RESEARCH ARTICLE

3D-Printed Degradable Anti-Tumor Scaffolds for Controllable Drug Delivery

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Abstract: In this study, porous polylactic acid/methotrexate (PLA/MTX) scaffolds were successfully fabricated by three-dimensional (3D) printing technology as controllable drug delivery devices to suppress tumor growth. Scanning electron microscopy and energy-dispersive spectrometer confirmed that MTX drug was successfully incorporated into the PLA filament. 3D-printed PLA/MTX scaffolds allow sustained release of drug molecules in vitro for more than 30 days, reducing systemic toxic side effects caused by injection or oral administration. In vitro cytotoxicity assay revealed that PLA/MTX scaffolds have a relatively high inhibitory effect on the tumor cells (MG-63, A549, MCF-7, and 4T1) and relatively low toxic effect on the normal MC3T3-E1 cells. Furthermore, results of in vivo experiments confirmed that PLA/MTX scaffolds highly suppressed tumor growth and no obvious side effects on the organs. All these results suggested that 3D-printed PLA/MTX scaffolds could be used as controllable drug delivery systems for tumor suppression.

Keywords: 3D printing; Polylactic acid; Methotrexate; Anti-tumor

1. Introduction
The mainstream treatment strategy for tumor is to remove the tumor tissue, supplemented by post-operative chemotherapy or radiation therapy. Despite the great progress in treatment for tumor, many adverse post-operative side effects, including the limited distribution of chemotherapy drugs at the target site and severe toxicity after radiotherapy, are unavoidable[1-3]. Therefore, developing a new drug delivery system that can overcome these limitations has become the focus of cancer research.

4-Amino-10-methylfolate (methotrexate [MTX]), an antitumor drug[4], has been widely used to treat various cancer diseases, such as head-and-neck tumors, breast cancer, and lung cancer[5-8]. In spite of its wide application in clinical practice, the treatment with MTX through injection or oral administration is still accompanied by some disadvantages, including non-tissue selectivity, the need for high dose, and high toxicity. In addition, MTX is resistant to bone marrow suppression and gastrointestinal cytotoxicity.

To avoid the shortcomings of injection and oral administration, it is necessary to design a drug delivery system to deliver MTX into the diseased area to achieve the optimal therapeutic effect[9]. Various strategies have been developed to control drug delivery using materials...
such as hydrogels, polymer micelles, and stimulus-responsive materials[18-22]. Three-dimensional (3D) printing technology, known as additive manufacturing (AM), has great potential in fabricating the personalized scaffolds[13-15]. Based on the patient’s computed tomography (CT) or magnetic resonance imaging (MRI), 3D models can be quickly and accurately established, making it possible to accurately print irregular models[16,17]. Moreover, the drug-loaded scaffolds made by 3D printing technology have unique advantages in personalization, spatial structure, drug components diversity, drug loading accuracy, and drug release sustainability[18-22].

Among various 3D printing technologies, fused deposition modeling (FDM), which was launched by Stratasys in 1992, has become one of the most popular technologies[23]. The technical advantages of FDM include the selectivity of a variety of applicable materials, customized high precision, and low cost[18]. As a typical heat dissipation technology for scaffolding, FDM uses a thermoplastic polymer filament, which is heated to the melting point, and then extruded from the nozzle, and deposited layer by layer to create a scaffold[24,25]. The thermoplastic materials used in FDM technology include polylactic acid (PLA), poly(ε-caprolactone) (PCL), poly(methyl methacrylate) (PMMA), polycarbonate (PC), and acrylonitrile butadiene styrene (ABS)[26-28]. Among these thermoplastic materials, PLA has been approved by the FDA as biomedical material due to its excellent biocompatibility[29,30]. Studies demonstrated that 3D printing is a powerful tool for manufacturing personalized scaffolds with specific geometries. Foulandian et al. reported that 3D-printed stents loaded with 5-fluorouracil (5-FU) drug were used to treat esophageal cancer. Incorporating anti-cancer drugs into endoluminal stents can provide a sustained release of drugs to esophageal malignant tissues while prolonging the retention of the stent and relieving dysphagia[31].

