Biocompatibility of poly-D, L-lactic-co-glycolic acid/type-I collagen/chitosan composite membrane as artificial spinal dura mater☆

Zhang Wei-hong, Yuan Wen, Wang Xin-wei, Liu Yang, Han Zhu

Abstract

BACKGROUND: Poly-D, L-lactic-co-glycolic acid (PLGA) that was characterized as absorbable, weak cytotoxicity, and adjustable hardness was ideal to be synthetized artificial spinal dura mater. Because of lacking of functional group at the surface of PLGA, it should be modified to fit the demand of satisfied biocompatibility.

OBJECTIVE: To study the biocompatibility of PLGA membrane modified by type-I collagen and chitosan.

DESIGN, TIME AND SETTING: Contrast observation study, which was carried out in the Biochemistry and Molecular Biology Laboratory Shanghai University of Traditional Chinese Medicine from May to December 2007.

MATERIALS: Porous PLGA membrane was provided by Jinan Banzheng Biology-Technology Co., Ltd., type-I collagen by Sigma Company, USA, chitosan by Shanghai Qisheng Biological Agent Medical Apparatus and Instrument Company, and L929 L cell by Cellular Institute of Shanghai Academy of Life Science, Chinese Academy of Science.

METHODS: PLGA membrane (P membrane), PLGA/type-I collagen composite membrane (PG membrane), PLGA/type-I collagen/chitosan (9:1) composite membrane (PGC 9:1 membrane) and PLGA/type-I collagen/ chitosan (5:5) composite membrane (PGC 5:5 membrane) were produced through a certain process.

MAIN OUTCOME MEASURES: Contact angle, absorption rate and cytotoxicity were tested. Morphological changes of L929 L cell cultured for 1, 3, and 7 days were observed under fiberscope.

RESULTS: Contact angle was shown as PG membrane < PGC 9:1 membrane < PGC 5:5 membrane < P membrane (P < 0.01); absorption rate was shown as P membrane < PGC 5:5 membrane < PGC 9:1 membrane < PG membrane (P < 0.01). L929 L cell was characterized as well distribution, expansion and appearance after inoculation of PG membrane, PGC 9:1 membrane and PGC 5:5 membrane. Cytotoxic experiment (MTT methods) showed that, on the 1st day, there was no significant difference in absorbency among groups (P > 0.05). On the 3rd and 7th days, there were significant differences between P membrane and PG membrane or PGC 9:1 membrane, and between PGC 9:1 membrane and PGC 5:5 membrane (P < 0.05). PGC 9:1 membrane could further improve cell adhesion and proliferation, and PGC 5:5 membrane could inhibit cell proliferation and differentiation.

CONCLUSION: Type-I collagen and chitosan appended to the exterior of PLGA can enhance the biocompatibility of membrane. In terms of biocompatibility, PLGA/type-I collagen/chitosan (9:1) composite membrane can be fit to the qualification as a type of material of artificial spinal dura mater.

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INTRODUCTION

Poly-D, L-lactic-co-glycolic acid (PLGA) is an important member of aliphatic polyester to ably degrade polymer materials and mainly apply in surgical repair and drug slow-release system[1]. Absorbable PLGA synthesized artificially is widely used in tissue engineering. The metabolites contain lactic acid and hydroxyacetic acid; however, the degradation product is the same as metabolite during tricarboxylic acid cycle that is in high biocompatibility and biological security. Up to now, it becomes the most usefully degradable biomedical material. PLGA that is characterized as absorbable, weak cytotoxicity, and adjustable hardness is ideal to be synthetized artificial spinal dura mater. Because of lacking of functional group at the surface of PLGA, it should be modified to fit the demand of satisfied biocompatibility. Type-I collagen is a major component of human spinal dura mater, and it is also a major component of extracellular matrix. It is characterized as unique biocompatibility, degradation and bioactivity; meanwhile, it is able to identify cellular signals and induce cell adhesion and growth. However, single collagen membrane is limited in weak mechanical strength and rapid degradation[2]. Chitosan is excellence of biocompatibility, mechanical function[3-4], hydrophilicity, and absorption rate, so it is an ideal implanted biomaterial. Shin *et al* [5] thought that microfibrous membrane of chitosan had good biocompatibility to MG63 cells of rabbit osteosarcoma but no any inflammatory reactions; while, it could promote regeneration of defected skull. Furthermore, mibrofibrous membrane can accelerate growth of epithelial cells, inhibit growth of fibroblasts, and prevent from adhesion and bacterial infection[6-7]. A large of chitosan can inhibit growth of fibroblasts, which may be related to amino on the surface of chitosan membrane. Therefore, we designed to improve biocompatibility of membrane, prolong degraded time of collagen, and promote adhesion, movement, and proliferation of fibroblasts by compounding collagen and chitosan in different proportions on the surface of PLGA. This study was aimed to study the biocompatibility of PLGA membrane, PLGA/type-I collagen membrane, and PLGA/type-I collagen/chitosan membrane.

