Ultrasound-Aided Targeting Nanoparticles Loaded with miR-181b for Anti-Inflammatory Treatment of TNF-α-Stimulated Endothelial Cells

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ABSTRACT: Gene therapy is an emerging therapeutic strategy used in clinics. Ultrasound-mediated gene transfection possesses great potential as a secure and available approach for gene delivery. However, transfection efficiency and targeting ability remain challenging. In this study, we developed a kind of ultrasound-aided and targeting nanoparticles for microRNA delivery. These nanoparticles carrying nucleic acids were prepared with cationic poly-(amino acid) encapsulated with perfluoropentane. The formulated nanoparticles were stabilized with negatively charged PGA–PEG–RGD peptide coating. Ultrasound imaging and specific gene transfection using this nanocarrier could be implemented simultaneously. Upon treatment with ultrasound irradiation, phase transition was induced in the nanoparticles and they generated acoustic cavitation, resulting in enhanced gene transfection against the endothelial cells. With the overexpression of miR-181b loaded by the nanoparticles, the TNF-α-stimulated endothelial cells were effectively rescued from the inflammatory state through the protection of cell viability and suppression of cell adhesion.

INTRODUCTION

Gene therapy is a potential therapeutic method for the treatment of diverse diseases, and the application of gene therapy coincides with the rapid development and progress of nanomedicines. Successful gene therapeutic approaches rely on safe, specific, and efficient gene delivery vehicles. Numerous gene delivery systems based on virus and nonvirus vehicles have been explored. However, because of the existing drawbacks, including indistinct immunological inflammation produced by virus vehicles and the poor gene transfection ability of nonvirus vehicles, more functional gene delivery strategies should be developed. During the last few years, microsized bubbles functioning as gene carriers have been combined with external stimuli such as ultrasonic irradiation to improve transfection efficiency. Ultrasound-aided gene delivery is a prospective strategy to enhance transfection capability through acoustic cavitation and sonoporation. This physical approach is generally recognized as a secure, noninvasive, and cost-efficient means of gene delivery. Atherosclerosis is the most common leading cause of cardiovascular diseases and has a high mortality worldwide. This chronic vascular disease is characterized by vulnerable plaque formation. Neovascularization is one of the crucial events in the formation of vulnerable plaques involving the emergence of new vascular endothelial cells and the production of a variety of inflammatory cytokines. Integrin αβ3 has been proposed as a useful marker of vulnerable plaques with high expression on neovascular endothelial cells. Integrin αβ3 can specially identify the short tripeptide motif, Arg–Gly–Asp (RGD). Based on the high affinity between RGD peptides and integrin αβ3, synthetic linear or cyclic RGD compounds were designed to serve as targeting ligand modifications on the surface of gene delivery vehicles.

In the past few years, studies have shown that the initiation of atherosclerosis involves the pathological activation and consequent dysfunction of the endothelial cells. The increase in inflammatory cytokines and adhesion molecules in endothelial cells induced by diverse inflammatory stimuli (such as tumor necrosis factor-α, TNF-α) is the primary pathological change. Among these changes, NF-κB signaling is a primary pathway relating to the inflammatory state. miR-181b plays a positive role in the mediation of the NF-κB signaling pathway in inflamed endothelium. miR-181b serves as a potent mediator of downstream signaling through direct targeting of importin-α3 (IPOA3), a protein critical for NF-κB translocation from the cytoplasm to the nucleus. miR-181b was reduced in the plasma of patients with coronary artery disease and ApoE−/− mice fed with a high-cholesterol diet. Overexpression of miR-181b could be effective in reducing...
the expression of genes related to NF-κB signaling, such as vascular cell adhesion molecule-1 (VCAM-1) and suppressing leukocyte adhesion to TNF-α-stimulated endothelial cells.23,24

In this study, we fabricated an ultrasound-aided gene delivery vehicle combining cationic polymers and targeting modification. The nanoparticle delivery system was developed based on our previous design of a biocompatible, cationic, and amphiphilic polymer.25–27 The morphological properties of nanoparticles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Cytotoxicity and gene transfection efficiency were measured, and ultrasound imaging was performed. Furthermore, gene transfection of miR-181b using this vehicle was studied by quantitative polymerase chain reaction (qPCR), and its function was monitored by cell viability and adhesion ability in the TNF-α-stimulated endothelial cell model.

