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Original Article

In vivo study on the histocompatibility and degradation behavior of biodegradable poly(trimethylene carbonate-co-D,L-lactide)

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The aim of this study was to explore the *in vivo* behavior and histocompatibility of poly(trimethylene carbonate-co-D,L-lactide) (PDLLA/TMC) and its feasibility of manufacturing cardiovascular stents. Copolymers with 50/50 molar ratio were synthesized by ring-opening polymerization with TMC and D, L-LA, or TMC and L-LA. Poly (L-lactide) (PLLA) was synthesized as a control. The films of the three polymers were implanted into 144 Wistar rats. At different time points of implantation, polymer films were explanted for the evaluation of degradation characteristics and histocompatibility using size exclusion chromatography , nuclear magnetic resonance , environmental scanning electron microscope , and optical microscope. Results showed that there were differences in the percentage of mass loss, molecular weight, shape and appearance changes, and inflammation cell counts between different polymers. With the time extended, the film's superficial structure transformed variously, which was rather obvious in the polymer of PDLLA/TMC. In addition, there were relatively lower inflammation cell counts in the PDLLA/TMC and poly(trimethylene carbonate-co-L-lactide) (PLLA/TMC) groups at different time points in comparison with those in the PLLA group. The differences were of statistical significance ($P < 0.05$) in the group of PDLLA/TMC vs. PLLA, and the group of PLLA/TMC vs. PLLA, but not within the PDLLA/TMC and PLLA/TMC groups ($P > 0.05$). These results suggested that the polymer of PDLLA/TMC (50/50) with favorable degradation performance and histocompatibility is fully biodegradable and suitable for manufacturing implanted cardiovascular stents.

Keywords biodegradation; biocompatibility; polymer; vascular stents; *in vivo*

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Introduction

Coronary artery disease has become a major threat to human life in the past decades, and percutaneous coronary intervention therapy with stent implantation is one of the main treatment options [1]. However, currently used bare metal stents and drug-eluting stents are known to affect the state of intima, blood dynamics, systolic and diastolic function of the target blood vessels due to their sustained mechanic action, as well as neointimal hyperplasia and thrombosis that may result in in-stent restenosis or lumen occlusion, thus increasing the incidence of major adverse cardiac events (MACE) [2–8].

Biodegradable polymers, in particular poly(D, L-lactide) (PDLLA), poly(L-lactide) (PLLA) and poly(trimethylene carbonate) have been largely used for biomedical applications because of their outstanding biodegradability, mechanical properties, and minor cytotoxicity and immunogenicity [9–14]. It is well known that stents implanted in the stenotic vessels should possess sufficient mechanical strength and elasticity in transverse and radial orientation to support and keep the vascular lumina with long-term patent. The biodegradation of the stents should match the healing of the injured vessels, so as to maintain an effective support, but not cause in-stent restenosis for the neointimal hyperplasia and thrombosis. Actually, all homopolymers commercially available, including PLLA, PDLLA, PGA (polyglycolic acid), or PCL (polycaprolactone), cannot possess both characteristics mentioned above. It is thus of great interest to find a balance between the stent biodegradation and mechanical properties. In our previous reported work, various homo- and copolymers were synthesized from L-lactide, D, L-lactide, trimethylene carbonate, and ϵ-caprolactone. Among them, PDLLA-TMC and PLLA-TMC present favorable *in vitro* degradable behavior, good cyto-compatibility, and mechanical properties

[15–18]. In this work, we studied the *in vivo* degradation and histocompatibility of PLLA/TMC and PDLLA/TMC copolymers to explore the feasibility of manufacturing fully biodegradable cardiovascular stents.

Materials and Methods

Reagents and raw materials

D,L-lactic acid, L-lactic acid, 1,3-propanediol, trimethylene carbonate diethylmaleate, dimethyl benzene, dibutyl tin dilaurate, metallic sodium, zinc dust, tin powder, and stibium trioxide (AR degree; National Pharmaceutical Group and Chemicals Co., Ltd, Shanghai, China), glycolic acid (AR degree; Shanghai Yuanyue Chemical Industry Co., Ltd, Shanghai, China), and zinc lactate and tin octoate (Sigma Aldrich, USA) were used without purification. Acetic ether, dichloromethane, methanol, and tetrahydrofuran (THF) (AR degree) were from Shanghai First Chemical Reagents Factory (Shanghai, China). Acetone and diethyl ether (AR degree) were from Shanghai Malu Pharmaceutical Factory (Shanghai, China). Type II collagenase (2.0 g/L) and trypase (2.5 g/L) were from Shanghai Xitang Biological Technology Co., Ltd (Shanghai, China).

