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Doxorubicin-loaded PLGA Microparticles with Internal Pores for Long-acting Release in Pulmonary Tumor Inhalation Treatment*

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Abstract Doxorubicin (DOX) loaded poly(lactic-*co*-glycolic acid) (PLGA) microparticles with internal pores (MP-D) were developed for long-acting release in pulmonary inhalation treatment. The PLGA microparticles exhibited favorable aerodynamic properties for pulmonary delivery. *In vitro* drug release profile suggested that MP-D have the advantage of long-term maintenance of drug concentrations. MTT assay demonstrated the *in vitro* anti-tumor efficiency of the DOX loaded PLGA microparticles. Furthermore, melanoma lung metastasis model was established to determine the *in vivo* anti-tumor efficiency. The mice treated with MP-D showed significantly fewer lesions than the untreated ones. The survival analysis indicated that MP-D prolonged the survival time of tumor-bearing mice. These results suggested that DOX loaded PLGA microparticles with internal pores have the potential to be used as long-acting release carriers in clinical lung cancer treatment.

Keywords: Doxorubicin; Poly(lactic-*co*-glycolic acid); Internal pores; Long-acting release; Pulmonary inhalation.

INTRODUCTION

Lung cancer retains the highest mortality rate among cancers, which is responsible of approximately 1.4 million annual deaths globally[1]. Most patients with lung cancer receive chemotherapy; however, the traditional route of administration has many disadvantages and side effects[2–4]. Recently, deliver drugs to the local tumor site directly by inhalation route generated tremendous research interest[5–7]. This *in situ* treatment presents a new proposal for the patients suffering from lung diseases, with fewer systemic effects and side effects[8–10].

With the maturity and innovation of inhalation therapies, there is an increasing demand for tailor-made inhalable drug carriers which are stable and effective for pulmonary delivery[3, 11]. Among the variety of drug carriers which have been developed to cope with this demand, inhalable dry-powder carriers provided an attractive option for inhalation therapies[12, 13]. Inhalable dry-powder carriers have been studied for the treatment

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of many pulmonary diseases such as asthma[14, 15], chronic obstructive pulmonary disease (COPD)[15, 16], cystic fibrosis[17], tuberculosis[18] and lung cancers[19].

Biodegradable poly(lactic-*co*-glycolic acid) (PLGA) and its derivatives were regarded as the most promising dry-powder carriers, due to their excellent biocompatibility and long-acting release of the inhaled drug[20–24]. Doxorubicin, used alone or as a component of combination therapy, is the most common chemotherapeutics drug in the treatment of a wide range of cancers[25–30]. A phase I study of inhaled doxorubicin for patients with metastatic tumors to the lungs showed the efficacy of inhaled therapy, and it is safe up to a dose of 7.5 mg/m^2 every 3 weeks[4]. Youn[10] *et al.* have developed doxorubicin loaded large and highly porous PLGA microparticles for inhalation treatment. Their porous large PLGA microparticles exhibited desirable aerosolization for pulmonary delivery; however, the microparticles released almost all drugs within 14 days[31–34]. In order to extend the release time and maintain the aerodynamic properties, carriers confining the pores in the interior area are required to be developed. Therefore, our strategy is to design doxorubicin loaded biodegradable PLGA microparticles with internal pores for long-acting release in pulmonary inhalation treatment. The properties for inhalation drug delivery of the PLGA microparticles were investigated by a series of *in vitro* experiments. Pulmonary metastases generated by injection B16F10 cells to the mice were utilized as the tumor model to determine the therapeutic effect *in vivo* of the PLGA microparticles.

EXPERIMENTAL

Materials

Poly(d,l-lactic-*co*-glycolic acid) (PLGA) (lactic acid:glycolic acid = 50:50, 0.2 dL/g) was provided by Changchun SinoBiomaterials Co., Ltd. Poly(vinyl alcohol) (PVA), ammonium bicarbonate (ABC) were purchased from Alfa Aesar. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, Ohio, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco (Grand Island, USA). Doxorubicin hydrochloride (DOX) was purchased from Beijing Huafeng United Technology Co., Ltd. *N*,*N*-Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Beijing Chemical Works (Beijing, China).