MATERIALS AND METHODS

Design

Contrast study.

Time and setting

This study was carried out in the Biochemistry and Molecular Biology Laboratory Shanghai University of Traditional Chinese Medicine from May to December 2007.

Materials

Material and instrument	Source
Porous PLGA membrane	Jinan Banzheng Biology-Technology Co., Ltd.
Type-I collagen	Sigma Company, USA
Chitosan	Shanghai Qisheng Biological Agent Medical Apparatus and Instrument Company
L929 L cell	Cellular Institute of Shanghai Academy of Life Science, Chinese Academy of Science
AE24o-I-0.2 mg electronic balance	Mettler Company, Switzerland
SL-200 contact angle apparatus	Shanghai
CO_2 incubator (INCUBATOR B5060EK/CO_2)	Heraeus Company, Germany

Methods

Preparation of porous PLGA membrane (P membrane): PLGA (0.5 g) was dissolved in chloroform (5 mL). After complete lysis, NaCl crystal with the particle diameter of 80-100 μm was added in the solution. The mixture was stirred with magnetic force, modeled, stood for 24-48 hours, demoulded, stood, and dried in the vacuum for 24 hours. Next, the mixture was maintained in double distilled water for 48 hours to remove NaCl, and dried in the atmosphere for 48 hours and vacuum for 48 hours. Finally, the sample was sterilized with ^{60}Co irradiation, and the final dosage was 25 kGy.

Preparation of PLGA/type-I collagen composite membrane (PG membrane): Type-I collagen (20 g/L) was mixed with P membrane at 10 ℃. And then, the mixture was air-dried in laminar-flowing table at 40 ℃, maintained in glutaraldehyde (0.25%) for 24 hours, dried in the vacuum for 24 hours, dripped in PBS, and washed. Other processes were as the same as P membrane.

Preparation of PLGA/type-I collagen/chitosan composite membrane (PGC membrane): Chitosan (20 g/L) was added in type-I collagen solution according to the volume proportion of 9/1 and 5/5. And then, the mixture was put in P membrane model. Other processes were as the same as above mentions. PLGA/type-I collagen/chitosan (9:1) composite membrane (PGC 9:1 membrane) and PLGA/type-I collagen/ chitosan (5:5) composite membrane (PGC 5:5 membrane) were produced through a certain process.

Absorption rate measurement: Membranes were weighted with electronic balance, dripped in distilled water at 37 ℃, and rapidly dried and weighted after 4 hours. The absorption rate (mean value out of six data in each group) was accumulated according to absorption rate = (post-hydrating mass – pre-hydrating mass)/pre-hydrating mass × 100%.

Contact angle determination: Contact angle was detected with contact angle apparatus at 20 ℃. Double distilled water drop was located at surface of the sample. Three points away from 5 mm each other were chosen with pendent drop method. Contact angle was accumulated with Q/2 tangent method.

Cytotoxicity test: ① Membranes were sheared into wafers with the diameter of 6 mm and put in 96-well culture plate. ② There were five groups, including P membrane, PG membrane, PGC 9:1 membrane, PGC 5:5 membrane, and blank control groups. ③ L929 L cells (4.0×10^6 /L) were put on membrane, six samples in each group. ④ RPMI1640 cell suspension and fetal bovine serum (10%) were added in each well. The samples were cultured in 0.05% CO_2 at 37 ℃ for 1, 3, and 7 days. ⑤ Culture solution was changed on the 1st, 3rd, and 5th days. ⑥ Morphological changes, adherence, and gross proliferation of fibroblasts were observed under microscope. ⑦ The primary culture medium was removed, and 1640 culture media (200 μL) and MTT (40 μL) were added in each well. Cells were incubated at 37 ℃ for 4 hours. Absorbency was measured at 490 nm wave with enzyme-linked immunosorbent assay.

Main outcome measures

Contact angle, absorption rate and cytotoxicity were tested.

Design, enforcement, and evaluation

This study was designed by the first author, enforced by all authors, and evaluated by the third and fourth authors. All evaluators underwent trainings.

Statistical analysis

SPSS 13.0 software was used by the first author in this study. Experimental data were expressed as Mean±SD and analyzed with one-way analysis of variance and 5×3 analysis of variance. $\alpha = 0.05$ on both sides was regarded as the test standard.