Polymer film preparation

The polymer films were prepared with the method of heated-melt and mould fixation as previously reported [15–18]. The three kinds of polymer materials and 10% (w/v) dichloromethane were melted into collosols by heating and cast into the culture capsules, and fixed into the films with dimensions of 0.4 mm × 10 mm × 10 mm overnight, respectively. Then the films were vacuum-dried, disinfected by ultraviolet light, wrapped up with aluminum foil sheet, and stored in vacuum at 4°C.

Nuclear magnetic resonance

The polymer chemical structure was determined by nuclear magnetic resonance (NMR) Bruker AV500 (Bruker BioSpin S.A.S., Wissembourg, France) at room temperature, with a resonance frequency of 500 Hz, using dimethyl sulfoxide as solvent and tetramethylsilane as internal standard. In consequence, the results of spectrogram flame ionization detector resolution was 0.245 Hz/point, with the scan width of 8 kHz and the dwell time of 2.04 s.

Size exclusion chromatography

The polymer molecular weight and its distribution were measured by size exclusion chromatography (SEC) using Alltima™ column (W. R. Grace & Co. Conn., Deerfield, USA). Measurements were performed on a Waters 515 apparatus (Waters Corporation, Milford, USA) equipped with a refractive index detector using tetrahydrofuran as solvent at a flow rate of 1.0 ml/min. Twenty microliters of 1.0% (w/v) solutions were injected for each analysis. Calibration was accomplished with polystyrene standards.

Environmental scanning electron microscopy

Environmental scanning electron microscopy (ESEM) with the standard mode was used to observe the surface and fracture cross-side morphology of films dried at vacuum, using a Philips XL30 ESEM (Philips, Amsterdam, the Netherlands) with tungsten filament. ESEM micrographs were obtained under reduced pressure at room temperature.

Experiment animals

Animal experiments were carried out in accordance with the Chinese legislations on Regulations for Experimental Animals Administration (The People's Republic of China Science and Technology Commission Publication No. 2, Chapter IV, 1988), and protocols were approved by the Shanghai Jiao Tong University Ethics Review Board (SYXK-Shanghai, 2006-0010). In addition, the handling of the animals conforms to the NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985). The 144 Wistar rats (equal in gender, 6 months, 180 g) were supplied by Shanghai Laboratory Animal Breed Base (Shanghai, China). The animals were divided into six groups (24 each) according to different time points: 2, 15, 30, 60, 90, and 180 days. Then each group was divided into three sub-groups (8 each) for PDLLA/TMC, PLLA/TMC, and PLLA. In each sub-group, four rats were used for *in vivo* study of biodegradation behavior and four rats for histocompatibility study. Rats were bred in the clean constant room-temperature environment, with gender ratio equal in each group.

Copolymer implantation and specimen collection

The films of PDLLA/TMC (50/50), PLLA/TMC (50/50), and PLLA were dipped into 75% ethanol solution for 15 min, and rinsed with 5% sodium chloride solution for 2 min. Rats were anesthetized through peritoneal injection with ketamine at 10 mg/kg. The lateral back skin was incised in longitude, and the subcutaneous tissue was dissected into a pouch about 4 cm × 5 cm in size where the three kinds of films were implanted and the incisions were sutured using aseptic technique. Subsequently, the animals were bred in the clean environment at constant room temperature without antibiotics administration until they were killed by euthanasia with intravenous injection of hypertonic potassium for the specimen collection.

Calculation of *in vivo* mass loss ratio of the polymers

The polymer films were explanted from the animals at different time points, and the surrounding tissues were removed. The films were then dipped into type II collagenase (2.5 g/L) solution and trypase (2.0 g/L) solution for 1 h respectively, rinsed

twice with deionized water, and weighed to obtain the wet weight (W_w). Then the films were dried in a vacuum oven for 1 week up to constant weight, to obtain the dry weight (W_d). The *in vivo* mass loss ratio of the polymers was calculated according to the following equation: mass loss (%)$=(W_i - W_d)/W_i) \times 100\%$, where W_i represents the initial weight of the polymers, and W_d represents the dry weight of the polymers. Each data point was obtained from triplicate samples.