Preparation of PLGA Microparticles

DOX-loaded PLGA microparticles with internal pores (MP-D) were prepared by a W/O/W double emulsion and solvent rapidly evaporation technique, using ammonium bicarbonate (ABC) as the porogen. PLGA was dissolved in 3 mL DCM at a concentration of 50 mg/mL, mixed with 0.5 mL deionized water containing 15 mg DOX. The mixture was vibrated for 2 min on a vortex to obtain the coarse emulsion. In addition, 0.1 mL ammonium bicarbonate solution (90 mg/mL) was added to the mixture and re-vortex for 1 min. This primary W/O emulsion was injected into 20 mL of ice-cold 1% PVA solution (*W*/*V*) and emulsified at 2000 r/min for 1 min utilizing a high shear mixed emulsion (BME 100LX, Shanghai Weiyu Mechano-electronic Manufacturing Co., Ltd, China). The emulsion was dropped into 40 mL of deionized water and gently stirred at room temperature for 4 h to evaporate DCM, followed by vigorous stirring at 60 °C for 0.5 h to completely decompose ABC. The microparticles were centrifuged at 3500 r/min for 3 min and washed three times with Milli-Q water. The microparticles were lyophilized and obtained as red powers. The blank microparticles (without drugs) were prepared in similar processes.

Characterization of PLGA Microparticles

The surface morphology of PLGA microparticles was investigated by scanning electron microscopy (SEM, FEI XL30 ESEM, Philips). The PLGA microparticles suspension at a concentration of 0.2 mg/mL was dropped onto a silicon chip. The silicon chip was completely dried overnight and sputter-coated with gold-palladium under argon condition. The image of PLGA microparticles were obtained at a 10 mm working distance.

The mass median aerodynamic diameters (MMAD) were calculated by the equation:

$$\mathrm{MMAD} = d \times (\rho/\rho_0 X)^{1/2}$$

where d was denoted as the geometric mean diameter, ρ was denoted as the tapped density, ρ_0 was denoted as the reference density (1 g/mL) and X was denoted as the dynamic shape factor (which is 1 for a particle). The tapped density was measured by a tapped density tester (Ningbo Rooko Instrument Co., Ltd, China).

Drug Loading and Drug Release

The drug loading content of DOX in microparticles was measured by a UV-Visible spectrophotometer at 480 nm at a concentration of 1 mg/mL in DMF. A calibration curve of 0–100 μg/mL DOX in the DMF was employed to calculate the drug loading content. The experiment was conducted in triplicate.

The drug loading content (DLC%) and the drug loading efficiency (DLE%) of DOX-loaded PLGA microparticles were calculated by the following equations.

DLC (%) = amount of DOX in microparticles/amount of DOX-loaded microparticles × 100%

Theoretical loading content (TLC %) = amount of DOX used for encapsulation/amount of DOX and PLGA used for encapsulation × 100%.

$$\text{DLE (\%)} = \text{DLC/TLC} \times 100\%$$

To investigate the drug release profile of MP-D, it was suspended in phosphate buffered saline (PBS, pH = 6.5 and pH = 7.4). The suspension was divided to 2 mL aliquots (1 mg/mL) and shaken at 100 r/min in an incubator. The microparticle suspension was centrifuged (12000 r/min, 5 min) at the regular time points (12 h, 1 d, 2 d, 3 d, 5 d, 7 d, 14 d) and the remaining particles were collected, lyophilized and analyzed as above.

Cell Culturing

B16F10 melanoma cell line was provided by Cancer Institute & Hospital, Chinese Academy of Medical Sciences (Beijing, China). The cells were cultured in the DMEM medium with 10% (*V*/*V*) fetal bovine serum (FBS) and 1% antibiotics penicillin-streptomycin. The cells were grown in a humidified atmosphere (containing 95% air and 5% CO_2) at 37 °C.

