Electronic Supporting Information for

Porous PLGA microparticles to encapsulate doxorubicin and polyethylenimine/miR-34a for inhibiting the proliferation and migration of lung cancer

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EXPERIMENTAL SECTION

Materials. The has-miR-34a eukaryotic expression plasmid pcDNA-miR-34a was constructed by GenePharma (Suzhou, China), amplified in *Escherichia coli* DH5α and purified using Plasmid Maxi kit (Axygen). The Quant-It™ PicoGreen dsDNA kit was purchased from Life Technologies (Grand Island, USA) to determine the plasmid concentration. PLGA-2A (50:50) was purchased from Lakeshore Biomaterials and used as received. Branched PEI25K, fluorescein isothiocyanate (FITC) and poly(vinyl alcohol) were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindile (DAPI) was purchased from Amersco (Solon, USA). Doxorubicin in the form of hydrochloride salt (>99% purity) was purchased from Huafeng Biotech. Co. (Beijing, China). The antibodies against procaspase-3, caspase-8, caspase-9, PARP, Bcl-2 and β-actin, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and HRP-labeled goat anti-mouse IgG were obtained from Abcam. Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Billerica, USA). Annexin V-FITC/PI apoptosis detection kit, cell cycle detection kit, BCA protein assay kit and caspase-3, 8 and 9 activity assay kits were purchased from Bestbio (Shanghai, China). All other chemicals were of the highest reagent grade commercially available and used as received.

Preparation of porous PLGA microparticles. The porous PLGA microparticles

loaded with doxorubicin and PEI25K/miR-34a were prepared using the water-oil-water emulsion solvent evaporation. Briefly, 100 mg of PLGA-2A was dissolved in 2 mL of dichloromethane and placed in an ice bath, followed by the dropwise addition of 0.2 mL of distilled water containing 1 mg of doxorubicin and PEI25K/miR-34a nanocomplex (1:1, w/w, 40 μg of pDNA). The mixture was emulsified in an ice bath by a homogenizer (8,000 rpm, 30 s), and 0.1 mL of ammonium bicarbonate solution (100 mg/mL) was added into the system. The mixture was further emulsified by a homogenizer at 4,000 rpm for 30 s to obtain the primary emulsion, which was poured into 50 mL of poly(vinyl alcohol) solution (0.5%, w/v) and homogenized at 4,000 rpm for 2 min. Subsequently, the emulsion was added to 50 mL of distilled water, and dichloromethane was evaporated under moderate magnetic stirring for over 3 h. Finally, porous microparticles were obtained by centrifugation at 8,000 rpm for 10 min, washed with distilled water three times, lyophilized and stored at -20 °C until use. Meanwhile, blank porous PLGA microparticles, porous PLGA microparticles loaded with PEI25K/miR-34a or doxorubicin, and FITC-labeled porous PLGA microparticles were prepared in a similar way except for the addition of different cargos.

Characterization of porous PLGA microparticles. The particle size and zeta potential of porous PLGA microparticles were measured by a laser scattering particle size analyzer (Beckman Coulter, USA) and Malvern Nano ZS90 Zetasizer (Malvern, UK), respectively. The aerodynamic diameter (d_{aero}) of porous PLGA microparticles was determined according to the guidelines for pulmonary drug delivery in Chinese

Pharmacopoeia (2010):

$$d_{aero} = \frac{d}{\gamma}\sqrt{\rho/\rho_a}$$

where d represents the geometric diameter, γ represents the shape factor (1.0), and ρ (g/mL) and ρ_a (1.0 g/mL) are particle bulk density and water mass density, respectively. The surface morphology of porous PLGA microparticles was observed by scanning electron microscopy (SEM, Micron PEI Philips) with an accelerating voltage of 5 kV. To determine the distribution of doxorubicin and miR-34a in microparticles, FITC-labeled porous PLGA microparticles were prepared and suspended in a 50% glycerol solution, and then examined by an LSM 710 confocal laser scanning microscope (Carl Zeiss Microscopy LLC, Germany).