Histopathology analysis

At different time points of implantation, the polymer films as well as the surrounding tissue were explanted and cut into two equal parts for each sample. One part was used in ESEM scan to observe the surface and structure changes, and the other was fixed in 4% methanol for 24 h, and made into paraffin section for hematoxylin and eosin (H&E) staining to observe the inflammatory reactions. MIQAS, the software for the quantitative analysis of medical images (Qiu Wei Biotechnology Co., Ltd, Shanghai, China), together with an Olympus BH2 optical microscope (Tokyo, Japan) and a JVC TK-C1481 BECdigital camera (Tokyo, Japan) were used in image collection and analysis. The figure style was TIF (tag image file). The number of inflammation cells in a slice was the average count in three randomly chosen areas of 0.017 mm^2 around the film and surrounding tissue. The histopathology analysis of the polymer films was carried out under blind conditions by a pathologist.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, USA) was used in the analysis. All the data were expressed as mean $\pm$ SD and analyzed by one-way analysis of variance for group comparison. Statistical significance was accepted at $P < 0.05$.

Results

General health state of animals

Twenty-four hours after implantation of the three polymers, all the Wistar rats ate food and acted normally, without fever, and no clear-cut swelling, bleeding, and oozing. Seven days later, the incisions healed primarily and no fatal accident occurred.

Mass loss changes

The *in vivo* degradation behavior of the three polymeric materials was quite different, as shown in **Fig. 1**. In the first 15 days, PDLLA/TMC, PLLA/TMC, and PLLA lost 6.9%, 6.1%, and 0.3% of their initial mass, respectively. After 60 days, the mass loss slightly increased to 10.2%, 6.74%, and 2.45%, respectively. Thereafter, the mass loss of PDLLA/TMC accelerated, and reached 27.6% and 89.0% after 90 and 180 days, respectively. In contrast, the mass loss of PLLA/TMC and PLLA was much lower. Therefore, the whole degradation rate of PDLLA/TMC is highest, followed by PLLA/TMC and PLLA.

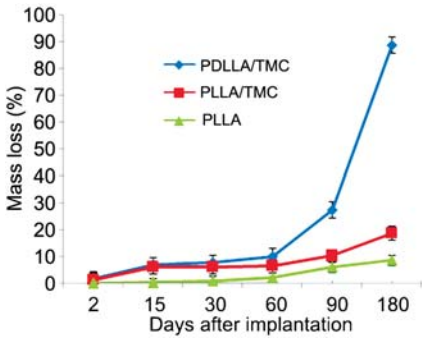

Figure 1 Mass loss ratio of PDLLA/TMC, PLLA/TMC, and PLLA polymer films The change curves of the mass loss ratio of the three kinds of polymers biodegradation *in vivo* at six different time points were shown. Mass loss (%)$=(W_i - W_d)/W_i \times 100\%$; W_i, the initial weight of the polymers; W_d, dry weight of the polymers after implantation. Three duplicate samples were used for each data point.

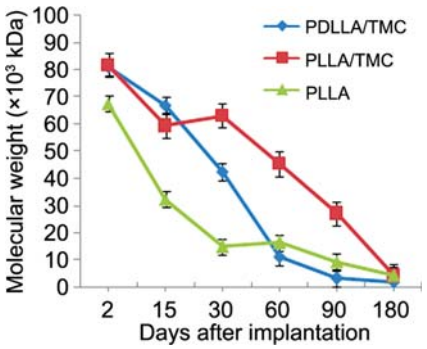

Figure 2 Molecular weight changes of PDLLA/TMC, PLLA/TMC, and PLLA polymer films The molecular weight profiles of the three kinds of polymers biodegradation *in vivo* at six different time points were measured by SEC. Three duplicate samples were used for each data point.

Changes of molecular weight

At the beginning, the molecular weight of the three kinds of polymer films implanted into the rats decreased rapidly. Later on, the decrease entered a slow stage, as shown in **Fig. 2**. The molecular weight of PLLA decreased rapidly from initial 72,000 to 68,000 kDa in the first 2 days and to 32,000 kDa 15 days later. Similar profiles of molecular weight decrease were obtained for PLLA/TMC and PDLLA/TMC copolymers, although PLLA/TMC appeared more resistant to hydrolytic degradation. Beyond 60 days, the molecular weight of PDLLA/TMC decreased below 10,000 kDa, in agreement with the rapid mass loss observed in this period.