RESULTS

Absorption rate and contact angle (Table 1)

Table 1 Absorption rate and contact angle ($\bar{x}\pm s$, n=6)

Membrane	Absorption rate (%)	Contact angle (°)
P	16.37±4.37	87.30±10.98
PG	30.70±5.51	71.05±6.54
PGC 9:1	28.90±4.80	73.04±9.88
PGC 5:5	24.23±3.82	79.54±8.02

PLGA: Poly-D, L-lactic-co-glycolic acid; P: PLGA membrane; PG: PLGA/type-Ⅰ collagen composite membrane; PGC 9:1:PLGA/type-Ⅰ collagen/chitosan (9:1) composite membrane; PGC5:5:PLGA/type-Ⅰ collagen/chitosan(5:5) composite membrane

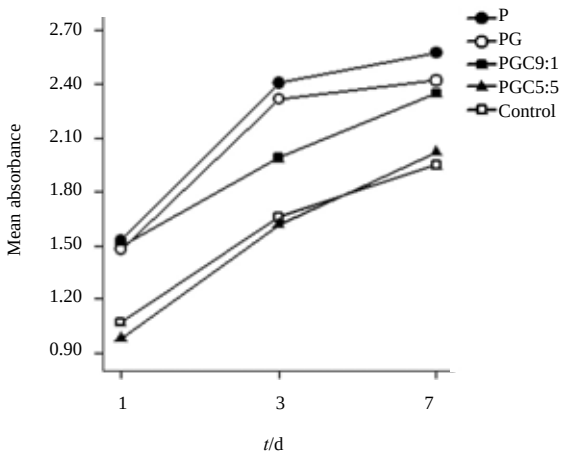
From Table 1, absorption rate was shown as P membrane < PGC 5:5 membrane < PGC 9:1 membrane < PG membrane ($P < 0.01$); contact angle was shown as PG membrane < PGC 9:1 membrane < PGC 5:5 membrane < P membrane ($P < 0.01$).

Morphological changes

Adherence of L929 fibroblasts occurred in PG membrane, PGC 9:1 membrane, and PGC 5:5 membrane three hours

after inoculation; additionally, 6 hours later adherence was rapidly accelerated, and L cell was characterized as well distribution, expansion and appearance. Complete adherence occurred 24-28 hours after inoculation, and L cells fully covered on the surface of membranes. In the P membrane, adherence started 4 hours after inoculated, rapidly accelerated after 8-10 hours, and completely finished after 36 hours.

MTT test (Figure 1)



PLGA: Poly-D, L-lactic-co-glycolic acid; P: PLGA membrane; PG: PLGA/type-Ⅰ collagen composite membrane; PGC 9:1: PLGA/type-Ⅰ collagen/chitosan (9:1) composite membrane; PGC5:5: PLGA/type-Ⅰ collagen/chitosan(5:5) composite membrane

Figure 1 Absorbency of membranes at different time points tested by MTT method

Figure 1 shows that on the 1st day, there was no significant difference in absorbency among groups ($P > 0.05$). On the 3rd day, there were significant differences between P membrane and PG membrane or PGC 9:1 membrane, and between PGC 9:1 membrane and PGC 5:5 membrane ($P < 0.05$); while, there were no significant differences between blank control group and PG membrane or PGC 9:1 membrane ($P > 0.05$), but significant differences between the other two groups ($P < 0.05$). On the 7th day, there were significant differences among P membrane, PG membrane, and PGC 9:1 membrane ($P < 0.05$), and there were also significant differences between PGC 9:1 membrane and PGC 5:5 membrane (P =0.000). Furthermore, there were significant differences between blank control group and other membrane groups (P =0.000).

DISCUSSION

Biocompatibility is a key member of tissue engineering. Cellular adhesion is a basis of cell migration, differentiation, and proliferation. Absorption rate can indirectly reflect hydrophilicity, *i.e.*, higher the absorption rate is, and stronger the hydrophilicity is. Furthermore, absorption rate is positively related to material porosity and aperture size. Results in this study suggested that absorption rate was ordered as P membrane < PGC 5:5 membrane < PGC 9:1 membrane < PG membrane ($P < 0.01$), this might be related to water absorbing capacity of type-I collagen > that of chitosan > that of PLGA[8].

