# Histocompatibility of poly (lactic-co-glycolic acid)/RNA Ⅲ inhibiting peptide sustained release microspheres *

Li Jing-dong, Zhang Xiao-bin, Hao Li-Bo, Xing Qing-chang, Wang Ji-fang

**Abstract**

**OBJECTIVE:** To evaluate the histocompatibility of poly (lactic-co-glycolic acid)/RNA III inhibiting peptide (PLGA/RIP) sustained release microspheres.

**METHODS:** The crude peptide comprising N to C-terminals was synthesized using Fmoc method. The crude synthetic RNAIII peptide was purified by reverse phase high performance liquid chromatography, followed by component harvesting according to ultraviolet absorption peak, and freeze-drying. PLGA/RIP sustained release microspheres with a diameter of 50–70 μm were prepared using liquid-phase multiple emulsion method. The histocompatibility of PLGA/RIP sustained release microscopes were preliminarily evaluated through the use of acute general toxicity test, MTT cytotoxicity test, intramuscular implantation test, sensitivity test, and pyrogen test.

**RESULTS:** Acute general toxicity test results showed that all included animals survived and presented with no toxicosis reaction and obviously changed body mass. MTT cytotoxicity test results revealed that the average relative growth rate of cells from two eluents was over 85%, with cytotoxicity grade 1, which indicates no cytotoxicity. Intramuscular implantation tests showed that at 4 weeks after implantation of RIP powder or PLGA/RIP microscopes, no obviously congested, degenerated, or necrotic tissue was observed. All RIP powder and a part PLGA/RIP microscopes were degraded. Fibroblasts accounted for a large proportion in all cells. No inflammatory cell infiltration, involving neutrophils and multinucleated giant cells, was observed. Sensitivity test results displayed that the average primary irritation index was 0.38, 0.33, and 0.31 in the eluent stock solution, 2% dinitoflruorobenzene, and physiological saline-administerd groups, respectively. Pyrogen test results showed that fervescence of each rabbit in the experiment was under 0.5 ℃ and the sum of fervescence was under 1.3 ℃.This is in coincidence with evaluation criteria of pyrogen test.

**CONCLUSION:** PLGA/RIP sustained release microspheres exhibit good histocompatibility.

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## INTRODUCTION

Artificial joint infection is a disastrous, intractable complication[1]. Staphylococcus aureus and staphylococcus epidermidis, which can generate biofilm, are the primary pathogenic bacteria that cause repeated artificial joint infection and delayed healing[2-3]. RNAIII inhibiting peptide (RIP) has been shown to be able to prevent the infections caused by all staphylococci used in the experiment including methicillin-resistant staphylococcus aureus and staphylococcus epidermidis[4] and to be an overall effective staphylococci inhibitor[5]. The present study synthesized RIP and prepared poly (lactic-co-glycolic acid)/RNA III inhibiting peptide (PLGA/RIP) sustained release microspheres to preliminarily evaluate histocompatibility of microspheres using acute general toxicity test, MTT cytoxicity test, intramuscular implantation test, sensitivity test and pyrogen test, according to International Organization for Standardization and national standard GB/T16886.1[6-7].

## MATERIALS AND METHODS

### Materials

A total of 44 adult healthy New Zealand rabbits, of either gender, weighing 2.5–3.0 kg, were provided by Laboratory Animal Center, Chinese PLA General Hospital. Experimental protocol was in accordance with animal ethical standard.

### Drugs and reagents

PLGA (η = 0.25) was provided by Shandong Institute of Medical Equipment, China. Dimethyl sulfoxide and MTT were purchased from Sigma, USA. Dulbecco's modified eagle's medium (DMEM) was purchased from Gibco, USA. Dinitoflruorobenzene (DFB) was from Chinese PLA Military Medical Academy, China.

### Methods

#### *Preparation of PLGA/RIP*

The crude peptide comprising N to C-terminals was synthesized using Fmoc technique. The crude synthetic RNAIII peptide was purified by reverse phase high performance liquid chromatography using Waters 600 high performance liquid chromatograph (Waters, USA). Following ultraviolet absorption detection at 214 nm, component collection according to ultraviolet absorption peaks, and freeze-drying procedures, RIP purified products were obtained. 10 mg RIP was dissolved in 100 μL water to form aqueous phase, and 100 mg PLGA 75/25 was dissolved in 0.7 mL methylene chloride to form oil phase. The aqueous phase was added into the oil phase to yield a primary emulsion (water in oil) under iced bath ultrasound. The primary emulsion was added into 10 mL external aqueous phase containing 1% PVA, stirred at 700 r/min for 4 hours, filtered, washed, and vacuum freeze-dried to prepare PLGA/RIP microscopes with a diameter of 50–70 μm. Following γ-ray radiation (250 kGy), these microscopes were stored at 4–8 ℃.