## Results and Discussion

### Synthesis and Characterization of Polymers.

The fluorinated poly-(β-benzyl-L-aspartate) C9F17−PBLA was synthesized through the reaction of ring-open polymerization according to our previous studies.25–27 C9F17−NH2 was used as an initiator of BLA-NCA. C9F17−PAsp was prepared through aminolysis by diethylenetriamine (DET) of the side chain of C9F17−PBLA. The 1H NMR spectra shown in Figure 1 indicated successful material synthesis. The integrin targeting anionic polymer was synthesized by conjugating cRGDfC peptides to poly-(glutamic acid)-g-poly-(ethylene glycol) (PEG-g-PGA) through fast reaction between sulphydryl and maleic amide under mild conditions.28

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c00823)

Figure 1. Chemical structures and 1H NMR spectrums of (A) C9F17−PBLA and (B) C9F17−PAsp.

The characteristic absorption peaks showed that the grafting ratio of PAG-g-PEG-RGD was 11.2% (Figure S1).

### Preparation and Characterization of Nanoparticles.

The perfluoropentane-loaded nanodroplets (PFP-NDs) were formulated through an oil-in-water emulsion process. The amphiphilic C9F17−PAsp and amphiphobic PFP liquid drop were well emulsified through the probe sonication. TEM image showed that PFP-NDs were almost spherical in shape and had a narrow size distribution (Figure 2A). The particle size
distribution measured by DLS illustrated that the diameters were approximately 340 nm and exhibited a narrow distribution (Figure 2B). A particle size less than 400 nm was necessary for the efficient delivery of the NDs into tissues through the enhanced permeability and retention effect.25,29

Although the average zeta potential of the PFP-NDs was 73.7 mV (Table 1), the excess positive charge on the nanoparticles might affect its in vivo stability and blood circulation.30 With the electrostatic bonding of LucDNA to the cationic PFP-NDs, the binary nanoparticles PFP-BNDs were formulated. The surface zeta potential of PFP-BNDs decreased slightly owing to the neutralization of the negatively charged LucDNA, while the diameter of PFP-BNDs was increased because of the loose electrostatic interaction between them. Subsequently, with the addition of anionic PGA-g-PEG-RGD at different C/N, the positive surface charge of ternary nanoparticles (RGD−PFP-TNDs) decreased from 43.8 mV to −11.6 mV (Figure S2). According to Zhou et al., a low positive charge was considered beneficial to cell growth and cell uptake.31 Therefore, the surface charge at C/N = 5/5 (14.4 mV) was selected for the following experiments. The diameter of RGD−PFP-TNDs decreased to 339 nm (C/N = 5/5) owing to the electrostatic adherence between nanoparticles and anion polymer. With the modification of PEG, the carriers had improved low-protein adsorption properties and in vivo stability.32

### In Vitro Ultrasonic Imaging.

To explore the positive influence of ultrasonic irradiation, exposure duration, duty cycle (DC), and ultrasonic intensity were taken into consideration. As shown in Figure 3, with the increase in ultrasonic intensity from 0.4 to 1.2 W/cm² and DC from 10 to 20%, gradually brighter and clearer ultrasound images were observed. This trend could be explained by the fact that with

![Table 1](https://dx.doi.org/10.1021/acsomega.0c00823)

Table 1. Characterization of Nanoparticles (Loading LucDNA, C/N = 5/5)

| sample       | size (nm) | PDI | zeta potential (mV) |
|--------------|-----------|-----|---------------------|
| PFP-NDs      | 342 ± 8   | 0.184 | 73.7 ± 0.7         |
| PFP-BNDs     | 443 ± 17  | 0.197 | 57.2 ± 1.0         |
| RGD−PFP-TNDs | 339 ± 8   | 0.138 | 14.4 ± 0.1         |

https://dx.doi.org/10.1021/acsomega.0c00823

ACS Omega 2020, 5, 17102–17110
more exposure to ultrasonic irradiation within certain degrees of intensity and DC, more PFP-loaded nanoparticles achieved successful phase transition through vaporization and cavitation. Sufficient phase transition of nanoparticles is crucial for clear ultrasonic imaging and efficient gene transfection, and excessive exposure and high ultrasonic intensity would cause bubble coalescence and rupture. The clearest ultrasonic imaging and highest gray-scale intensity were obtained at an ultrasonic intensity of 1.2 W/cm², DC of 20%, and exposure duration of 60 s. These optimized ultrasonic parameters were adopted for the following studies.

