

Co-delivery of Andrographolide and Notch1-targeted siRNA to Macrophages with Polymer-based Nanocarrier for Enhanced Anti-inflammation

Teng Wu^{a,b}, Min Tan^a, Heng-Ye Gong^a, Yong Wang^a, and Xin-Tao Shuai^{a,b*}

^a PCFM Lab of Ministry of Education, School of Materials Science and Engineering, Sun Yat-Sen University, Guangzhou 510275, China

^b Center of Biomedical Engineering, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China

Abstract Chronic inflammatory responses induced by macrophages play a pivotal role in the progression of atherosclerosis. In the present study, a multifunctional nanocarrier based on poly(ethylene glycol)-block-poly(L-aspartic acid) grafted with diethylenetriamine, lysine and cholic acid (PEG-PASp(DETA)-Lys-CA₂) polymer was synthesized for co-delivery of andrographolide and siRNA targeting Notch1 gene to alleviate the inflammatory response in macrophages. The nanocarrier exerted low cytotoxicity as well as high performance in drug/siRNA co-delivery. *In vitro* studies demonstrated the co-delivery of andrographolide and Notch1 siRNA not only significantly inhibited lipopolysaccharide (LPS)-activated interleukin-6 (IL-6) and monocytes chemotactic protein 1 (MCP-1) expression as well as blocked nuclear factor- κ B (NF- κ B) signal activation, but also interfered the Notch1 gene expression and increased anti-inflammatory cytokines such as interleukin-10 (IL-10) and arginase-1 expression obviously in macrophages. These results suggested that the combination therapy based on Notch1 siRNA and andrographolide co-delivered nanocarrier, *i.e.* suppressing the expression of pro-inflammatory cytokines while simultaneously increasing anti-inflammatory factors expression, be a feasible strategy for atherosclerosis treatment.

Keywords Macrophages; Anti-inflammatory; Andrographolide; Notch1 siRNA

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INTRODUCTION

Despite the great advantages of clinical therapy, atherosclerosis-related cardiovascular diseases are still the leading cause of mortality and morbidity around the world^[1, 2]. Instead of the main therapeutic strategies of lipid metabolism regulation, currently, several pre-clinical and clinical researches demonstrate that inhibiting the chronic inflammatory of atherosclerosis shows great potentials in atherosclerosis therapy^[3].

As the chronic inflammatory disease, recent research considered macrophages participate all atherosclerosis-related processes including initiation, progression as well as regression of atherosclerotic lesions formation^[4]. Macrophages have been triggered to increase the expression of monocytes chemotactic protein 1 (MCP-1) and release several chemokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α), which lead to the inflammatory microenvironment in plaques and finally promote the progression of atherosclerosis^[5]. Foam cells, also developed from macrophages *via* the endocytosis of oxidized low-

density lipoprotein (oxLDL), can lead to further inflammatory response and the development of atherosclerotic plaques^[6]. Therefore, macrophage-targeting strategy can be an effective means for atherosclerosis treatment.