Structural transformations of the films

With the *in vivo* degradation, the smooth and orderly superficial structure of the three films transformed into wrinkle,

distort, sagged, full of holes and hollows, as shown in **Fig. 3**. There were no visible holes detected with relative smooth structure on the PDLLA/TMC film surface after 2 and 30 days of degradation *in vivo*. In contrast, holes appeared more on the surface of the other two films with relative smooth and orderly structure. After 90 days, the PDLLA/TMC film showed visible cracks and wrinkles. Notable sags, gaps, and holes were visible after 180 days. At the same terms, the surface of PLLA/TMC and PLLA films kept relative smooth and orderly although some gaps were observed.

In vivo gross anatomy observation

At the time point of 2 weeks, there were no films distort and enwrapping, no visible tissue congestion and edema surrounded with the three polymer films except for some fiber proliferation. On Day 30, all the films were covered with a thin and translucent fiber coating with a loosen adhesion, without evident deformation, distortion, and disintegration. After 90 days, the coating fiber membranes around the three polymer films thickened. The fiber membranes adhering to the films of the PDLLA/TMC and PLLA/TMC were tighter and more difficult to separate in comparison with that of the PLLA. Moreover, the PDLLA/TMC and PLLA/TMC polymer films appeared deformed and wrinkled excluding the PLLA film whose surface appeared whitish for the light descent. By the time of 6 months, the surrounding tissues grew into the films of PDLLA/TMC and PLLA/TMC and their volumes decreased obviously, which made their shapes look like spindle or rice. It was difficult to separate them out, and the film of PLLA broke into fragments.

H&E staining of the films

The H&E staining images of the three kinds of films were shown in **Fig. 4**. After 2 days and 15 days, a great number of inflammatory cells, mainly lymphocytes and neutrophils, and a few macrophages were infiltrated within the films and surrounding tissues, with a few proliferated fibroblasts and secreted collagen connecting them. After 30 days, the number of inflammatory cells within the loose collagen fibers decreased, and the films were encapsulated obviously by the numerous neo-formative fibroblasts and connective fibrous tissue. By Day 90 and 180, the number of inflammatory cells, mainly lymphocytes, reduced to a further low degree with thickened and compacted collagen fibers. After 180 days, the PDLLA/TMC film boundaries and its surrounding tissue obscured with atypical hyperplasia, where the collagen fibers became thinner gradually. In contrast, the boundaries of PLLA/TMC and PLLA films remained clear with much limpid membranous fiber covering them, without obvious tissue degeneration, necrosis, and atypical hyperplasia.

Comparison of the three films' surrounding tissue reaction using optical microscope showed that the inflammation reaction around the PDLLA/TMC and PLLA/TMC films were milder than that of the PLLA, and the surrounding cells of the PDLLA/TMC and PLLA/TMC films arranged much orderly within wrapping thinner fiber membranes than those of the PLLA. Moreover, the films' surrounding inflammatory response within the two groups of PDLLA/TMC and PLLA/TMC had no obvious differences.

Inflammatory cell counting in the surrounding tissues of three films

There were few inflammation cells in the PDLLA/TMC and PLLA/TMC groups at different times, especially at the later 90 days, when compared with the PLLA group ($P < 0.05$). There was no statistical significance between PDLLA/TMC and PLLA/TMC groups (**Fig. 5**).

Discussion

The *in vivo* mass loss of PDLLA/TMC, PLLA/TMC, and PLLA polymer films with time can be divided into three stages. First, the rapid increasing stage occurred at the beginning of the several days with fast mass loss for PDLLA/TMC and PLLA/TMC. The initial mass loss could be assigned to the release of residual monomers or oligomers. If there was a sub-acute inflammation response in the first stage, most likely a change of the tissue pH occurred, which could also contribute to initial degradation. On the other hand, pH decrease on polymer degradation is able to trigger and sustain inflammation. It has been reported that disodium hydrogenphosphate could improve the biocompatibility of degrading PDLLA at the point of viscous disintegration by stabilizing the microenvironmental pH value of the implants for several weeks and reducing adverse tissue reactions [16]. Also, the motion and friction that the film was subjected to in the subcutaneous area may be another reason. After sub-acute inflammation response for about 14 days, the degradation went into the stationary stage, in which the mass loss rate was obviously very slow. The explanation may be that with time extending the inflammatory response regressed, the proliferative fiber membranes covered the films, and various factors present in the degradation process participated, and therefore the microstructure and molecular constitution of the polymers remained relatively stable to maintain their optimal mechanical properties, and their molecular weight reduction did not reach the level of the polymer substance fully dissolved. Another explanation could be that the pH rose again on decreasing inflammation and this decelerated the polymer degradation. Finally, the degradation process was the acceleration stage. The hydrolysis degradation of