**Cytotoxicity Assay.** The cytotoxicity of the gene delivery nanoparticles was evaluated by the MTT assay on human umbilical vein endothelial cells (HUVECs). The potential effect of cell growth caused by ultrasonic irradiation was also taken into consideration. As shown in Figure 4, with increased nanoparticle concentrations, the cell survival rates remained higher than 85%. This result indicated that the RGD–PFP-TNDs and ultrasonic irradiation had negligible effects on the viability of HUVECs. As a result, this gene delivery vehicle could be considered safe.

**Competitive Inhibition Experiment.** The main function of PFP-TNDs modified with RGD peptides is to enhance the selective internalization of nanoparticles to endothelial cells through receptor-mediated cell uptake. To investigate the targeting effect of RGD-modified PFP-TNDs, HUVECs were pre-incubated with free cRGDfC peptides at different concentrations to block the RGD receptors on the cytomembrane. As the concentration of free cRGDfC peptides increased, the cellular uptake efficiency and luciferase expression decreased in the RGD–PFP-TNDs groups. In contrast, the TNDs without RGD modification maintained the same cell uptake and gene transfection efficiency (Figure 5). Similar results were reported when the competitive free RGD peptides were pre-incubated with cells. This effect was attributed to the blocking of the available receptor-mediated cell uptake of RGD–PFP-TNDs. Furthermore, the mean fluorescence intensity (MFI) of the PFP-TNDs group was higher than that of the RGD–PFP-TNDs at 50 μg/mL concentration; we believe that as the free RGD concentration increased to a certain degree, additional RGD modified on PFP-TNDs also affected the interaction between nanoparticles and cells, which might have resulted in the decreased cell uptake efficiency compared with that observed in the non-RGD-modified sample.

**In Vitro Gene Transfection.** According to the investigation of the targeting efficiency of RGD and the optimization of the ultrasonic irradiation parameters above, the RGD–PFP-TNDs loaded with LucDNA were prepared and transfected into HUVECs with ultrasonic irradiation. With ultrasound irradiation, the transfection efficiency of both PFP-TNDs/LucDNA and RGD–PFP-TNDs/LucDNA increased significantly. This effect was attributed to the enhancement of the LucDNA uptake and expression by the phase transition of the nanoparticles under ultrasound irradiation. The subsequent cavitation and sonoporation effect of nanoparticles produced better cell permeability, allowing more LucDNA to enter the cell. The transfection efficiency was further improved by RGD modification (Figure 6). This improvement arose from the affinity between RGD–PFP-TNDs and integrin αvβ3 on HUVECs. The transfection efficiency of RGD–PFP-TNDs did not show a significant difference from the control.

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**Figure 3.** Ultrasound images and corresponding gray-scale intensities of RGD-PFP-TNDs triggered by ultrasound irradiation with different parameters. (A) Different ultrasonic DCs and time durations (intensity = 1.2 W/s); (B) different ultrasonic intensities (DC = 20%; time duration = 60 s).

**Figure 4.** Cell viability of HUVECs treated with different concentrations of PFP-TNDs or RGD–PFP-TNDs loading LucDNA with or without ultrasonic irradiation.

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Lipofectamine 2000 (LF2K) group \( (p = 0.248) \), which is widely used as the golden standard for transfection evaluation with high efficiency.\(^{35}\) This result suggested that the targeting nanoparticle combined with ultrasound irradiation might serve as an efficient strategy for gene delivery.

**In Vitro Therapeutic Gene Transfection Study.**

According to the exploration and verification above, under the selected optimal parameters \((C/N = 5/5, \text{ ultrasonic frequency} = 1 \text{ MHz}, \text{ ultrasonic intensity} = 1.2 \text{ W/cm}^2, \text{ DC} = 20\%, \text{ time duration} = 60 \text{ s})\), RGD–PFP-TNDs could serve as safe and efficient vehicles for gene transfection. Consequently, the following studies using the therapeutic gene miR-181b were carried out under optimal parameters.