In vitro Cytotoxicity Assays

The *in vitro* cytotoxicities of the MP-D and the blank microparticles were assessed by MTT assay. B16F10 cells were seeded into a 96-well plate at 2.0×10^4 cells per well and pre-incubated for 24 h. MP-D and the blank microparticles with different concentrations were dispersed in a PBS (pH = 7.4) solution, and 20 μL of the suspension was added to each well. The B16F10 cells were subjected to MTT assay after incubated for another 48 h. The absorbance was measured at 492 nm by an ELISA microplate reader (Bio-Rad 680). The cell viability (%) was calculated as:

$$\text{Cell viability (\%)} = (A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} was denoted as the absorbance of the cells treated by microparticles and A_{control} was denoted as the absorbance of the untreated cells.

In vivo Antitumor Efficiency

C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing) and the experimental protocol was in accordance with the regulations of Northeast Normal University. The animals were housed in groups of 5 with independent air supply system in an air-conditioned room under a 24 h day and night cycle. The mice were acclimatized for 1 week with sufficient food and water before the experiments. Pulmonary metastases tumor model was generated by the subcutaneous intravenous injection of B16F10 cells (1.0×10^4, 100 μL in PBS) in the tail of each mouse.

Ten of the metastases-bearing mice were randomly divided into two groups of five. One group was insufflated with MP-D into the trachea using a dry power insufflators (Penn-century, USA) at 5 days after B16F10 cells injection, and the other group was treated with blank PLGA microparticles. Five mice without injecting with tumor cells were treated as negative controls. At day 21 after B16F10 implantation, the mice were euthanized and lungs were excised. The average body weight of each group was measured every 5 or 6 (for the last time) days. Lungs of different groups were fixed with paraformaldehyde solution (4% in PBS), embedded

into paraffin and sectioned. The sections were stained with hematoxylin and eosin (H&E) and observed by a microscope (Nikon TE2000U).

The other metastases-bearing mice were randomly divided into two groups of ten. One group was treated with MP-D as above and the other group was untreated control. The survival rates of the animals were determined.

Statistical Analysis

Each experiment was conducted in triplicate and expressed as means ± SD. Statistical significances were analyzed utilizing the Student's t-test. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered highly significant.

RESULTS AND DISCUSSION

Characterization of PLGA Microparticles

DOX loaded PLGA microparticles (MP-D) were prepared through double emulsification method. Ammonium bicarbonate was decomposed into carbon dioxide and ammonia during the emulsification and the heated agitation. As shown in Fig. 1, MP-D featured wrinkled surfaces and internal pores. The "closed" nanopores microparticles were more beneficial for prolonged drug release than the "opened" ones. The geometric mean diameter and MMAD of MP-D were (5.21 ± 0.95) μm and (2.58 ± 0.47) μm, respectively. Overall, the PLGA microparticles exhibited low density and large specific surface due to their internal and external structures, and these features were beneficial for pulmonary delivery[35].

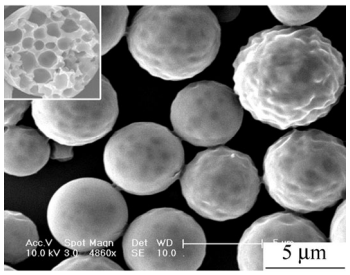

Fig. 1 SEM image of PLGA microparticles with internal pores (inset)

Drug Loading and Drug Release

The drug loading content of MP-D was measured to be (5.54 ± 0.08)% and drug loading efficiency was calculated to be (60.95 ± 0.88)%.

The *in vitro* drug release of MP-D was conducted in PBS solutions (pH = 6.5 and pH = 7.4). As illustrated in Fig. 2, the PLGA microparticles drug delivery system can provide effective protection and long-acting release of the inhaled drug. Doxorubicin sustained released from the biodegradable matrix PLGA and approximately 50% of the drugs were accumulatively released in 14 days. This long-acting release has the advantage of reducing dosing frequency and improving patient adherence. Moreover, the PLGA microparticles release drugs faster in acidic condition because the degradation of PLGA is acid-catalyzed, which can be useful in targeted drug delivery to tumors where the pH is reduced to 6.5. Thus, MP-D has the advantage of long-term maintenance of drug concentrations.

