the Petri dishes, with the objective 4X（Scale bar＝500 μm）.

In Fig. 4, although two adjacent DRG explants make a connection to each other, they could not induce all the axons to grow on one direction. So some assistances are needed, such as NGF and supporting cells, but they are proved to be hard to control[19]. And some researchers tried the strategies of electrical stimulation[20], mechanical force[21], and 3D-structrue surface to induce[22] the growth of axons. All those methods need DRG axons grow and adhere to the substrate strongly. So our work has an essential significance for their research. In our experiment, the PDL-coated cases allowed the greatest number of axons, but not as rough as that in the blank cases. To optimize it, the method of coupling PDL with some other objects was proposed. Tian et al.[23] developed a scaffold material: hyaluronic acid (HA)–PDL copolymer hydrogel by incorporating the PDL into the HA–PDL hydrogel to modulate the adhesion between the neuronal cell and the neural network formation. That nerve scaffold was implanted in rat brain and proved to be potential for long-term implantation and well-connected with host nerve.

In Fig. 5, the axons growing with a considerable length hold promise to be an ideal donor for repairing lesions on major nerve. What's more, when the Aclar film is replaced by electron chips or MEAs, it proposes a possibility to connect amputees' residual limb with artificial limbs by tissue-electron chip[3]. Nowadays, some researchers believe that a double neural interface of motor-sensory can be achieved with the nerve – MEA by applying nerve tissue engineering[3,8]. Being a very important step of it, culturing the neurons or explants in different substrates with strong adhesion, is worth more attention. All in all, PDL as a coating material has showed a great potential in applied in culturing DRG explants for nerve tissue engineering, through much research need to do.

5　Conclusions

In the paper, the methods of isolating the DRG explants from the spinal cord of SD rat, and coating the Aclar film with PDL and PLL respectively have been described in detail. Then the strategy of culturing the DRG explants cultured on the coated Aclar film was introduced. An experiment was designed to investigate growth of the DRG explants, especially, the DRG axons cultured on the two coated-Aclar film respectively. The results of experiment prove PDL is better in coating the Aclar film for culturing the DRG explants. We also find that PDL-coated Aclar substrates allow the greatest

number of axons. It maximizes the total amount of tissue available to form the core element of a living nerve construct, which promises to be applied in nerve tissue engineering or stretch by mechanical force to construct a donor for major nerve lesions.

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