The purpose of this research is to prepare porous PLA/MTX scaffold with a controllable MTX release. PLA/MTX filaments with different MTX concentrations (MTX mass fraction: 0.5 wt%, 1.5 wt%, and 2.5 wt%) were prepared by melt mixing and extruding method. PLA/MTX scaffolds were printed by FDM using prepared PLA/MTX filament. The morphology, composition, and structure of printed PLA/MTX scaffold were investigated by scanning electron microscopy (SEM) and energy-dispersive spectrometer (EDS). The biocompatibility of printed PLA/MTX scaffolds and the inhibitory effect on tumor cells were evaluated in vitro by mouse embryo osteoblast precursor cells (MC3T3-E1), human osteosarcoma cells (MG-63), human breast cancer cells (MCF-7), human lung cancer cell lines (A549), and mouse breast cancer cells (4T1). In addition, the subcutaneous xenograft model was used to explore the inhibitory effect on tumor tissues and the toxicity to normal tissues and organs.

2. Materials and methods

2.1. Materials

PLA (MW: 500 kDa) was purchased from Sigma-Aldrich (Darmstadt, Germany), and MTX was purchased from Bio Basic Inc. (Markham, Ontario, Canada). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Japan). LIVE/DEAD® Viability/Cytotoxicity Kit (Live/Dead) was purchased from Thermo Fisher Scientific (L-3224). The fetal bovine serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM), RPMI-1640, penicillin-streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Grand Island (New York, USA). All the reagents were used without further treatment.

2.2. Preparation of the PLA and PLA/MTX composite filaments

The mixture of PLA and MTX was melted and extruded using granulators. PLA/MTX and PLA filament were prepared by the 3D printing consumable extruder (SHSJ, Songhu Machinery Co., Ltd., Dongguan, China) with a 1.75 ± 0.05 mm constant diameter at 220°C and cooled by water, and the screw speed was 45 rpm.

2.3. Fabrication of PLA/MTX scaffolds

The 3D printing bracket was designed using Mimics software and SolidWorks2015 software. STL files were converted to a format (geode) recognizable by 3D printer (ShanRui DK2, Guangzhou, China) using CURA software. An ink cartridge was added to the 3D printer to transport the PLA/MTX composite filaments, and the filaments were drawn and melted (210°C) and extruded through a nozzle (0.4 mm) to print layer by layer.

2.4. Characterization of PLA/MTX scaffolds

The structures and aperture sizes of PLA/MTX scaffold were characterized using field emission SEM (SEM, Zeiss Supra55, Germany). Energy-dispersive X-ray analysis (JXA-8230, JEOL, Japan) was then used to perform elemental analysis of the PLA/MTX scaffold surfaces to assess the distribution of MTX in the PLA matrix.

The high-precision digital density meter (ED-1000, Shanghai Tuxin Electronic Technology Co., Ltd.) was used to measure the porosity. The porosity calculation formula is as follows:

\[
\text{Porosity} = \left(1 - \frac{m}{\rho} \right) \times 100\%
\]

Where, \(V\): outer volume, \(m\): mass, and \(\rho\): density.
2.5. Drug loading capacity and drug release

The 500 mg of PLA/MTX scaffolds were placed in a beaker containing 3 mL CHCl₃ to fully dissolve, and then, phosphate-buffered saline (PBS) was used to extract the drug. The absorbance of the PBS was determined by ultraviolet (UV) spectrophotometer at 305 nm.

The 500 mg of scaffold was placed in a 5 mL centrifuge tube containing 3 mL PBS and then shaken at 37°C with a speed of 100 r/min. The absorbance of the scaffolds was determined by UV spectrophotometer at 305 nm and calculated by the following formula.

\[
\text{Loading efficiency} \% \ (\text{LE} \%) = \left( \frac{\text{Weight of encapsulated drug}}{\text{total mass of the PLA/MTX scaffold}} \right) \times 100\%
\]

\[
\text{Entrapment efficiency} \% \ (\text{EE} \%) = \left( \frac{\text{Weight of encapsulated drug}}{\text{Weight of total added drug}} \right) \times 100\%.
\]

2.6. In vitro experiments

(1) Cell lines

Mouse embryo osteoblast precursor cells (MC3T3-E1), human osteosarcoma cells (MG-63), human breast cancer cells (MCF-7), human lung cancer cell lines (A549), and mouse breast cancer cells (4T1) were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM (or RPMI) containing 10% FBS and 100 U/mL penicillin-streptomycin.

(2) Cytotoxicity assay

Cells were cultured using 24-well plates with a density of 1 × 10⁴ cells/well. When the cells adhered and spread in plates, the scaffolds were immersed into the culture medium. The CCK-8 assay was performed on days 1, 3, and 5. Briefly, the culture medium of the sample was replaced with 100 μL DMEM (RPMI) containing 10% FBS and 100 U/mL penicillin-streptomycin.