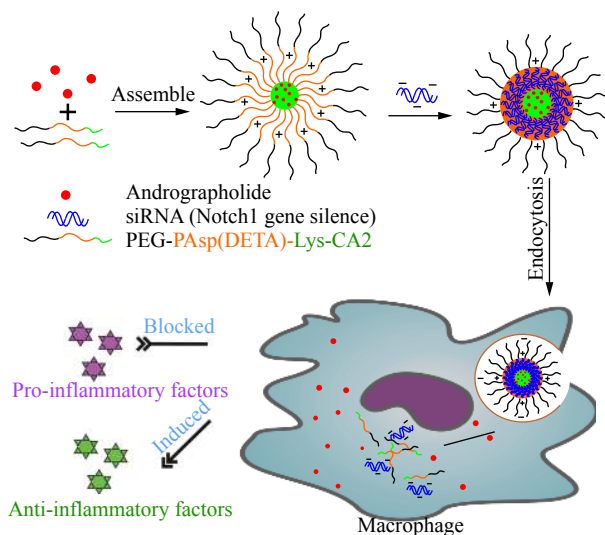
Ameliorating the inflammatory response in atherosclerosis lesions is the significant strategy which has been widely used in pre-clinical and clinical researches for atherosclerosis treatment. Andrographolide (Andro), a natural labdane diterpenoid from *Andrographis paniculata*, has been officially approved for respiratory infection and fever treatment as a traditional Chinese medicine for decades of years. Recently, mechanism research showed that andrographolide exhibited great anti-inflammatory activity *via* blocking the nuclear factor- κ B (NF- κ B) signaling pathway^[7]. Further studies also indicated the great potential of Andro on treating anti-inflammation-related diseases such as cancer, diabetes and *etc*^[8–10]. On the other hand, increasing the expression of anti-inflammatory cytokines is another strategy that has been reported to benefit atherosclerosis treatment. Currently, some researches indicated that blocking Notch1 gene expression was helpful for alleviating the progression of atherosclerosis *via* upregulating the levels of anti-inflammatory cytokines such as interleukin (IL-10) and arginase-1 expression^[11–13]. Thus, combination therapy of suppressing the expression of pro-inflammatory cytokines while simultaneously increasing

* Corresponding author: E-mail shuaixt@mail.sysu.edu.cn

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the anti-inflammatory ones may promote the atherosclerosis treatment.

In recent years, nanocarrier-mediated drug delivery has provided great potentials for combination disease treatment such as cancer^[14], hepatic fibrosis^[15] and cerebral ischemic stroke^[16] by the co-encapsulation of many therapeutic agents, *e.g.* small molecule drugs and genes^[17–19]. In the present study, a multifunctional nanocarrier based on amphipathic polymer of poly(ethylene glycol)-block-poly(L-aspartic acid) grafted with diethylenetriamine, lysine and cholic acid (PEG-PAsp(DETA)-Lys-CA₂, abbreviated as PALC) was synthesized to co-deliver andrographolide and Notch1 siRNA into macrophages (Scheme 1), with the purpose of obtaining a synergistic anti-inflammatory effect in atherosclerosis treatment *via* combination of inhibiting pro-inflammatory factors such as IL-6 and MCP-1 as well as increasing the expression of inflammatory resist factors such as IL-10 and arginase-1.



Scheme 1 Schematic illustration of the formation of Andro and Notch1 siRNA co-loaded nanoparticle and its multi-functions on macrophages

EXPERIMENTAL

General Considerations

The following reagents were purchased from Sigma-Aldrich and used as received: *N*-hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU), *N,N*-diisopropylethylamine (DIPEA), di-*tert*-butoxycarbonyl-L-lysine (Boc-Lys(Boc)-OH), α -methoxy- ϵ -hydroxy-poly(ethylene glycol) (PEG-OH, $M_n = 2$ kDa), anhydrous dimethyl sulfoxide (DMSO) and anhydrous dimethylformamide (DMF). Diethylenetriamine (DETA), trifluoroacetic acid (TFA), and cholic acid (CA) were purchased from Alfa Aesar China Co., Ltd. (Tianjin, China). Dialysis bags (MWCO: 3.5 kDa, 14 kDa) were purchased from Shanghai Green Bird Technology Development Co., Ltd., China. Chloroform (CHCl₃), acetic ether, petroleum ether, and dichloromethane (DCM, CH₂Cl₂) were dried over CaH₂ and then distilled under ambient pressure. Diethyl ether was of