The cell counting kit-8 (CCK-8) assay of HUVECs incubated with various concentrations of inflammatory inducement TNF-\(\alpha\) was performed as primary investigation. The proliferation of HUVECs was demonstrated as a dose-dependent effect, and an obvious decrease in cell viability was observed when the TNF-\(\alpha\) concentration reached 10 ng/mL (Figure S3). Therefore, HUVECs treated with TNF-\(\alpha\) (10 ng/mL) were selected as the inflammatory stimulation for the following studies.

**Characterization of Nanoparticles Loading miR-181b.**

RGD–PFP-TNDs/miR-181b were prepared via the same procedure as described above except being loaded with miR-181b mimic. The diameter of RGD–PFP-TNDs/miR-181b was 368 nm, and the zeta potential was 16.3 mV (Table S1); neither the size distribution nor the zeta potential showed an obvious change. The diameter of RGD–PFP-TNDs/miR-181b was slightly greater than that of RGD–PFP-TNDs/LucDNA. This difference could be influenced by the electrostatic binding between the nanoparticles and different nucleic acids.

**Intracellular Distribution of miR-181b.**

Figure 6. Luciferase expression against HUVECs transfected with LucDNA loaded by RGD–PFP-TNDs or PFP-TNDs, with or without ultrasonic irradiation. \(^{*} p < 0.05.\)

Intracellular distribution of miR-181b was performed via the same procedure as described above except being loaded with miR-181b mimic. The diameter of RGD–PFP-TNDs/miR-181b was 368 nm, and the zeta potential was 16.3 mV (Table S1); neither the size distribution nor the zeta potential showed an obvious change. The diameter of RGD–PFP-TNDs/miR-181b was slightly greater than that of RGD–PFP-TNDs/LucDNA. This difference could be influenced by the electrostatic binding between the nanoparticles and different nucleic acids.

To confirm the efficient gene delivery and intracellular distribution of miR-181b transfected by nanoparticles, confocal laser scanning microscopy (CLSM) was performed. As shown in Figure 7, we found that the green fluorescent miR-181b mimics labelled by carboxyfluorescein (FAM) were well distributed in the cytoplasm around the nucleus stained with 4',6-diamidino-2-phenylindole (DAPI), which indicated that the miR-181 mimics were well taken up by HUVECs. With the modification...
of RGD, the RGD−PFP-TNDs group showed higher uptake of the gene than the PFP-TNDs group, which was consistent with flow cytometry results and gene transfection studies. Since the study reported by Maubant et al. confirmed that the affinity between RGD peptides and integrin αvβ3 also existed in inflammatory endothelial cells,15 we believe that RGD−PFP-TNDs could be used as promising vehicles for the transfection of miR-181b mimics into HUVECs.

Quantitative PCR Assays. Expression of miR-181b in the mimic group was approximately 110-fold higher than that in the untreated group (Figure 8A). This result indicated that successful transfection of the therapeutic gene miR-181b was achieved by RGD−PFP-TNDs and ultrasonic irradiation. Then, the gene expressions of IPOA3, NF-κB p65, and VCAM-1 in TNF-α-stimulated HUVECs after incubation with functional nanoparticles were also detected by qPCR analyses (Figure 8). The TNF-α-stimulated blank group without gene transfection showed much higher expression of all the three measured genes than the blank control group. Expression of IPOA3, NF-κB p65, and VCAM-1 of the mimic group was obviously decreased (p < 0.05). These results could be explained by the overexpression of miR-181b, inhibiting the activation of NF-κB signaling. The direct targeting of the 3′-untranslated regions of IPOA3 was blocked, which was important for NF-κB cytoplasmic-nuclear translocation.21,22

As a result, the expression of NF-κB p65 decreased. The downstream molecule VCAM-1 was also suppressed. Sun et al. reported the similar changes about the genes in TNF-α-stimulated HUVECs transfected by miR-181b with a commercialized reagent.24 The expression in the negative control (NC) group was close to that in the TNF-α-stimulated group. These results may indicate that miR-181b mimics transfected by RGD−PFP-TNDs were successfully expressed in HUVECs and mediated the NF-κB genetic pathway.