2.7. In vivo anti-tumor effect

(1) Subcutaneous xenograft model

The 4T1 cells (2 × 10⁶) were subcutaneously injected into the back of the BALB/c mice to establish the subcutaneous xenograft model. After surgery, celecoxib (Dalian Meilun Biotechnology, China) was given to relieve postoperative pain (10 mg/kg, gavage, q.d./2 days) and ceftriaxone sodium (Dalian Meilun Biotechnology, China) to prevent post-operative infection (20 mg/kg, tail vein injection, q.d./2 days). The mice were housed under the standard conditions.

2.8. Statistical analysis

All the experiments were performed in triplicate. Results were expressed as mean ± standard deviation (SD). Bonferroni post-test was performed to assess statistical significance. Statistical analysis was performed using non-parametric Kruskal–Wallis tests. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Morphological, composition, and structural analysis

The morphology, microstructure, and compositions were investigated by SEM and EDS (Figure 1). According to Figure 1A-1, SEM image of the PLA/MTX scaffold showed a relatively smooth surface and had open big pores in uniform size with integrated lines (755 ± 0.7 μm) among the big pores. Moreover, the printed PLA/MTX scaffolds were porous, with the porosity and pore size at 29.7% and 309 ± 0.5 μm, respectively.

The EDS spectra are shown in Figure 1B, and the peaks of C, N, and O were observed in the spectrum of PLA/MTX scaffold. The appearance of N peak confirmed that MTX was introduced into PLA matrix. The color map obtained from EDS analysis in Figure 1A shows the N (blue) distribution in the scaffold structure, which proves that MTX was well distributed in the PLA matrix. The evenly dispersion of MTX in the scaffold provides the possibility for MTX to be released along with PLA degradation.
3.2. Characterization of drug loading (LE%) and encapsulation efficiency (EE%) in PLA/MTX scaffolds and drug release profiles in vitro

Before assessing the release profiles of MTX from PLA/MTX scaffolds with different drug concentrations, the LE% and EE% were calculated by measuring the concentration of drug absorbance in the PBS. The EE% of MTX was 98.1% and the LE% of MTX was controlled at 0.5%, 1.5%, and 2.5%.

The release profiles of MTX from PLA/MTX scaffolds are shown in Figure 2. Approximately 25% of MTX was released within the first 24 h, and about 50% of MTX was released within the first 7 days. 3D-printed PLA/MTX scaffolds allow sustained release of drugs in vitro more than 30 days. In addition, with the increase of drug content, the release trend of MTX is basically similar. Therefore, the drug release could be controlled by adjusting MTX content in scaffolds. This result proved that PLA/MTX scaffolds had a sustained release effect.

Figure 3 shows the comparison of MTX release profiles of 3D-printed PLA/MTX scaffolds of varying drug contents and cast PLA/MTX with 0.5% of MTX over 5 days. The drug release pattern of cast PLA/MTX sample is similar to that of 3D-printed porous PLA/MTX scaffold. However, the drug release rate of the cast PLA/MTX sample is slower than that of 3D-printed PLA/MTX scaffold. In cast PLA/MTX sample, the MTX drug molecules were wrapped tightly by PLA material to obstruct the release of drug molecules from the polymer matrix. The release of MTX from 3D-printed porous PLA/MTX scaffold is more stable than cast sample.

3.3. In vitro cytotoxicity

Figure 4 shows the in vitro inhibitory effect of PLA/MTX scaffolds on human lung cancer cell lines (A549), human osteosarcoma cells (MG-63), human breast cancer cells (MCF-7), and normal mouse embryo osteoblast precursor cells (MC3T3-E1). As shown in Figure 4A-C, the inhibitory effect increases as the drug content increases. The growth of tumor cells (A549, MG-63, and MCF-7) has been significantly inhibited at 24 h. At the same time, the inhibitory effect increased as the administration time was extended to the third and 5th days. Each PLA/MTX scaffold has a 95% inhibition rate of tumor cells at the 120 h. The above results indicate that the implantable PLA/MTX scaffold showed strong anti-tumor efficacy compared with PLA group and control group. The toxicity of PLA and PLA/MTX scaffolds on normal MC3T3 cells was also investigated (Figure 4D). The results showed that PLA scaffold had little effect on the growth of normal cells,
while PLA/MTX scaffold had some effect on normal cells, but the effect was limited.