In vitro Cytotoxicity Assays

To investigate the anti-tumor efficacy of DOX loaded PLGA microparticles, the cytotoxicity of MP-D and blank PLGA microparticles without drugs (MP-B) in B16F10 cell line was investigated. As illustrated in Fig. 3, MP-D exhibited manifest cytotoxicity to B16F10 cells while MP-B exhibited no obvious cytotoxicity. MTT assay showed that 80% cells were killed when the concentration of the microparticles was 0.05 mg/mL. These results suggest that MP-D has the potential for cancer treatment *in vivo*.

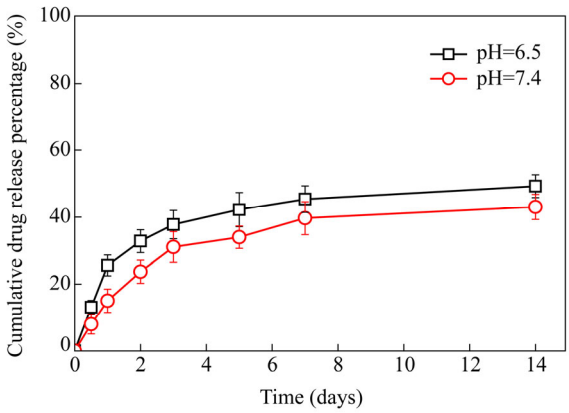

Fig. 2 *In vitro* drug release of PLGA microparticles with internal pores at different pH values

In vivo Anti-tumor Efficacy

The *in vivo* anti-tumor efficacy of MP-D was assessed using B16F10 experimental metastasis model, and compared with untreated control group. The morphology of the lungs excised from the mice was investigated by macroscopic observation, as exhibited in Fig. 4. The lungs of the negative controls showed normal physiological appearance (line 1). However, the surfaces of the untreated tumor-bearing mice lungs were covered with melanomas (line 2). The lungs excised from the treated tumor-bearing mice (line 3) showed favorable therapeutic effects, performed as fewer lesions than the untreated ones.

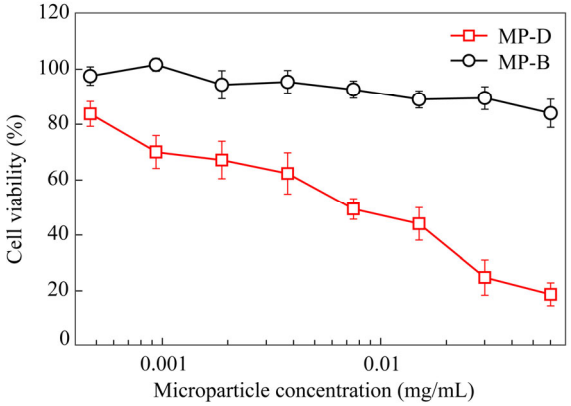

Fig. 3 *In vitro* cytotoxicity of DOX loaded PLGA microparticles and blank PLGA microparticles without drugs in B16F10 cells for 48 h

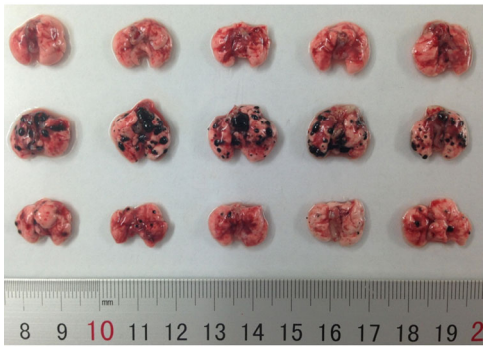

Fig. 4 Macroscopic observation of lungs obtained from the mice (Line 1: negative control group; Line 2: untreated tumor-bearing mice; Line 3: MP-D treated tumor-bearing mice)

Moreover, the average lung weights of the mice also demonstrated the therapeutic effect of MP-D. The weights of the lungs in the tumor-bearing mice were greatly increased due to the encroachment of the malignant melanoma. As illustrated in Fig. 5, the average lung weight of the tumor-bearing mice treated with MP-D (238 ± 19.24 mg) was significantly lower than that of the untreated ones (384 ± 62.29 mg). As a comparison, the average lung weight of the control group was (210 ± 21.21) mg. Meanwhile, no obvious changes occurred to the average body weights of the mice during the therapeutic period (Fig. 6). These results suggest that MP-D can effectively exert their pharmacological effect and inhibit tumor growth in the lung region.