CCK-8 Assay and Cell Adhesion Assay. Overexpression of miR-181b had a positive function in anti-inflammatory action was explored. Cell viability was monitored by CCK-8 assay, and adhesion capability was evaluated by the adhesion of THP-1. The cell viability of the TNF-α-stimulated blank group and NC group decreased (Figure 9A) because of the obvious effect of TNF-α stimulation. The cell viability of the miR-181b group was rescued to a level close to that of the untreated group. This result revealed that overexpression of miR-181b could provide protection of the HUVECs and suppress the TNF-α stimulation. Figure 9B showed that fewer THP-1 cells were stuck to the surface of TNF-α-stimulated HUVECs after the overexpression of miR-181b. This result demonstrated that miR-181b could play a positive role in suppressing the adhesion capability of TNF-α-stimulated HUVECs. Our results were in accordance with the observation reported by the study of Maubant et al.
CONCLUSIONS

In this study, we designed an integrin αβ₃-targeting, ultrasound-triggered, phase-transition nanoparticle system for efficient gene delivery. The targeting nanoparticles exhibited stable physical properties, low cytotoxicity, and high cellular uptake efficiency. With the phase transition triggered by ultrasound radiation, both the efficiency of gene transfection and ultrasound imaging were improved. Furthermore, after the successful transfection of the therapeutic gene miR-181b, the TNF-α-stimulated endothelial cells obtained efficient rescue from an inflammatory state through the protection of the cell viability and suppression of the leucocyte adhesion. In conclusion, this integrin αβ₃-targeting, ultrasound-triggered, phase-transition nanoparticle system loading miR-181b could serve as a potential therapeutic strategy for treating TNF-α-stimulated endothelial cells in atherosclerosis.

MATERIALS AND METHODS

Materials. β-Benzyl-l-aspartate N-carboxyanhydride (BLA-NCA) was obtained from Beijing HWRK Chem (China). Deuterium oxide (D₂O), dimethyl sulfoxide-d₆ (DMSO-d₆), MTT formazan, 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,9-heptadecan-2-yl-1H-fluorononylamine (C₉F₁₇-NH₂) were purchased from Sigma-Aldrich (China). N-Methyl-2-pyrrolidone (NMP), N-hydroxy-succinimide (NHS), dimethylformamide (DMF), dichloro-ethane (EDC), dichloromethane (DCM), DET, and Rose Bengal were purchased from Aladdin Industrial (China). Poly(γ-glutamic acid) (γ-PGA, 5 kDa) was purchased from Nanjing Sai Tai Biologics Technology (China). The cRGDFC [cyclic (Arg-Gly-Asp-d-Phe-Cys)] peptides were obtained from Ruxi Biologics Technology (China). NH₂-poly(ethylene glycol)–Mal (NH₂–PEG–Mal, 3.5 kDa) was purchased from Jenkem Biological Technology (China). PFP was purchased from Strem Chemicals (USA) and stored at −20 °C. LucDNA (the pGL4.13 vector encoding the luciferase reporter gene luc2), luciferase assay reagent, and 5X reporter lysing buffer were obtained from Promega (USA). LF2K, DAPI, TNF-α agent, and TRIZol reagent were purchased from Thermo Fisher Scientific (China). The Label IT Tracker intracellular nucleic acid localization kit was purchased from Mirus (USA). miR-181b mimic, NC agents, FAM-stained miR-181b mimic, the Bulge-Loop miRNA qRT-PCR starter kit, and the Ribo mRNA qRT-PCR starter kit were purchased from Ribobio Biotechnology Co. Ltd. (China). The CCK-8 kit was purchased from Beyotime Biotechnology (China).

For cell culture, HUVECs were purchased from ScienCell (USA) and cultured in endothelial cell medium (ECM) supplemented with 5% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin-streptomycin antibiotic, and 1% (w/v) endothelial cell growth supplement (ECGS) in a 5% CO₂ environment at 37 °C. THP-1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (China) and cultured in RPMI medium 1640 supplemented with 10% (v/v) FBS, 1% (w/v) penicillin-streptomycin antibiotic, 1% (w/v), and 0.05 mM β-mercaptoethanol (100 mL) in a 5% CO₂ environment at 37 °C.