#### *Eluent preparation*

PLGA/RIP microsphere powder was leached for 72 hours at 1 g/L using physiological saline at 37 ℃ to generate PLGA/RIP eluent stock solution. 50% density eluent was prepared by adding the same volume of sterile physiological saline.

***Acute general toxicity test***

Eighteen healthy adult New Zealand rabbits of either gender, weighing approximately 3.0 kg were intraperitoneally administered either 5 mL/kg eluent stock solution ($n$ = 6), or 5 mL/kg 50% density eluent ($n$ = 6), or 5 mL/kg sterile physiological saline ($n$ = 6). Immediately, as well as at 24, 48, and 72 hours after administration, dieting and activities of animals were observed, and toxicity was recorded (no-toxicity, mild toxicity, moderate toxicity, severe toxicity, death).

***MTT cytotoxicity test***

293 cell strains (Chinese PLA Military Medical Academy, China) were well growing cells after 48-hour passage culture. Cell culture medium was used as negative control. $3\times10^8$/L cell suspension was prepared using DMEM containing 10% fetal bovine serum and inoculated into a 96-well plate at a concentration of $2.5\times10^4$/well (200 μL/well). 20 μL eluent stock solution and 50% density eluent was added separately. Following 72-hour incubation at 37 ℃ in a 5% $CO_2$ incubator, 20 μL MTT (5 g/L) was added to each well, after which, there was 4-hour incubation at 37 ℃. Subsequent to culture removal, 150 μL dimethyl sulfoxide, and 30-minute vibration was performed. Absorbance at 500 nm was determined through the use of ELX800uv ELISA reader. Absorbance determination was run in triplicate to obtain the mean value. Relative growth rate (RGR) was calculated as follows: RGR = (Absorbance $_{\text{experimental group}}$/Absorbance $_{\text{negative control group}}$) ×100%. Finally, respective cytotoxicity grade was designated according to following criteria: grade 0 : RGR≥100%; grade 1: 75%－99%; grade 2: 50%－74%; grade 3: 25%－49%; grade 4: 1%－24%; grade 5: RGR ≤ 0.

***Intramuscular implantation test***

Eight healthy adult New Zealand rabbits, half number in male and female, weighing 2.5–3.0 kg, were randomly and evenly divided into two groups. Following anesthesia by injection of 1.5% sodium pentobarbital (30 mg/kg) *via* the ear vein, local hair shaving, routine sterilization, and cloth paving, back skin and subcutaneous tissue along each side of spine were cut open through two 1.5-cm incisions to bluntly separate bilateral sacrospinalis. Then, 10 mg PLGA/RIP microspheres or RIP powder were implanted respectively, sarcolemma was sutured, implanted materials were fixed and labeled, and finally incisions were routinely sutured. At 1, 2, 3, and 4 weeks after surgery, 1 rabbit from each group was sacrificed per week. A muscular tissue block containing implanted materials was harvested, fixed with 10% formalin, and routinely sliced. No muscular irritation response was observed in gross observation or hematoxylin-eosin stained section observation.

***Sensitivity test***

Twelve adult New Zealand rabbits, half number in male and female, were randomly administered either eluent stock solution (experiment group, $n$ = 4), or 2% DFB (positive control group, $n$ = 4), or physiological saline (negative control group, $n$ = 4). At 24 hours prior to surgery, median back skin (approximately 10 $cm^2$) was shaved. Following ethanol sterilization, intradermal injection within the shaved region was made through 6 symmetry sites, with a site-site distance of 2 cm. Immediately as well as at 24, 48, and 72 hours after administration, focal skin reactions were observed and scored according to erythema, eschar, and edema degrees. 0–4 points were respectively designated for erythema and edema. The total score of skin responses was 8 points. The average primary irritation index was calculated according to the following two formulas: primary irritation index = total score of erythema, edema/ total number of injection sites, and the average primary irritation index = the sum of primary irritation index of all animals/ total number of all animals. A score of 0–0.4 indicated no irritation, a score of 0.5–1.9 demonstrated mild irritation, a score of 2.0–4.9 denoted moderate irritation, and rabbits with a score of 5.0–8.0 suggested severe irritation.