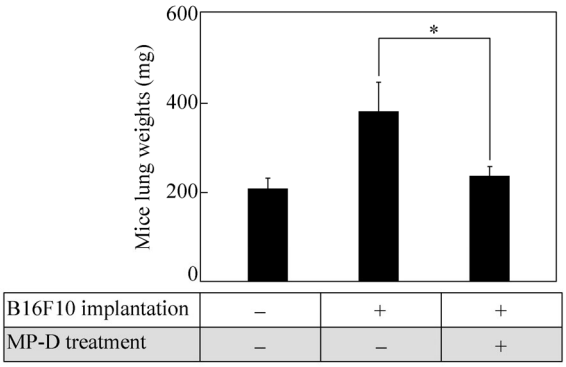

Fig. 5 Average weights of lungs obtained from the mice ($^{*}p < 0.005$)

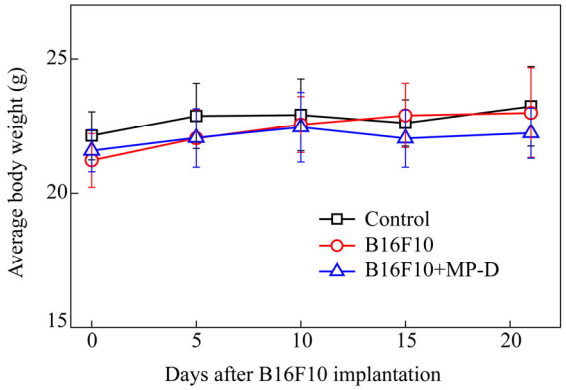

Fig. 6 Average body weights of the mice at 0, 5, 10, 15 and 21 days after B16F10 implantation

Histopathology Study

Histopathology was studied by observing the hematoxylin and eosin (H&E) stained sections under an optical microscope. As shown in Fig. 7, great numbers of alveolus were encroached by melanoma in lungs of the untreated melanoma-bearing mice, which explained the increased mass of the lungs. The melanoma-bearing mice treated with MP-D exhibited barely tumor lesions, which demonstrated the excellent therapeutic effect of the DOX loaded PLGA microparticles.

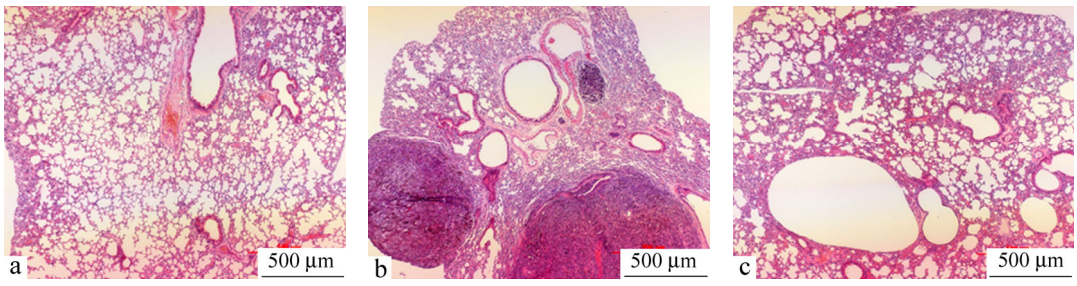

Fig. 7 Histological study of lung tissues in the three groups: (a) negative control group, (b) untreated tumor-bearing mice and (c) MP-D treated tumor-bearing mice

Survival Rate

The therapeutic effect seen in tumor growth by macroscopic observation also correlated with the survival rates. The survival rates of tumor-bearing mice treated with MP-D and the untreated ones are plotted in Fig. 8. All the untreated mice died after 29 days while the mice treated with MP-D showed better survival profile due to 50% of

the mice in this group survived for more than 36 days. These results suggest that MP-D could inhibit the tumor growth and decrease the death rate.