Both hydrophilicity and wettability can be improved by contact angle that means the included angle between material surface and liquid droplet. The smaller the contact angle is, and the greater the wettability is. Customarily, $\theta > 90°$ is regarded as no wettability, and $\theta < 90°$ as wettability. This study demonstrated that contact angle was ordered as PG membrane < PGC 9:1 membrane < PGC 5:5 membrane < P membrane ($P < 0.01$). Size of contact angle was related to surface energy and surface cleaning degree of material and surface tension of liquid droplet. Contact angle of P membrane was larger due to a lot of pore structure and rough surface and stronger surface free energy of high molecular polymer. PG membrane was fully covered by type-I collagen, so its contact angle was 71.345° that was similar to pure collagen. We considered that the difference of contact angle between PG member and pure collagen was associated with rough surface of membrane[9]. When chitosan was compounded with membranes in different proportions, contact angle was increased. That was to say, higher the chitosan was, and larger the contact angle was. However, we also thought that size of contact angle could not be used as the only index to determine hydrophilicity, because conclusions might be different in wet environment. Both collagen and chitosan are modified in order to improve material intensity, shorten degradation time, and weaken immunity. At present, glutaraldehyde (0.25%) is a common cross-linking agent in clinical application. Low dosage or repeated irrigation may relieve effects of cross-linking agent on L929 cells. Cytotoxicity test showed that both collagen and chitosan could improve adhesion rate of L929 cell in an early stage and shorten time of complete adherence. Absorbency tested by MTT method suggested that, as compared to P membrane, PGC 9:1 membrane could improve proliferation of L929 cell on the 3rd and 7th day; PGC 5:5 membrane could obviously accelerate adhesion on the 1st day but inhibit growth of L929 cell on the 3rd and 7th days. Possibly, a lot of amino and hydroxyl of chitosan that were located on the surface of chitosan (5:5) and type-I collagen inhibited growth of fibroblasts in a later stage. Therefore, content of chitosan is a key factor to modify PLGA membrane, and it is significant for improving biocompatibility by an ideal proportion between type-I collagen and chitosan.

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聚乳酸-聚乙醇酸共聚物/Ⅰ型胶原/壳聚糖复合人工硬脊膜的生物相容性☆

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摘要

背景：聚乳酸-聚乙醇酸共聚物（poly-D,L-lactic-co-glycolic acid, PLGA）具有可吸收、细胞毒性小及硬度可调性的特点，符合作为人工硬脊膜材料的基本条件。但由于 PLGA 的表面缺乏功能性基团，生物相容性难以达到满意的要求。

目的：通过加入Ⅰ型胶原和壳聚糖对 PLGA 进行表面改性，观察其作为人工硬脊膜材料的生物相容性。

设计、时间及地点：对比观察实验,于 2007-05/12 在上海中医药大学生化与分子生物学实验室完成。

材料：多孔 PLGA 膜由济南岱罡生物科技有限公司提供，Ⅰ型胶原蛋白由美国 Sigma 公司提供，壳聚糖由上海其胜生物制剂医疗器械公司提供，L929 小鼠成纤维细胞由中国科学院上海生命科学院细胞所提供。

方法：制作多孔 PLGA 膜、PLGA/Ⅰ型胶原复合膜（简称 PG 膜）、PLGA/Ⅰ型胶原/壳聚糖(9∶1)复合膜(简称 PGC9∶1 膜)、PLGA/Ⅰ型胶原/壳聚糖（5∶5）复合膜（简称 PGC5∶5 膜)。

主要观察指标：各膜接触角、吸水率测定。L929 小鼠成纤维细胞体外培养 1，3，7 d 后纤维镜下观察细胞形态变化，并采用 MTT 法测定细胞毒性。

结果：接触角分别为：PG 膜< PGC 9∶1 膜< PGC 5∶5 膜< 多孔 PLGA 膜，各组间比较差异有显著性意义（$P < 0.01$)。吸水率分别为：多孔 PLGA 膜< PGC 5∶5 膜< PGC 9∶1 膜< PG 膜，各组间比较差异有显著性意义（$P < 0.01$)。L929 成纤维细胞在 PG 膜、PGC 9∶1 膜、PGC 5∶5 膜接种后细胞分布均匀，伸展及形态良好。细胞毒性实验显示：第 1 天，各组间吸光度值差异无显著性意义（$P > 0.05$)。第 3，7 天，多孔 PLGA 膜与 PG 膜、PGC 9∶1 膜各组间，PGC 9∶1 膜与 PGC 5∶5 膜组间差异有显著性意义（$P < 0.05$)。PGC 9∶1 膜可进一步改善复合膜的细胞黏附及增殖，PGC 5∶5 膜可抑制细胞增殖与分化。

结论：PLGA 膜的表面复合Ⅰ型胶原和壳聚糖可提高复合膜的生物相容性；PLGA/Ⅰ型胶原/壳聚糖（9∶1）复合膜在生物相容性方面基本符合人工硬脊膜的材料要求。

关键词：PLGA ；硬脊膜；壳聚糖；Ⅰ型胶原；生物相容性

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