Determination of drug loading and encapsulation efficiency. Briefly, 10 mg of porous PLGA microparticles were suspended in 1 mL of dimethylsulfoxide (DMSO), and diluted to 10 mL using 50% methanol. The supernatant was collected and analyzed by a reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a Symmetry® C18 column (4.6×250 mm) to determine the drug loading and encapsulation efficiency of doxorubicin. The elution was performed using a 50:50 mixture of solution A (0.01 M sodium acetate buffer, pH 3.0) and solution B (methanol) at a flow rate of 1.0 mL/min. To determine the loading and encapsulation efficiency of miR-34a, 10 mg of porous PLGA microparticles were treated with 100 μL of DMSO, and 400 μL of distilled water was then added into the system. The mixture was centrifuged at 12,000 rpm for 10 min, and the supernatant was collected to measure the plasmid concentration using Quant-

It™ PicoGreen dsDNA kit.

***In vitro* drug release.** Briefly, 10 mg of porous PLGA microparticles were suspended in 1 mL of phosphate buffered saline (PBS, pH 7.4), and the mixture was incubated in a rotary shaker at 37 °C. At predetermined time points, the supernatants were collected and centrifuged at 8,000 rpm for 5 min, and then an equivalent volume of PBS was added into the system. The concentrations of doxorubicin and plasmid in the supernatants were determined using RP-HPLC and Quant-It™ PicoGreen dsDNA kit as described above, respectively. Meanwhile, the supernatants were subjected to agarose gel electrophoresis for detecting the status of miR-34a.

Cell proliferation assay. The A549 cells were seeded onto 96-well plate at a density of 9.0×10^3 cells/well and incubated in DMEM for 24 h. Then 20 μL of release supernatant of porous PLGA microparticles at 3, 8, 14 and 30 days was added into the well. After 24 or 48 h, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well, and the plate was incubated for an additional 4 h. Afterwards, MTT solution was removed from each well, and 200 μL of DMSO was added to dissolve the formazan crystals. The plate was incubated for 10 min, and the absorbance at 492 nm was measured using a GF-M3000 microplate reader (Shandong, China). The cell viability (%) was calculated as $A_{\text{sample}}/A_{\text{control}}$, where A_{sample} and A_{control} were the absorbance values of treated and untreated cells, respectively. For morphologic observation, the cells were seeded in 6-well plate at a density of 2.5×10^5 cells/well, incubated in DMEM for 24 h and treated with 50 μL of release supernatant at 30 days for 48 h. The cells were then harvested,

washed with PBS and fixed with 70% ethanol at 4 °C for 5 min. The fixed cells were stained with DAPI solution (1 μg/mL) at room temperature for 10 min, and observed through an IX71 fluorescence microscopy (Olympus, Tokyo, Japan).

Cell apoptosis analysis. Briefly, A549 cells were seeded in 6-well plate containing 2 mL of DMEM at a density of 2.5×10^5 cells/well, and incubated at 37 °C overnight. The medium was aspirated, and 2 mL of medium containing 100 μL of release supernatant at 30 days was added. After 24 h, the cells were harvested, washed twice with PBS and suspended in binding buffer according to the manufacturer's protocols. The cells were then mixed with Annexin V-FITC and PI, and incubated at room temperature for 10 min in the dark. The cell apoptosis was determined by analyzing 15,000 gated cells using FACSCalibur (BD Biosciences, Mountain View, USA).

Cell cycle analysis. The A549 cells were seeded in 6-well plate at a density of 2.5×10^5 cells/well, and incubated for 24 h before treatment. 50 μL of release supernatant at 30 days was added into the well, and incubated for an additional 24 h. Afterwards, the cells were collected, washed with PBS twice, suspended in 0.5 mL solution containing 10 μL of RNase A (25 μg/mL) and 10 μL of PI (50 μg/mL), and incubated at 37 °C for 30 min in the dark. The cell cycle was measured by analyzing 15,000 gated cells using FACSCalibur (BD Biosciences, Mountain View, USA) and ModFitLT 2.0 (Verity Software House, Topsham, ME, USA).