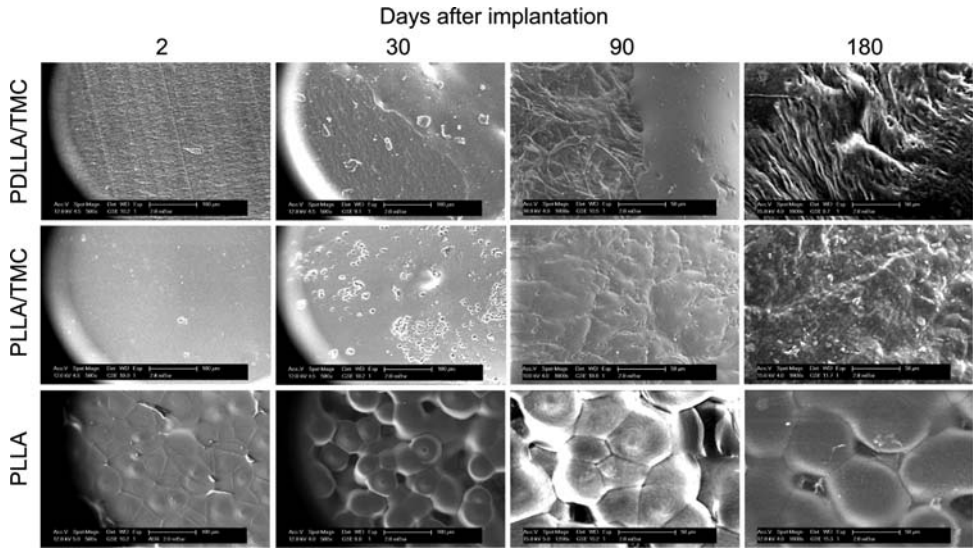

Figure 3 Structural transformations of the PDLLA/TMC, PLLA/TMC, and PLLA films under ESEM The superficial microstructure changes of the three kinds of polymer films biodegradation *in vivo* at four different time points (500 folds enlarged for 2 and 30 days, 1000 folds enlarged for 90 and 180 days, respectively) were showed within the images obtained by the ESEM under reduced pressure (5 Torr) at room temperature.

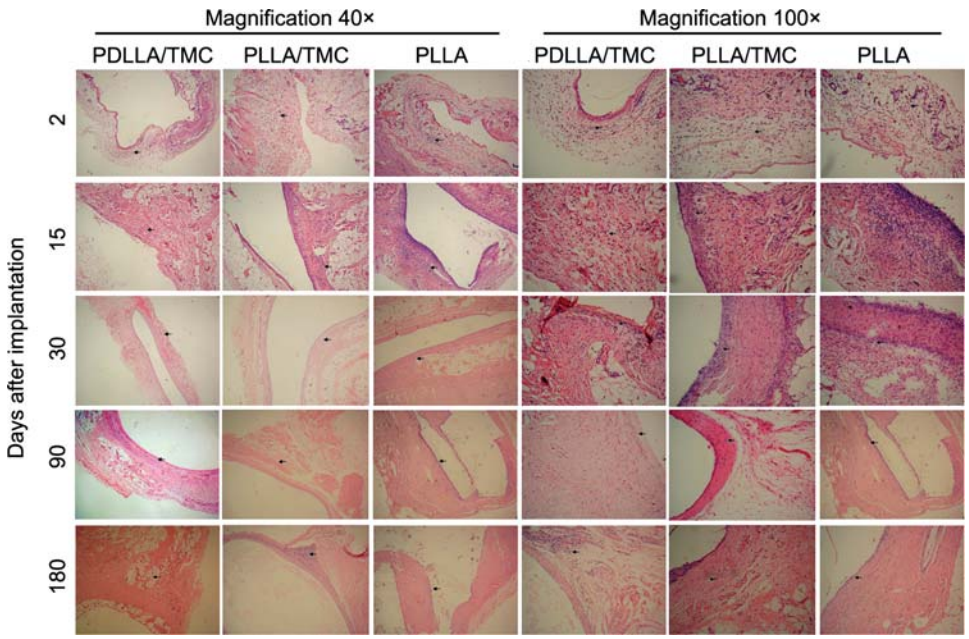

Figure 4 H&E staining of the PDLLA/TMC, PLLA/TMC, and PLLA films The H&E staining images of the three kinds of polymer films biodegradation *in vivo* and their surrounding tissues at five different time points were showed with 40 folds enlarged (left three columns) and 100 folds enlarged (right three columns) images, respectively, in which the black arrows represent inflammatory cells.