As shown in Figure 5, the toxicity of PLA and PLA/MTX scaffolds on cancer cells was also investigated by Live/Dead fluorescence staining of mouse breast cancer 4T1 cells. Due to its excellent biocompatibility, PLA scaffold group has low cytotoxicity within 120 h. The PLA/MTX scaffold groups showed a lower cell survival rate compared with PLA group and control group, and the inhibitory effect increased with the prolonged culture time. With the gradual release of MTX from the scaffold, the cell viability in the PLA/MTX scaffold group decreased. With the increase of drug concentration and culture time, the number of living cells gradually decreased and the number of dead cells gradually increased. The total induction of apoptosis of 4T1 cells by PLA/MTX scaffolds increased from 52.89% to 99.63% (Figure 4C).

Thus, compared with the PLA and control groups, the PLA/MTX scaffolds showed strong inhibitory effect on tumor 4T1 cells.

3.4. In vivo anti-tumor effect

As shown in Figure 6, the anti-tumor effects of PLA/MTX scaffolds were evaluated by subcutaneous xenograft model of 4T1 tumor-bearing Balb/c mice compared with intraperitoneal drug injection of MTX. The minor body weight changes in implant groups (Figure 6A) proved that the implanted scaffolds were well tolerated by the mice. Weight loss occurred in the 0.5% PLA/MTX and 2.5% PLA/MTX groups at the beginning due to surgical anesthesia, surgical implantation, and other operations, but the growth status in the middle and later stages was the same as the control group (Figure 6A). Throughout the experiment, the overall growth of mice in the negative control group was good, but one mouse in the control group was paralyzed due to excessive compression of the spine by the tumor. In the early stage, the growth state of the injection group was the same as that of the control group. In the later stage, there were some adverse phenomena, such as weight loss, bad hair, small and sticky stool, and scorched yellow urine. There was no significant difference in organ coefficient of heart, liver, lung, and kidney among groups. The organ coefficient of spleen in injection group was much lower than the other three groups, which was found at the time of dissection (Figure 6B). The tumor sizes in Figure 6C can directly show that PLA/MTX scaffold implantation significantly inhibited the tumor growth compared with control group. The anti-tumor effects of 0.5% PLA/MTX scaffold with low concentration of drug are similar to

![Figure 4](image-url) Evaluation of anticancer effect in vitro. In vitro proliferation of MCF-7(A), MG-63 (B), A549 (C), and MC3T3 (D) cells after culturing in the polylactic acid/methotrexate (0.5%, 1.5%, and 2.5%) scaffolds and the tissue culture plate substrate (as control) for 1, 3, and 5 days.
that of injection group. The anti-tumor effects of 2.5% PLA/MTX scaffold with high concentration of drug were relatively better than that of 0.5% PLA/MTX scaffold with low concentration of drug (Figure 6D). The mean tumor volume of the control group was above 1300 mm³, which was much larger than tumor volume in the 0.5% PLA/MTX and 2.5% PLA/MTX groups (<400 mm³).

As shown in Table 1, PLA/MTX scaffold groups were more effective than the MTX injection in controlling tumor growth. The anti-tumor effect of the 2.5% PLA/MTX scaffold group was also significantly different from that of the 0.5% PLA/MTX group ($P < 0.05$). The main reason is that the injected drug is eliminated by renal metabolism in a short time, resulting in a short plasma half-life (5-8 h) and low drug concentration in target tissues. The tumor growth inhibition value (TGI) of high concentration group could reach 89.26%, which was higher than 74.04% in injection group. The results indicated that PLA/MTX scaffold had a better therapeutic effect than the intraperitoneal injection of drugs.

![Figure 5. Evaluation of anticancer effect in vitro. (A) Live/Dead fluoresce staining of 4T1 cells on polyactic acid/methotrexate (PLA/MTX). The cells were cultured in DMEM for 12 h, 24 h, 72 h, and 120 h. Viable cells are stained green while dead cells red. (B) In vitro proliferation of 4T1 cells after culturing in the PLA/MTX (0.5%, 1.5%, and 2.5%) scaffolds and the tissue culture plates substrate (as control) for 1, 3, and 5 days. (C) Inhibition rate at different MTX concentrations.](image)

| Group          | Tumor weight (g) | TGI   |
|----------------|------------------|-------|
| 2.5% PLA/MTX   | 0.1557±0.024     | 89.26%|
| 0.5% PLA/MTX   | 0.33524±0.042    | 76.88%|
| Injection      | 0.3763±0.018     | 74.04%|
| Control        | 1.45±0.3         | -     |

TGI, tumor growth inhibition value.