Western blotting analysis. The A549 cells were seeded in 6-well plate at a density of 2.5×10^5 cells/well and subsequently cultured in DMEM for 24 h. 50 μL of release

supernatant at 30 days was added into the well, and incubated for an additional 24 h. The cells were harvested, washed with PBS twice and lysed with RIPA lysis buffer on ice for 2 h. The lysates were centrifuged at 12,000 rpm for 10 min to obtain the supernatants. An equal amount of proteins was subjected to electrophoresis on SDS-PAGE and transferred to PVDF membrane by electroblotting. The membrane was blocked with PBS containing 5% non-fat milk and 0.1% Tween-20 (PBST) at room temperature for 1 h, and then incubated with desired antibodies (antibodies against procaspase-3, caspase-8, caspase-9, PARP, Bcl-2 and β-actin) at 4 ^{o}C overnight. Afterwards, the membrane was washed with PBST twice and incubated with HRP-labeled secondary antibody at room temperature for 1 h, and specific proteins were detected by enhanced chemical luminescence (ECL, Transgene).

Caspase-3, 8 and 9 activity assay. The activities of caspase-3, 8 and 9 were determined according to the protocols of activity assay kits. Briefly, A549 cells were treated with 50 μL of release supernatant at 30 days for 48 h, and then harvested, washed with PBS twice, and lysed in 100 μL of lysis buffer supplied in the kits. The suspension was centrifuged at 10,000 g for 10 min (4 ^{o}C), and 10 μL of supernatant was used to measure the changes of absorbance at 405 nm for detecting the caspase-3, 8 and 9 activities.

Wound healing assay. The A549 cells were seeded on 6-well plate at a density of 2.5×10^{5} cells/well and cultured to 90% confluence. The cell monolayer was subjected to a mechanical scratch wound using a sterile pipette tip, and the cells were washed with

PBS twice. The cells were then incubated with 50 μL of release supernatant at 30 days for different time. The images of wound area were captured with IX71 fluorescence microscopy (Olympus, Tokyo, Japan), and three representative zones were selected to calculate the average width of cell migration through Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA).

Transwell migration assay. The A549 cells were seeded in 6-well plate at a density of 2.5×10^5 cells/well and subsequently cultured for 24 h. 50 μL of release supernatant at 30 days was added into the well, and incubated for an additional 24 h. The cells were harvested and added to the upper chamber with 8-μm pores at a density of 1.0×10^6 cells/well, while 600 μL of DMEM containing 1% BSA was placed in the lower chamber. The cell migration was conducted at 37 °C for 48 h, and non-migrating cells on the top of membrane were removed by mechanical wiping. The cells migrating to the lower surface of membrane were fixed with 75% ethanol, stained with 0.1% crystal violet and detected in a random objective field (200× magnification) using an IX71 fluorescence microscopy (Olympus, Tokyo, Japan).

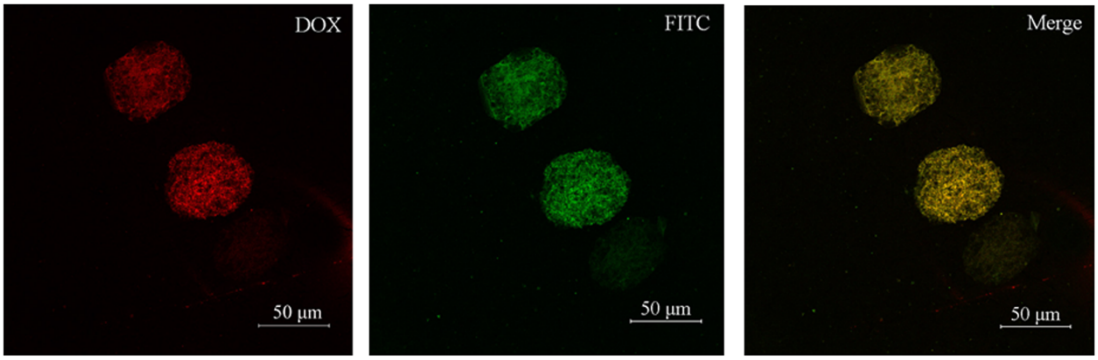

Fig. S1 The distribution of doxorubicin and miR-34a in FITC-labeled porous PLGA microparticle MP-4 determined by confocal laser scanning microscopy.