Synthesis and Characterization. Synthesis and Characterization of the Cationic Polymer. Polymer C₆F₂₅-PAsp (DET) was synthesized according to our previous study. BLA-NCA and C₆F₂₅-NH₂ were dissolved in anhydrous DMF and reacted after addition of anhydrous DCM. Reaction was sustained for 72 h with stirring in a dry N₂ atmosphere at room temperature. After dialysis, lyophilization and dissolved in NMP, the product was obtained through aminolysis reaction with DET. The products were dissolved in DMSO and detected by proton nuclear magnetic resonance spectroscopy (¹H NMR, 400 MHz Bruker Avance III). The molecular weight and degree of polymerization were calculated.

Synthesis and Characterization of PGA–PEG–RGD. First, γ-PGA (40.0 mg) and NH₂–PEG–Mal (111.9 mg, 10% grafting ratio of the carboxyl of the PGA) were dissolved in phosphate-buffered solution (PBS 5 mL, pH 8.5, 0.05 M). Second, EDC (6.5 mg, 1.1 equiv of PEG) and NHS (3.9 mg, 1.1 equiv of PEG) predissolved in PBS (5 mL) were quickly added as catalysts and the mixture was stirred for 24 h. After dialysis, the resultant PGA-g-PEG mixture was redissolved in PBS (pH 7.2, 0.1 M) and cRGD peptide (35.8 mg, 2 equiv of PEG) were added. Reaction was performed with stirring for another 24 h. After dialysis and lyophilization, the product was stored at −20 °C for use. PGA–PEG–RGD was dissolved in D₂O to confirm its chemical structure through ¹H NMR.

Preparation of Targeting Nanoparticles. Targeting nanoparticles were prepared in three steps. First, cryopreserved PFP (30 μL, 3%, v/v) was quickly added to precooled C₆F₂₅–PAsp (DET) solution (5 mg/mL, 1 mL), and a liquid drop was formulated at the bottom of the bottle. The emulsification was accomplished with a probe-type sonicator (Misonix S4000). The machine was under a selected procedure (4 °C, 22% amplitude, 1 s on, 1 s off, 90 s). Product PFP-NDS [PFP/C₆F₂₅–PAsp (DET) nanoparticles] were stored at 4 °C. Second, the prepared PFP-NDS and plasmid LucDNA were blended for 30 min at 4 °C for electrostatic adsorption. Then, PFP-BNDS (PFP-NDS/LucDNA) (N/P ratio = 20) were formulated. Finally, the targeting nanoparticles RGD–PP-TNDs (PFP-BNDS/PGA–PEG–RGD) were fabricated through mixing PFP-BNDS and PGA–PEG–RGD with different C (PGA–PEG–RGD)/N (cationic polymer) ratios from 2/5 to 10/5. The mixture was incubated for another 30 min.

Characterization of the Nanoparticles. The characterization of the prepared nanoparticles included particle size, zeta potential, and morphology. The zeta potential and size were detected by DLS (Malvern Zetasizer Nano ZS90). Size distribution and visualized morphology were studied by TEM (JEM 1400) after staining with 1% uranyl acetate.

In Vitro Ultrasound Imaging. To determine the acoustic droplet vaporization efficiency of RGD–PP-TNDs under ultrasonic irradiation, ultrasound imaging experiments were performed. The RGD–PP-TNDs prepared as above were diluted to 400 μg/mL and filled in plastic Pasteur pipettes sealed with hemostatic forceps. A therapeutic ultrasound machine (SGENE-1000, China) was used to obtain the RGD–PP-TNDs irradiated with a fixed frequency of 1 MHz based on our previous work. The different parameters included...
exposure duration from 10 to 120 s; DC of 10, 20, and 50%; and ultrasonic intensity from 0.4 to 2.4 W/cm². Then, the pipe containing nanoparticles was rapidly immersed in a degassed water bath tank (37 °C) equipped with a black background. Ultrasound imaging was performed through the scanner system of the clinical equipment, Aplio500 system (Toshiba, Japan), equipped with a 12LS convex transducer, a fixed frequency (6.5 MHz), and mechanical index (0.5). All the images were digitally recorded. ImageJ (National Institutes of Health, USA) was employed for the subsequent analysis of the greyscale values.