PDLLA/TMC was rapid in this stage, because the used poly rac-LA unit for synthesis is heterotactic or tactic stereoblock while atactic configuration cannot prevent water molecular from entering into the polymer substance [17]. Once the polymer substance weight declined, mass dissolved gradually for the hydrolysis, and most of the ester bond fractured not to maintain its structure properties, then the tissue enzymatical or non-enzymatic acidic hydrolysis or both of them acted on the surface of the films making their superficial microstructures disintegrated gradually, emerging major pores, rifts, sinus, and collapse. Further, with the decreasing of polymers molecular weight and the dissolving of substance over the surface cracks, the velocity of the substance dissolution accelerated. These should be the reasons of the polymer mass loss displaying a phenomenon of rapid acceleration after the stationary phase. It can be inferred that, once the polymers are manufactured into cardiovascular stents and implanted into the vessels and adherent to their walls, the stents would soon be encapsulated by vessel endothelial cells, avoiding the rapid erosion of the blood flow, with similar degradation process and mass loss rate to the films embedded in the

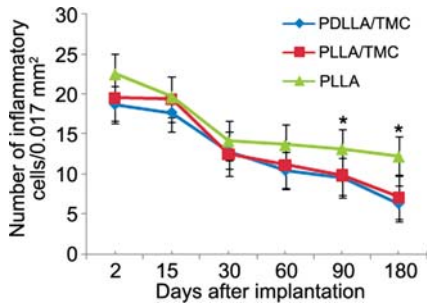

Figure 5 Inflammatory cell counts The inflammatory cell counts per 0.017 mm^2 of the three kinds of films and surrounding tissues at six different biodegradable time points were showed. The number was the average value of three randomly chosen areas in a slice by a pathologist under blind conditions. $*P < 0.05$ compared with PDLLA/TMC or PLLA/TMC.

subcutaneous. Also, it would be likely, in case of vascular stents implanted, that the blood circulation will maintain a more constant pH at the polymer surface, thus preventing a rapid degradation in the primary stage. Therefore, when the polymers were made into stents and implanted into the vessels, their degradation characters *in vivo* need to be further studied.

The polymers mass loss lagged significantly to their molecular reduction, which displayed that most of the degradation processes were concurrent on the entire copolymer matrix confined to the ontology hydrolysis category of ester fracture. During the degradation, when the polymer molecular weight decreased enough to be dissolved in the liquid, the mass loss phenomenon appeared. The similar trends of the polymer mass loss and biodegradation rates indicated that the polymer degradation *in vivo* was mainly the hydrolysis process, also correlated to various factors such as enzymes, blood circulation, and stress [19–23].

Mass loss of the PDLLA/TMC film embedded in the subcutaneous approached 90% after 180 days, which was much higher than those of the PLLA/TMC and PLLA films. It has been reported that the degradation kinetics and water ingestion depend on the degree of crystallinity of the polymers. Amorphous structure facilitates water molecular as well as some small molecular weight oligomers to enter into the polymer and access to the substance, while crystal structure inhibits the process [24,25]. Because of their highly crystalline structure, the *in vivo* hydrolysis process of PLLA/TMC and PLLA polymers are quite slow. On the contrary, for its amorphous configuration structure, the mass loss of PDLLA/TMC is faster. Although there are other factors that may affect and slow down the degradation process of PLLA/TMC and PLLA polymers *in vivo*, such as molecular weight changes, autologous dissolution, surface corrosions, and bio-enzymes, it can be seen that the PLLA/TMC and PLLA polymers had no favorable degradation behaviors *in vivo* due to their crystalline structure and relatively strong hydrophobic effect.