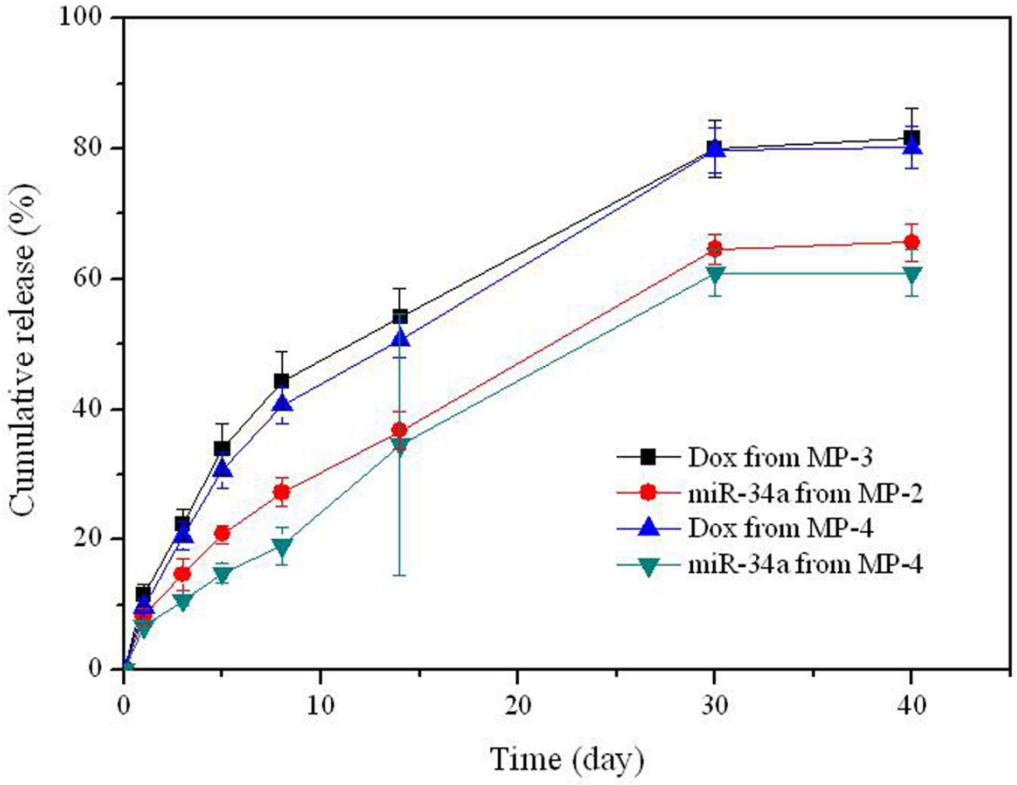

Fig. S2 The release profiles of doxorubicin and miR-34a from porous PLGA microparticles MP-2, MP-3 and MP-4. The data were expressed as mean value ± SD, n=3.

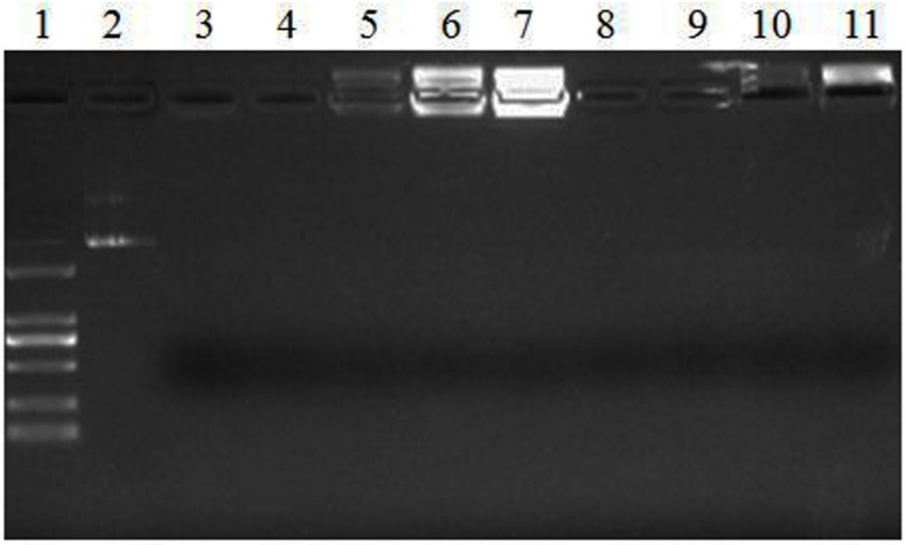

Fig. S3 Agarose gel electrophoresis of PEI25K/miR-34a nanoparticles released from porous PLGA microparticles. Lane 1: DL2000 maker; lane 2: naked plasmid pcDNA-miR-34a; lane 3: MP-1 microparticle; lane 4-7: release supernatants from MP-2 at 3, 8, 14 and 30 days; lane 8-11: release supernatants from MP-4 at 3, 8, 14 and 30 days.