The hematoxylin and eosin (H and E) staining results in Figure 7 showed that some area of tumor necrosis appeared in the sections of 0.5% PLA/MTX group, 2.5% PLA/MTX group, and injection group. The nuclei of cancer cells enlarged, produced cavities and the cells showed apoptosis and necrosis. Eventually, the nucleus breaks, creating a “ghost” area. There was no obvious tumor necrosis in the control group. No obvious metastasis and inflammatory were found in the 0.5% PLA/MTX group and 2.5% PLA/MTX group, while the control group showed metastases. The H and E staining
Figure 6. Evaluation of therapeutic efficiency of scaffolds in vivo. (A) Time-dependent body weight of the mice after different treatments. (B) Organ coefficients. (C) Time-dependent tumor growth curves of the mice with different treatments. (D) Photographs of the harvested tumors on day 21 from mice with different treatments.

Figure 7. H & E staining results of experimental endpoint. The representative H and E staining images of the tumor, heart, liver, spleen, lung, and kidney from Balb/c mice with indicated treatment. Explanation about the circled area: In the tumor slices of 0.5% polylactic acid/methotrexate (PLA/MTX), 2.5% PLA/MTX, and injection group, the circled area indicates that tumor necrosis occurred in some areas, forming a “ghost” area. In the control group, the structure of tumor cells in the circled area was stable, the nuclei were clearly visible and arranged regularly. In the control group, metastases and increased inflammatory cells appeared in the circled area of lung sections. There were more inflammatory cells (lymphocytes, leukocytes and monocytes, etc.) in the liver slices of injection group. Scale bars represent 100, 250, 500, and 1000 μm.
of the spleen showed that the injection group had obvious white hyperplasia and more inflammatory cells (B-cell monocytes, etc.). There was no obvious nephrotoxicity found in all four groups.

4. Discussion

As shown in Figure 8, under the guidance of computer-aided design (CAD) and 3D printing technology, PLA/MTX drug-loaded scaffold was prepared by FDM printer. Then, PLA/MTX drug-loaded scaffold was implanted into the mouse body. With the release of MTX, the growth and reproduction of tumor cells were inhibited. The drug release from the PLA/MTX scaffold showed a sustained and slow process due to the degradation process of PLA. The CCK-8 assay demonstrated that the PLA material did not exert severe cytotoxic effects on 4T1, A549, MCF-7, MG-63, and MC3T3 cells. In vitro cellular experiments revealed that the printed PLA/MTX scaffolds have a relatively high inhibitory effect on the tumor cells MG-63, A549, MCF-7, and 4T1, and lower toxic effect on the normal cells MC3T3-E1. The inhibition rates of three different concentrations of PLA/MTX scaffolds on cancer cells were more than 95% after cultured for 5 days.

After 3 weeks of observation, PLA/MTX scaffold with different MTX concentrations group had a higher tumor inhibition rate. We found that PLA/MTX scaffold was better than MTX injection in controlling tumor growth. This was attributed to an enrichment of the released MTX around the tumor tissue and lasting therapeutic MTX levels for prolonged time. The intraperitoneal delivery of MTX needed injection of drug every 2 days while PLA/MTX scaffold was implanted only once for the treatment, indicating that the PLA/MTX drug delivery system was more efficient than traditional injection method.

In a word, 3D-printed PLA/MTX scaffolds maintained a high tumor inhibition rate and significantly reduced systemic drug toxicity compared with intraperitoneal injection. The implanted PLA/MTX scaffolds can release and maintain therapeutic drug levels at the tumor site for prolonged time while reducing systemic drug exposure to healthy tissues. Moreover, this implantable scaffold only requires 1 time implantation, which greatly reduces the frequency of drug administration. Therefore, PLA/MTX scaffold as a controllable drug delivery system has great potential for suppressing tumor growth.

5. Conclusions

We developed a novel 3D-printed drug delivery scaffold for tumor therapy. Morphological and structural analysis results showed that MTX was successfully incorporated into the PLA filament. PLA/MTX scaffolds can maintain MTX release for more than 30 days. In vitro cytotoxicity experiments confirmed that PLA/MTX scaffolds have a relatively high inhibitory effect on tumor cells while showing less toxicity to normal cells. In addition, in vivo results demonstrated that the PLA/MTX scaffolds highly suppressed tumor growth with negligible side effects on the normal tissues and organs. 3D-printed PLA/MTX scaffolds could be used as a controllable drug delivery system and have the great potential for suppressing tumor growth.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors’ contributions

P.Z., C.H., and Y.M. conceived the project. Y.M. and C.H. conducted the scaffold fabrication and characterization. C.H. and C.G. conducted the cell and animal study. P.Z. reviewed the experimental results and advised the organization of the main contents. P.Z., G.L., H.L, C.G., and Y.M. wrote the manuscript.

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