Competitive Inhibition Experiment. To analyze the targeting efficiency of cRGDC peptides for integrin αβ₃, a competitive inhibition study was performed on HUVECs. For the cellular uptake experiment, we conjugated fluorescent to LucDNA according to the protocol of the Label IT Tracker intracellular nucleic acid localization kit. HUVECs were seeded in a 6-well plate at a density of 2 × 10⁵/well and incubated for 12 h. The free cRGDC peptides (at a concentration of 25 or 50 μg/mL) were dissolved in ECGS/serum-free culture medium. HUVECs were preincubated for 1 h. After replacement of the medium, RGD–PFP-TNDs or PFP-TNDs loading the fluorescent-labelled LucDNA were added. HUVECs were incubated for 6 h. The cells were washed with PBS (0.01 M, pH = 7.4) three times. Then, cells were collected after digestion by trypsin. Thereafter, the collected cells were centrifuged at 5 min at 1500 rpm and resuspended in cold PBS. MFI was analyzed by flow cytometry using a FACS-Callibur instrument (Becton Dickinson, US) at an excitation wavelength of 488 nm. In addition to the uptake assay, gene transfection assay for the competitive inhibition experiment was also performed.

Cytotoxicity Study. HUVECs were seeded in 96-well plates at a density of 5 × 10³ cells/well for 12 h. RGD–PFP-TNDs or PFP-TNDs at different concentrations from 10 to 50 μg/mL were added. Ultrasonic treatment was followed. Twenty-four hours later, MTI solution (5 mg/mL) was added (20 μL/well) to each well. HUVECs were incubated for 4 h at 37 °C. Then, the supernatant was removed. DMSO (150 μL) was injected into each well. Absorbance was measured (absorption wavelength 570 nm) by a microplate reader (BioTek Synergy 4, USA). Five duplicate wells were run in each group. Cell viability was normalized as the absorbance value of the treatment group/absorbance value of the NC group (untreated cells).

Gene Transfection. HUVECs were seeded in a 24-well plate at 5 × 10⁴/well and incubated with ECM for 12 h. Cells were incubated for 6 h with ECGS/serum-free ECM after the addition of RGD–PFP-TNDs/LucDNA. It is worth mentioning that 30 min after the addition, the cells were exposed to the ultrasonic irradiation from the bottom of the well. Then, the medium was replaced with fresh ECM without ECGS. Luciferase assay was performed after a total 36 h infection. Cells were washed twice with PBS. Reporter lysis buffer (100 μL each well) was added following 1 h of incubation. Then, cells were harvested and centrifuged at 4 °C for 10 min at 12,000 rpm. Fifteen microliters of the obtained supernatant were used for BCA protein assay to measure the protein concentration. Twenty microliters of the supernatant were used to measure the luciferase activity in terms of relative light units (RLU). The final gene transfection efficiency of luciferases was exhibited in terms of RLU/mg. Experiments were repeated three times.

In Vitro Therapeutic Gene Transfection Studies. Characterization of Nanoparticles Loading miR-181b. Targeting therapeutic nanoparticles RGD–PFP-TNDs loading miR-181b mimic were prepared through a three-step process as described above. Note that, for the second step, PFP-BNDs were formulated through the electrostatic adsorption of miR-181b mimic or NC (the concentration of the miR-181b mimic or NC was 100 nM on according to the manufacturer’s protocols). Then, the physical characters of the therapeutic nanoparticles including size and zeta were measured with DLS.

CLSM for Intracellular Distribution. CLSM was performed to observe cellular uptake and intracellular distribution of fluorescence-labelled FAM-miR181b mimic transfected by RGD–PFP-TNDs and PFP-TNDs against HUVECs. HUVECs were seeded on specialized culture dishes at a density of 2 × 10⁵ cells/mL. Subsequently, the cells were treated with RGD–PFP-TNDs/FAM-miR181b or PFP-TNDs/FAM-miR181b and ultrasonic trigger. After 4 h, the cells were washed twice with PBS. Then, the cells were fixed with 4% paraformaldehyde and stained with DAPI. The final fluorescence images (FAM-miR181b, employed excitation wavelength 488 nm, emission wavelength 520 nm; DAPI, employed excitation wavelength 405 nm, emission wavelength 461 nm) were obtained by CLSM (Leica TCS SPS, Germany).