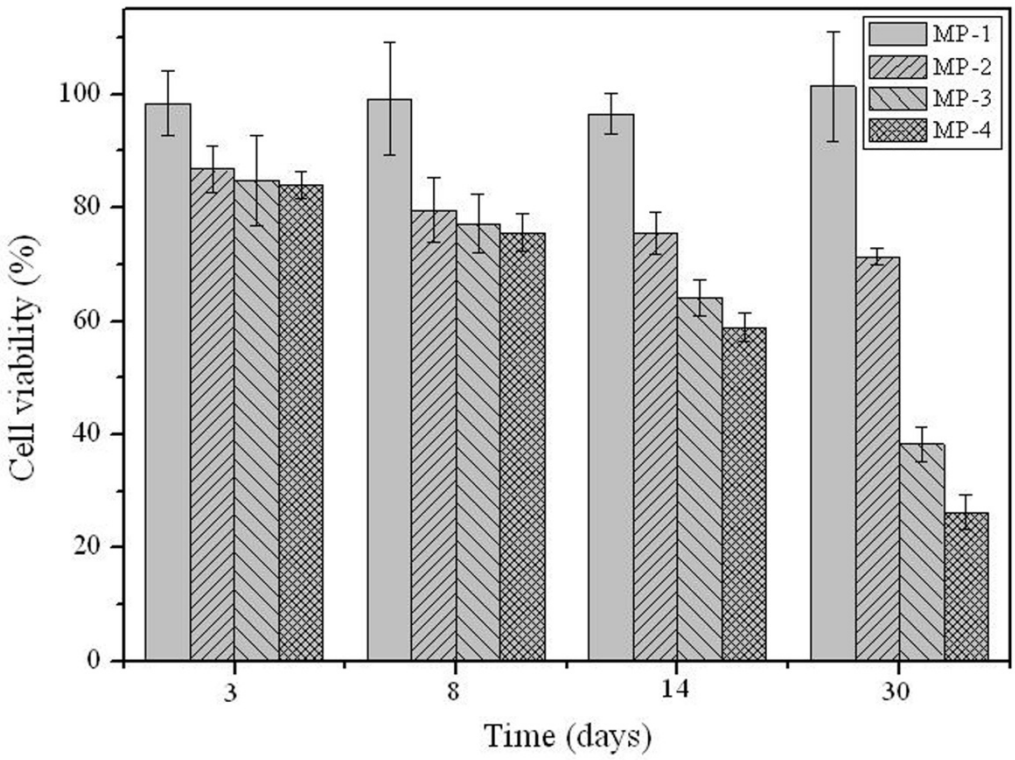

Fig. S4 Cell viability of A549 cells after treating with the release supernatants at 30 days from porous PLGA microparticles for 48 h. Data were represented as mean value ± SD, n=4.