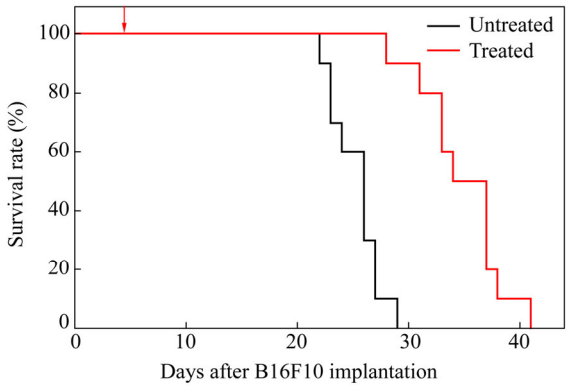

Fig. 8 Kaplan-Meier plot for survival rate of tumor-bearing mice untreated and treated with MP-D (The arrow represents the administration of MP-D)

CONCLUSIONS

In conclusion, we developed PLGA microparticles with internal pores as sustained-release carriers for pulmonary inhalation treatment of melanoma metarstatic tumor. Drug release profile confirmed the long-acting release of the MP-D. *In vitro* cell experiments suggested that MP-D have the ability to kill cancer cells at proper concentrations. Moreover, MP-D exhibited excellent ability to inhibit tumor in the lungs. The group treated with MP-D showed remarkably fewer lesions than the untreated group. Finally, these DOX loaded PLGA microparticles with internal pores would provide a new scenario for lung cancer treatment.

REFERENCES

1 Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. and Forman, D., Ca-Cancer J. Clin., 2011, 61(2): 69
2 DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L., Alteri, R., Robbins, A.S. and Jemal, A., Ca-Cancer J. Clin., 2014, 64(4): 252
3 Ungaro F., d' Angelo, I., Miro, A., La Rotonda, M.I. and Quaglia, F., J. Pharm. Pharmacol., 2012, 64(9): 1217
4 Otterson, G.A., Villalona-Calero, M.A., Sharma, S., Kris, M.G., Imondi, A., Gerber, M., White, D.A., Ratain, M.J., Schiller, J.H., Sandler, A., Kraut, M., Mani, S. and Murren, J.R., Clin. Cancer Res., 2007, 13(4): 1246
5 Germershaus, O., Schultz, I., Luhmann, T., Beck-Broichsitter, M., Hogger, P. and Meinel, L., Eur. J. Pharm. Biopharm., 2013, 85(1): 61
6 Xu, C.N., Tian, H.Y. and Chen, X.S., Chinese J. Chem., 2014, 32(1): 13
7 Bruinenberg, P., Serisier, D., Cipolla, D. and Blanchard, J., Am. J. Resp. Crit. Care, 2010, 181
8 Cipolla, D., Shekunov, B., Blanchard, J. and Hickey, A., Adv. Drug Deliv. Rev., 2014, 75C: 53
9 Kim, I., Byeon, H.J., Kim, T.H., Lee, E.S., Oh, K.T., Shin, B.S., Lee, K.C. and Youn, Y.S., Biomaterials, 2013, 34(27): 6444
10 Kim, I., Byeon, H.J., Kim, T.H., Lee, E.S., Oh, K.T., Shin, B.S., Lee, K.C. and Youn, Y.S., Biomaterials, 2012, 33(22): 5574
11 Chow, A.H.L., Tong, H.H.Y., Chattopadhyay, P. and Shekunov, B.Y., Pharm. Res., 2007, 24(3): 411
12 Lee, S.H., Teo, J., Heng, D., Ng, W.K., Chan, H.K. and Tan, R.B.H., Eur. J. Pharm. Biopharm., 2013, 83(2): 275
13 Islam, N. and Gladki, E., Int. J. Pharmaceut., 2008, 360(1–2): 1
14 Roche, N., Dessanges, J.F. and Dubus, J.C., Rev. Mal. Respir., 2005, 22(4): 605