Real-Time qPCR Assay. HUVECs were seeded in 6-well plates at a density of 2 × 10⁵ cells/well. RGD–PFP-TNDs/miR-181b were added, and the cells underwent ultrasonic exposure as described above. HUVECs were suspended in the TRizol reagent. Total RNA was isolated. Reverse transcriptions of miR-181b were operated by using a Bulge-Loop miRNA qRT-PCR starter kit. A Bulge-Loop miRNA qRT-PCR primer set was used for qPCR analysis with the ABI 7500 Real-Time PCR System (Applied Biosystems, USA) following the manufacturer’s instructions. The U6 RNA expression was used as the endogenous control for analysis of miR-181b. Quantitative measurements were calculated according to the ΔΔCt method. All samples were measured in triplicate.

To explore the influence of the therapeutic gene miR-181b of the TNF-α-stimulated HUVECs, qPCR assays were operated to investigate the gene expression of the targeting spot of miR-181b (IPOA3) and downstream NF-κB signaling pathways (NF-κB p65, and VCAM-1). All the transfection procedures were the same as described above, except a 6 h TNF-α stimulation was supplemented before isolating the total RNA. Correspondingly, reverse transcriptions of mRNA of IPOA3, NF-κB p65, and VCAM-1 were operated by using a Ribot mRNA qRT-PCR starter kit. qPCR analysis was performed using a Bulge-Loop mRNA qRT-PCR primer set. The GAPDH mRNA expression was used as the endogenous control.

CCK-8 Assay. To evaluate the viability of the HUVECs responding to inflammatory-induced TNF-α, CCK-8 assay was performed. HUVECs were seeded in 96-well plates at a density of 5 × 10⁵ cells/well. The cells were incubated with TNF-α of different concentrations (2.5–50 ng/mL) for 6 h and then washed twice with PBS. The cells were incubated with fresh culture medium, and CCK-8 liquid drop (20 μL each well) was added. Then, the cells were incubated for another 2 h without light. Absorbance was measured (absorption wavelength 450 nm). To explore the therapeutic function of the TNF-α-stimulated HUVECs, another CCK-8 assay was performed. The same transfection procedures were employed as described above. After 36 h of incubation, the 6 h induction of a certain
concentration TNF-α was performed. Finally, absorbance was measured as described above.

**Cell Adhesion Assay.** To verify the efficiency of miR-181b in suppressing the adhesion ability of TNF-α-stimulated HUVECs, an adhesion assay was performed. The operation of transfection of miR-181b and TNF-α stimulation on HUVECs were performed as described above. For the adhesion assay, after washing twice with PBS, HUVECs were incubated with THP-1 cells (suspended in serum-free medium at 5 × 10^5 cells/mL) for 1 h. Then, nonadherent THP-1 cells were removed. HUVECs were gently washed twice with serum-free medium. Finally, 100 μL of 0.25% Rose Bengal sodium salt dissolved in PBS (0.01 M, pH = 7.4) was injected into each well. Cells were dyed for 10 min (room temperature). After another two washes with PBS, a mixture of PBS and absolute ethyl alcohol at 1:1 (v/v) was added (200 μL). Cells were incubated for 2 h. Finally, absorbance was measured (absorption wavelength 570 nm).37

**Statistical Analysis.** SPSS 22.0 software was used. The results are expressed as the mean ± standard deviation. Statistical comparisons were performed by Bonferroni’s means comparison test and two-sample Student’s t-tests. P < 0.05 was considered statistically significant.

**ACKNOWLEDGMENTS**

This work was supported by the National Natural Science Foundation of China (51773231 and 81601500), the Natural Science Foundation of Guangdong Province (2016A03013315 and 2014A030312018), the Project of Key Laboratory of Sensing Technology and Biomedical Instruments of Guangdong Province (2011A060901013) and Science and Technology Planning Project of Guangdong Province of China (20160904).

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**ASSOCIATED CONTENT**

1. **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00823.

1H NMR spectra of PGA-g-PEG-RGD, characterization of nanoparticles, and viability of TNF-α stimulated cells (PDF)

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**Author Contributions**

D.L. and J.L. contributed equally to this work. All the authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.
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