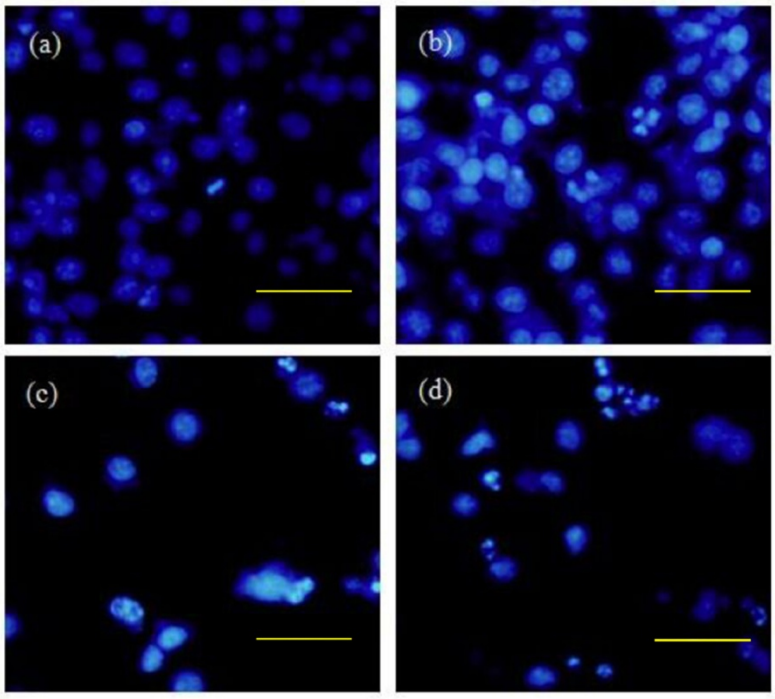

Fig. S5 Morphologic observation of A549 cells treated with the release supernatants at 30 days from porous PLGA microparticles MP-1 (a), MP-2 (b), MP-3 (c) and MP-4 (d) for 48 h. Scale bar: 10 μm.

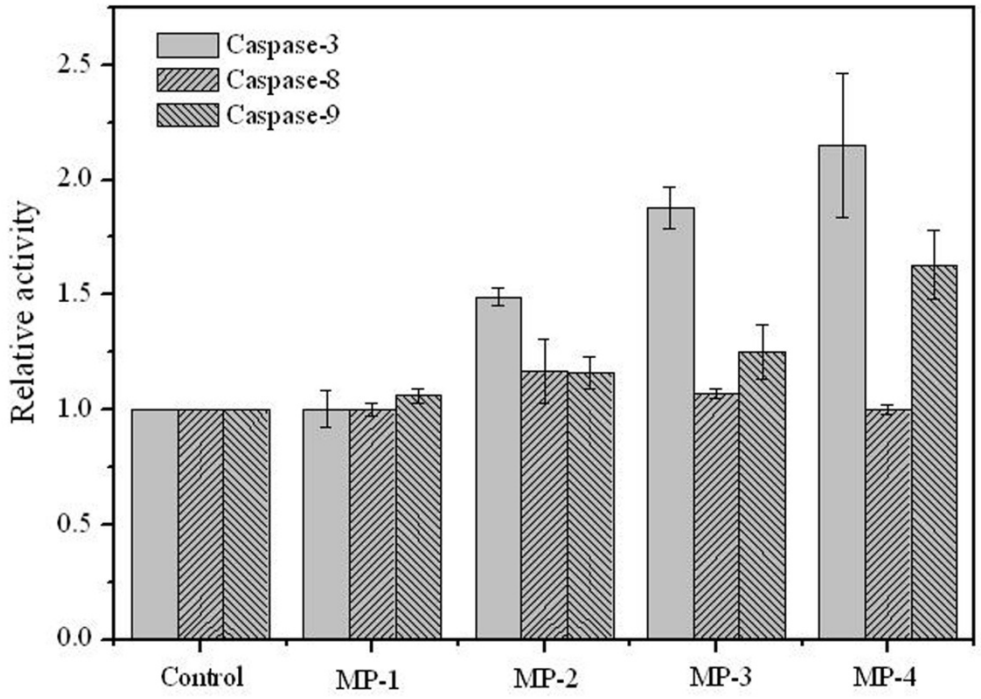

Fig. S6 Relative activity of caspase-3, caspase-8 and caspase-9 in A549 cells treated with the release supernatants at 30 days from porous PLGA microparticles. The data were expressed as mean value ± SD of three experiments.