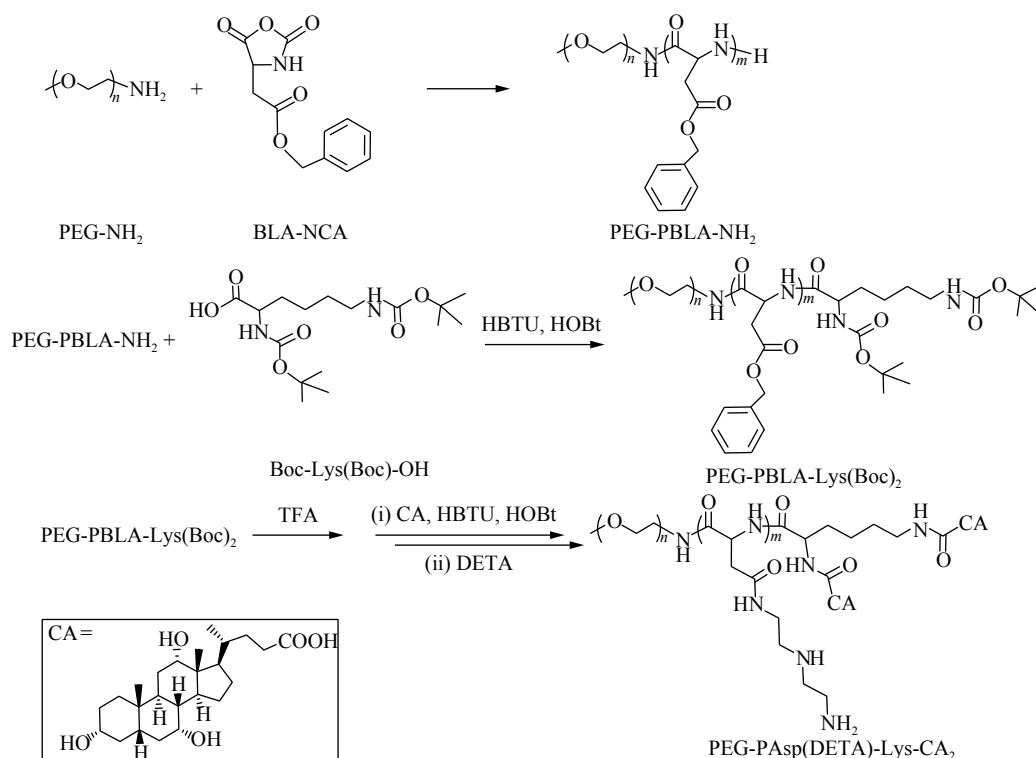
analytical grade and purchased from Guangzhou Chemical Reagent Factory, China. Methoxy-poly(ethylene glycol)-amino (PEG-NH₂) and *N*-carboxyanhydride of β -benzyl-L-aspartate (BLA-NCA) were synthesized as previously reported^[20]. Andrographolide and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation and cytotoxicity assay kit were purchased from Sangon (Shanghai, China). The primary antibodies against p65 and phosphorylated (p)-p65 were purchased from Santa Cruz Biotechnology.