***Pyrogen test***

Prior to testing, rabbits were raised under the same environment and fed with the same animal feeds for 7 days. During this period, rabbit body temperature was daily measured. Six rabbits that exhibited normal body temperature, consciousness, appetite, and excretion, and not decreased body mass were used and administered either eluent stock solution ($n$ = 3) or 50% density eluent ($n$ = 3) at 37 ℃ *via* the ear vein, at a dose of 1 mL/kg, twice per week. Prior to administration, body temperature was measured as basal value. After initial administration, body temperature was measured every other 1 hour, totally 3 times. The fervescence was calculated as the difference between the highest among 3 temperature values and the basal value.

**Statistical analysis**

Statistical analysis was performed using SPSS 11.0 software. $t$ test was utilized for comparison among groups. $P < 0.05$ denoted statistical significance.

## RESULTS

**Acute general toxicity test**

Following intraperitoneal administration, 3 groups of rabbits presented with depressed state, delayed activity, no convulsion or respiratory depression. Twenty-four hours later, all animals exhibited normal activity and dieting, no dyspnea. During experimentation, toxic reactions were not observed, no animals died, and body mass was not changed.

**MTT cytotoxicity test** (Table 1)

The average RGR in two kinds of eluent was over 85%, with cytotoxicity grade 1, which indicates no cytotoxicity. Significant difference existed when compared with negative control group ($P > 0.05$).

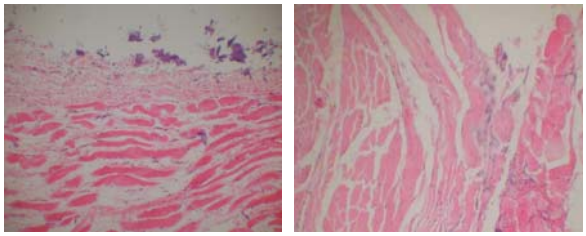
Table 1 Results of cytotoxicity test, as detected by MTT assay ($\bar{x}\pm s$)

| Group | Absorbance value | Relative growth rate(%) | Grade of cytotoxicity |
|---|---|---|---|
| Eluent stock solution | 1.602±0.188 | 88.60 | 1 |
| 50% density eluent | 1.845±0.223 | 90.22 | 1 |
| Negative control | 1.956±0.176 | 100.00 | 0 |

**Intramuscular implantation test**

At 4 weeks after implantation of RIP powder or PLGA/RIP microscopes, the surrounding muscle tightly wrapped the materials, and no obviously congested, degenerated, or necrotic tissue was observed. All RIP powder and a part PLGA/RIP microscopes were degraded, showing irregular appearance. The fibrous connective tissue coating formed

around the materials which were not completely degraded and provided the space for ingrowth of connective tissues including fibers and vessels. Fibroblasts accounted for a large proportion in all cells. In addition, there were a small number of lymphocytes and macrophages. Hematoxylin-eosin staining results showed that cell inflammation involving neutrophils and multinucleated giant cells was not observed around RIP powder or PLGA/RIP microscopes (Figure 1).



a: × 40 b: × 60

Figure 1 Morphological observation following intramuscular implantation (Hematoxylin-eosin staining)

**Sensitivity test**

At 1 hour after administration of eluent stock solution, 4 rabbits from the experiment group exhibited slight erythema and mild edema. At 24 hours, erythema and extremely mild edema were still present in 1 rabbit, but these symptoms disappeared in the remaining 3 rabbits. At 48 hours, erythema and edema thoroughly disappeared. At 1, 24, and 48 hours, 4 rabbits from the negative control group showed similar symptoms to the experiment group. At 1 hour after sensitization, all rabbits from the positive control group exhibited slight erythema and mild edema, but these symptoms disappeared 24 hours later. The average primary irritation index was 0.38, 0.33, and 0.31 in the experiment, positive control, and negative control groups, respectively. There was no significant difference among 3 groups.

**Pyrogen test**

Prior to experimentation, the body temperature of rabbits was 38.3–38.6 ℃. The change of body temperature after eluent administration is shown in Table 2. Results demonstrated the fervescence of each rabbit in the experiment was under 0.5 ℃ and the sum of fervescence was under 1.3 ℃.This is in coincidence with evaluation criteria of pyrogen test. These results suggest that the compound material contained the pyrogen, which satisfied the requirements of living body, and exhibited good biocompatibility.