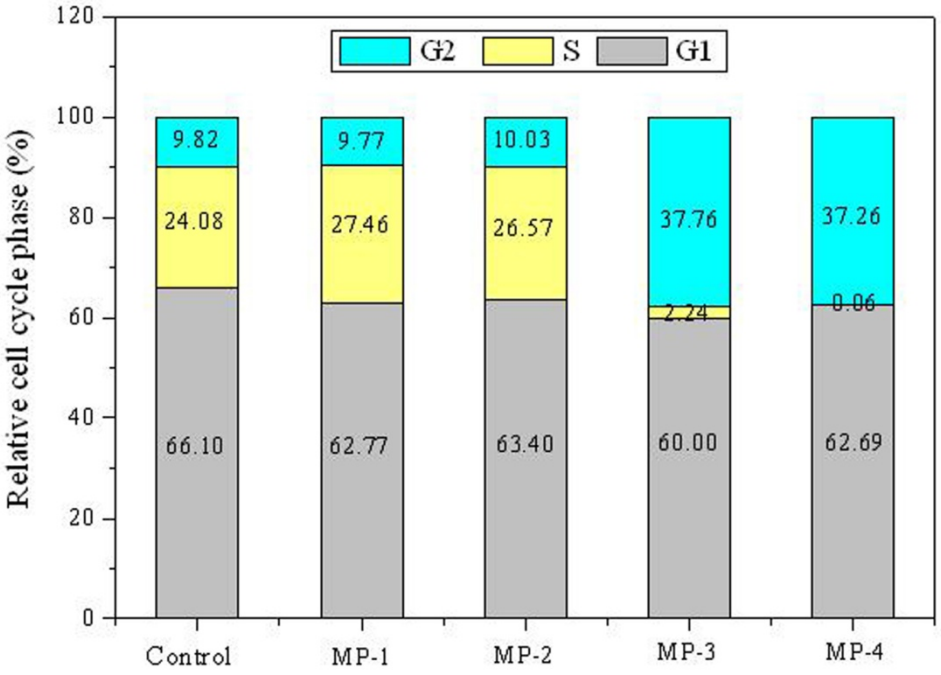

Fig. S7 Relative distribution of cell population in the cell cycle phases of A549 cells treated with the release supernatants at 30 days from porous PLGA microparticles for 24 h.

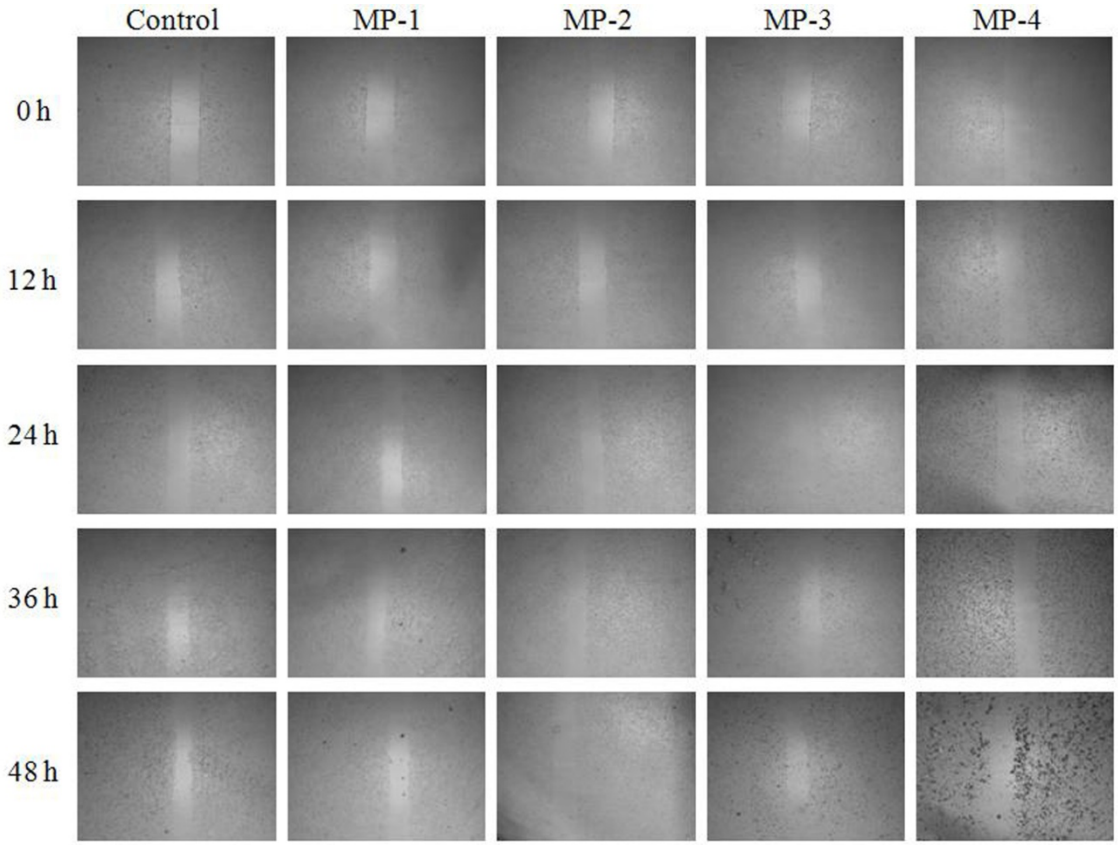

Fig. S8 Wound healing assay of A549 cells treated with the release supernatants from porous PLGA microparticles at 30 days.