15 Onoue, S., Sato, H., Ogawa, K., Kojo, Y., Aoki, Y., Kawabata, Y., Wada, K., Mizumoto, T. and Yamada, S., Eur. J. Pharm. Biopharm., 2012, 80(1): 54
16 Tashkin, D.P., Rennard, S.I., Martin, P., Ramachandran, S., Martin, U.J., Silkoff, P.E. and Goldman, M., Drugs, 2008, 68(14): 1975
17 Westerman, E.M., De Boer, A.H., Le Brun, P.P.H, Touw, D.J., Roldaan, A.C., Frijlink, H.W. and Heijerman, H.G.M., J. Cyst. Fibros., 2007, 6(4): 284
18 Chan, J.G.Y., Chan, H.K., Prestidge, C.A., Denman, J.A., Young, P.M. and Traini, D., Eur. J. Pharm. Biopharm., 2013, 83(2): 285
19 Meenach, S.A., Anderson, K.W., Hilt, J.Z., McGarry, R.C. and Mansour, H.M., Eur. J. Pharm. Sci., 2013, 49(4): 699
20 Yang, Y., Bajaj, N., Xu, P., Ohn, K., Tsifansky, M.D. and Yeo, Y., Biomaterials, 2009, 30(10): 1947
21 Rawat, A., Majumder, Q.H. and Ahsan, F., J. Control. Release, 2008, 128(3): 224
22 Park, S., Kwag, D.S., Lee, U.Y., Lee, D.J., Oh, K.T., Youn, Y.S., and Lee, E.S., Polym. Adv. Technol., 2014, 25(1): 16
23 Chen, A.Z., Zhao, C., Wang, S.B., Liu, Y.G. and Lin, D.L., J. Mater. Chem. B, 2013, 1(23): 2967
24 Fan, J.B., Huang, C., Jiang, L. and Wang, S.T., J. Mater. Chem. B, 2013, 1(17): 2222
25 Wang, F., Wang, Y.C., Dou, S., Xiong, M.H., Sun, T.M. and Wang, J., ACS Nano, 2011, 5(5): 3679
26 Chu, Y.F., Yu, H., Ma, Y.Y., Zhang, Y.T., Chen, W.H., Zhang, G.Y., Wei, H., Zhang, X.Z., Zhuo, R.X. and Jiang, X.L., J. Polym. Sci., Part A: Polym. Chem., 2014, 52(13): 1771
27 Esteve, P.O., Chin, H.G. and Pradhan, S., J. Biol. Chem., 2007, 282(4): 2615
28 Liao, C.D., Sun, Q.Q., Liang, B.L., Shen, J. and Shuai, X.T., Eur. J. Radiol., 2011, 80(3): 699
29 Fan, H., Hu, Q.D., Xu, F.J., Liang, W.Q., Tang, G.P. and Yang, W.T., Biomaterials, 2012, 33(5): 1428
30 Zhang, L.Z., Zhang, Y.J., Wu, W. and Jiang, X.Q., Chinese J. Polym. Sci., 2013, 31(5): 778
31 Lee, J., Oh, Y.J., Lee, S.K. and Lee, K.Y., J. Control. Release, 2010, 146(1): 61
32 Kim, H., Lee, J., Kim, T.H., Lee, E.S., Oh, K.T., Lee, D.H., Park, E.S., Bae, Y.H., Lee, K.C. and Youn, Y.S., Pharm. Res., 2011, 28(8): 2008
33 Fan, J.B., Song, Y.Y., Wang, S.T., Jiang, L., Zhu, M.Q. and Guo, X.L., RSC Adv., 2014, 4(2): 629
34 Qian, Q.P., Huang, X.P., Zhang, X.Y., Xie, Z.G. and Wang, Y.P., Angew. Chem. Int. Ed., 2013, 52(40): 10625
35 Edwards, D.A., Hanes, J., Caponetti, G., Hrkach, J., BenJebria, A., Eskew, M.L., Mintzes, J., Deaver, D., Lotan, N. and Langer, R., Science, 1997, 276(5320): 1868