Comparison on the superficial morphology of the three kind films at different time points showed that the surface characteristics of PDLLA/TMC polymer changed obviously, with more sags and holes, and wider and deeper cracks. Especially in the later terms they appeared more chaotic and disorderly. The polymer surface corroded variously and changed with its utmost mass loss and degradation rate, which indicated that the deformation correlated with the mass loss. Also, the small pores on the film surface testified that the degradation process of low molecular substance was peel dissolving. During explantation, we found that the three kinds of polymer films embedded in the subcutaneous transformed and curled variously at the time point of 60 days. Though they are the mild changes, they might affect the maintenance of the PDLLA/TMC polymer's inherent mechanical properties. Using special modification techniques, the support power of the PLLA stents can reach 0.21–0.25 MPa equivalent to that (0.20–0.22 MPa) of the ordinary metal stainless-steel stents, indicating sound mechanical properties of polymer scaffolds [26]. Therefore, by modification techniques to change the components and constitute a proportion of the PDLLA/TMC polymer, we believe that the balance between the mechanical properties and degradable behaviors could be found to meet the demands of fully biodegradable cardiovascular stents.

The inflammatory reactions caused by the implanted materials were aseptic and non-specific inflammatory response. The initial mild reactions around the films were acute and sub-acute aseptic inflammation due to operational stimulus, mechanical injuries, and implants themselves. Polymer fragments, oligomers, monomers, and released polymerization catalysts may arouse inflammatory response of surrounding tissue and gathering of inflammation cells. With time and wrapping of films by fiber capsular, the transudation and redness representing acceptable histocompatibility responses to extraneous materials disappeared.

According to gross observation, H&E staining appearances and the inflammatory cell counts, the inflammatory response of PDLLA/TMC polymer were less than those of PLLA, but similar to those of PLLA/TMC. Compared with the other two kinds of polymers, during the degradation process PDLLA/TMC films had more cracks and lager apertures on the surface, which facilitated the entrance of various cells into them, but more difficult to separate the films out for the tightly connected fibers, especially in the later terms. After 90 days, the PDLLA/TMC and PLLA/TMC films showed various curling deformation, some of them looked like spindle for the tissue wrapping, which indicated that the materials possessed relatively inferior rigid structure and superior elasticity. On the contrary, the PLLA film possessed a relatively good rigid structure, thus resulting in difficult deformation but easy brittleness. Therefore, the embedded PLLA film within the

subcutaneous fractured and cracked due to the endured friction force. Consequently, the PLLA film pieces as extraneous materials caused relatively heavier inflammation reactions and more inflammatory cells gathering than those of the other two kinds of films. Our findings were coincident with previous data [27].

The molecular weight of polymer degradation products is also an important factor causing inflammation reaction. In particular, products of moderate molecular weight could increase the aseptic and non-specific inflammatory response, while the high molecular weight of PLLA degradation products could delay but not eliminate the response [28,29]. As PLLA is a crystallized structure with relatively hydrophobic property, it could delay but not prevent the dissolution process of low molecular weight degradation products in the early terms. Meanwhile, the crystal morphous structure of polymer intensified the friction between the implants and the surrounding tissues and aggravated the inflammation reaction. In the early terms, the molecular weight of PLLA polymer decreased faster and plenty of moderate and low-molecular-weight degradation products were dissolved, and the aseptic inflammation reaction within the surrounding tissues could be observed through the H&E staining. The moderate- and molecular-weight degradation products apart from the PDLL/TMC and PLLA/TMC were slower than that of PLLA, so their inflammatory responses were much less than that of the PLLA. In addition, the accumulated PLLA degradation products and the changed regional microenvironmental pH-value might affect the growth and function of body cells, such as cytotoxicity on the endothelial cell and even apoptosis. It has been proved that suppressing the aseptic inflammation by adding alkaline calcium carbonate, calcium bicarbonate, sodium bicarbonate, or hydroxyapatite into the polymers could balance or buffer the regional acid microenvironments caused by degradation products [30,31]. In this study, the degradable products of HCO_3^- from TMC within PDLLA/TMC played a neutral role in the regional acid microenvironment caused by PDLLA degradation, and reduced the inflammation reaction. The H&E staining displayed that the number of inflammatory cell counts in tissues surrounding the PDLLA/TMC was less than that of the polymer PLLA ($P < 0.05$), indicating favorable biocompatibility of PDLLA/TMC. In conclusion, PDLLA/TMC (50/50) with favorable degradation properties and histocompatibility may be suitable for manufacturing fully biodegradable cardiovascular stents.

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