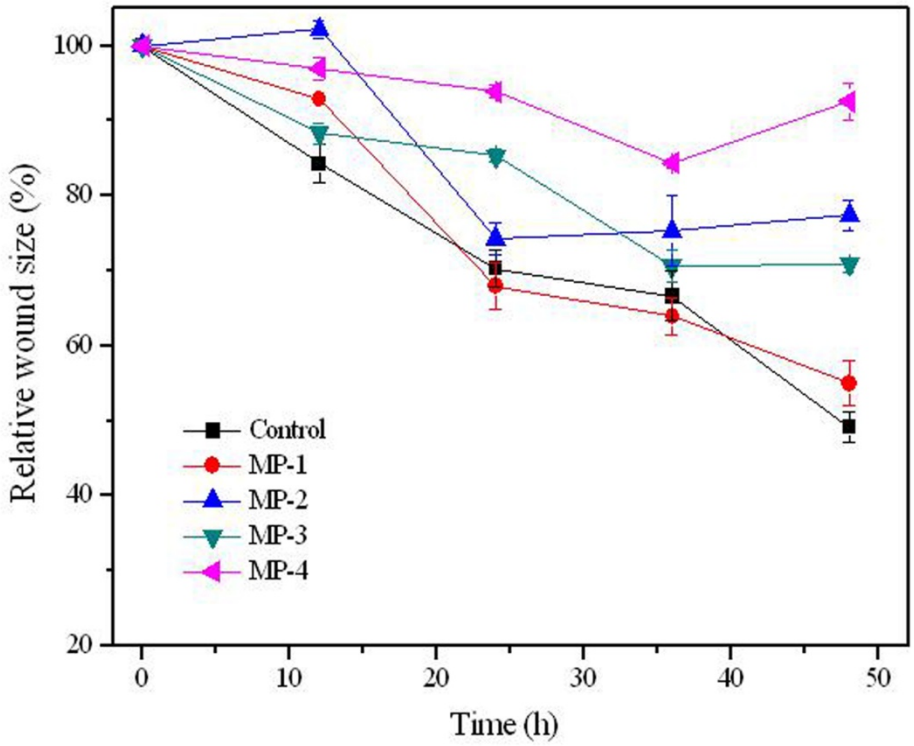

Fig. S9 Quantitative wound size of A549 cells treated with the release supernatants from porous PLGA microparticles at 30 days.

Table S1. Particle size and zeta potential of PEI25K/miR-34a nanoparticles and porous PLGA microparticles. The data were expressed as mean value ± SD, n=3.

Entry	Sample	Particle size[a]	Aerodynamic diameter[b]	Zeta potential (mV)	Drug loading for doxorubicin (%, w/w)	Drug loading for miR-34a (%, w/w)	Encapsulation efficiency for doxorubicin (%)	Encapsulation efficiency for miR-34a (%)
1	PEI/miR-34a	172.4 ± 76.3 nm	—	+40.7 ± 22.1	—	—	—	—
2	MP-1	46.7 ± 23.6 μm	8.58 ± 1.20 μm	-2.2 ± 1.9	—	—	—	—
3	MP-2	43.4 ± 19.5 μm	9.67 ± 0.87 μm	+34.4 ± 6.3	—	0.025 ± 0.001	—	63.6 ± 1.3
4	MP-3	48.9 ± 22.4 μm	9.17 ± 1.05 μm	-8.9 ± 8.7	0.823 ± 0.050	—	81.7 ± 1.4	—
5	MP-4	45.8 ± 21.3 μm	9.05 ± 1.18 μm	-4.0 ± 2.6	0.773 ± 0.030	0.013 ± 0.001	77.2 ± 0.9	33.5 ± 1.0

[a]Determined by Beckman Laser Scattering Particle Size Analyzer or Malvern Nano ZS90 Zetasizer.

[b]Determined according to the guidelines for pulmonary drug delivery in Chinese Pharmacopoeia (2010).