Table 2 Results of pyrogen test

| Group | Number | Body temperature prior to administration (℃) | Body temperature after administration (℃) | | |
|---|---|---|---|---|---|
| | | | 1 h | 2 h | 3 h |
| Eluent stock solution | 1 | 38.5 | −0.2 | 0.2 | 0.3 |
| | 2 | 39.3 | 0.1 | 0.2 | −0.2 |
| | 3 | 38.8 | 0 | 0.2 | 0.4 |
| 50% density eluent | 4 | 38.5 | 0.2 | 0.2 | −0.2 |
| | 5 | 38.6 | 0 | 0.2 | 0.2 |
| | 6 | 39.1 | 0.1 | 0.3 | 0.3 |

## DISCUSSION

Biomaterials should possess biocompatibility to ensure the application safety in the clinic[8]. There have been two methods today to evaluate the biocompatibility: hemocompatibility and histocompatibility; the former reflects the mutual adaptative degree between biomaterial and blood, and the latter reflects the nutual adaptative degree between biomaterials and tissues with exception of blood[9]. Both polylactic acid (PLA) and PLGA possess good biocompatibility and safety[10-11]. Previous studies have reported that simple RIP possesses good histocompatibility and safety, but few studies are reported with regard to the toxicity of PLGA/RIP to human body[12]. The present study evaluated the histocompatibility of PLGA/RIP using *in vivo* experiments and detected cytoxicity using MTT assay.

General toxicity test is used to determine the toxicosis reactions of biomaterials or their eluents following body absorption[13]. The present acute general toxicity test results demonstrated that following implantation of materials, rabbits generally survived, no adverse events occurred, and obviously changed body mass was not observed. These results priliminarily indicate that the prepared PLGA/RIP microspheres possess good biocompatibility but without marked toxicity and stimulation.

MTT cytoxicity test, a represent for *in vitro* measuring biomaterial safety, has been become one of important indices in the evaluation system of biomaterials and medical equipment[14] and is an indispensable procedure for each tested material prior to clinical application[15]. Toxic substance released on the surface of eluent or material will influence cell growth[16]. Cytoxicity can be precisely determined by dynamic observation of cell morphology and detection of cell number change. Greater toxicity of tested material indicates greater cell inhibition[17]. The present study determined RGR in PLGA/RIP eluent stock solution and 50 % density eluent. Results showed that all testing samples exhibited cytoxicity at grade 0, suggesting that testing samples possess good cell biocompatibility but exhibited no cytotoxity.

Intramuscular implantation test is one of indispensable methods for detecting biocompatibility of biomaterials[18]. It enables the direct observation of immune response of animal organisms to antigens and chemical substances in the materials[19]. Intramuscular implantation test results from this study showed that following implantation of RIP powder or PLGA/RIP, focal red swelling was not observed, and incisions well healed. By 4 weeks after implantation, there were only a few lymphocytes and macrophages around the materials which had been wrapped by fibers, and no obvious inflammatory cell infiltration was observed. These results suggest that PLGA/RIP microscopes barely exhibit antigenicity but possess good histocompatibility.

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# 聚乳酸乙醇酸/RNA Ⅲ抑制肽缓释微球的组织相容性*

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**摘要**

**目的：**通过实验评价聚乳酸乙醇酸/ RNA Ⅲ抑制肽缓释微球的组织相容性。

**方法：**采用 Fmoc 法由 C 端至 N 端先合成粗品肽；采用反相液相色谱法对 RNA Ⅲ抑制肽粗品进行纯化分析，按紫外吸收峰收集组分，冷冻干燥，得到 RNA Ⅲ抑制肽纯品。再采用液相复乳法制备直径 50~70 μm 的聚乳酸乙醇酸/RNA Ⅲ抑制肽微球。以急性全身毒性试验、MTT 细胞毒性试验、肌肉内植入试验、过敏试验、热原试验对其组织相容性进行初步评价。

**结果：**①急性全身毒性试验结果显示 3 组动物无中毒反应，无动物死亡，体质量无明显变化。②MTT 细胞毒性试验结果显示两种浸提液的平均细胞增殖率均大于 85%，细胞毒等级 1 级，不具有细胞毒性。③肌肉内植入试验结果显示 RNA Ⅲ抑制肽粉末和聚乳酸乙醇酸/RNA Ⅲ抑制肽微球植入 4 周后，组织未见明显充血、变性或坏死。RNA Ⅲ抑制肽粉末完全降解，微球部分降解，主要细胞成分为纤维母细胞，未见中性粒细胞及多核巨细胞等炎症细胞浸润。④过敏试验结果显示 3 组动物的平均原发刺激指数分别为 0.38、0.33 和 0.31，3 组间无显著差别。⑤热原试验结果显示每种材料测试的 3 只新西兰大白兔中，体温升高均在 0.5 ℃以下，并且体温升高总度数在 1.3 ℃以下，符合热原实验的评价标准。

**结论：**聚乳酸乙醇酸/RNA Ⅲ抑制肽微球具有良好的组织相容性。

**关键词：**聚乳酸乙醇酸/RNA Ⅲ抑制肽微球；组织相容性；动物实验
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