Polymer Synthesis

The amphipathic polymer of PALC was synthesized *via* multi-step reactions outlined in Scheme 2 according to our previous study^[16]. Firstly, the copolymer of ethylene glycol and β -benzyl L-aspartate (PEG-PBLA) were synthesized by ring-opening polymerization of BLA-NCA using PEG-NH₂ as an initiator^[21]. In brief, 1.00 g of PEG-NH₂ (0.5 mmol) was dissolved with 60 mL of anhydrous CHCl₃, and 3.74 g of BLA-NCA (15 mmol) dissolved in 6 mL of anhydrous DMF was then added under the protection of N₂ gas. The solution was kept stirring for 72 h at 35 °C followed by precipitated into excessive cool diethyl ether. The precipitate was filtered, washed with diethyl ether, and vacuum-dried until a constant weight was attained (PEG-PBLA: $M_n = 7.1$ kDa, calculated from ¹H-NMR spectrum). Secondly, 64 mg of Boc-Lys(Boc)-OH (0.185 mmol, 1.5 equiv.) was coupled onto the *N*-terminal of PBLA using HBTU (0.185 mmol, 1.5 equiv.) and HOBt (0.185 mmol, 1.5 equiv.) as coupling reagents in DMF overnight^[22]. The targeted molecules were precipitated and washed three times by cold diethyl ether. Subsequently, the Boc groups were removed by treating with TFA for 30 min, the mixture solution was precipitated into cold diethyl ether, and the precipitate was filtered, washed with diethyl ether, and vacuum-dried to get PEG-PBLA-Lys. Thirdly, CA molecules (0.235 mmol, 3.0 equiv.) were coupled onto the *N*-terminal of PEG-PBLA-Lys *via* amidation reaction with HBTU (3.0 equiv.) and HOBt (3.0 equiv.) as coupling reagents. The reaction proceeded in DMF overnight and the product was precipitated and washed by cold methanol followed by filtering and vacuum-drying to get PEG-PBLA-Lys-CA₂. Finally, PEG-PAsp(DETA)-Lys-CA₂ was synthesized by aminolysis of PBLA with DETA. In brief, 0.60 g of PEG-PBLA-Lys-CA₂ (0.066 mmol) and 10.71 mL of DETA (99 mmol, about 50 equiv. to benzyl group) were dissolved in 20 mL of anhydrous DMSO, and then the reaction was conducted under stirring for 2 h at 35 °C. The mixture was dialyzed (MWCO: 14 kDa) against methanol for 2 days and deionized water for 2 days, respectively, and then freeze-dried to obtain final polymer PEG-PAsp(DETA)-Lys-CA₂ ($M_n = 8.0$ kDa, calculated from ¹H-NMR spectrum, total yield: 54%).

Preparation of Nanoparticle Co-loading Andrographolide and siRNA

The hydrophobic andrographolide-loaded micelle was firstly prepared as reported before^[16]. Andrographolide (2 mg) and 20 mg of polymer (PEG-PLL-Lys-CA₂) were co-dissolved into 2 mL of DMSO and chloroform (*V/V* = 1:1). Under



Scheme 2 The synthetic strategy of PEG-PAsp(DETA)-Lys-CA₂

sonication (VCX130, Sonics, USA, 40% power level), the mixture solution was added dropwise into 20 mL of deionized water. After the chloroform was removed by rotary evaporation, the solution was dialysed (MW cutoff: 14 kDa) against water for 2 days followed by ultrafiltration using a MILLIPORE centrifugal filter device (MW cutoff: 100 kDa). Then, the sample was filtered through a syringe filter (pore size: 450 nm) to remove large aggregates and get the final andrographolide-loaded nanoparticle (A-NP).

Notch1 gene targeting siRNA or scrambled siRNA (SCR) was dissolved in RNase-free water and then mixed with A-NP according to the designed N/P ratio (the molar ratio of polymer nitrogen to RNA phosphorus). The mixture was vibrated vigorously for 30 s followed by being kept at room temperature for 30 min to form the siRNA or SCR and andrographolide co-loaded nanoparticles (A/siR-NP or A/SCR-NP).

Characterizations of the Polymer and Nanoparticles

¹H-NMR spectroscopy was carried out on a Varian Unity 400 MHz spectrometer using DMSO-d₆ as a solvent. Dynamic light scattering (DLS) measured by a 90 Plus/BIMAS equipment (Brookhaven Instruments Corporation, USA) was used to evaluate the sizes and zeta potentials of nanoparticles, which was carried out at 25 °C. For the zeta potential measurement, a standard electrophoresis mini-cell from Brookhaven was used. The data for particle size and zeta potential were collected on an auto-correlator with a detection angle of scattered light at 90° and 15°, respectively. The data from five measurements were averaged to obtain the mean ± standard deviation (SD) for each sample. Transmission electron microscopy (TEM) was performed

using a Hitachi model H-7650 TEM operated at 80 kV. Samples were prepared by adding a drop (5 µL, 0.5 mg/mL) of the sample solution on a copper grid coated with amorphous carbon and then blotted with a filter paper after 1 h. Uranyl acetate solution (10 µL, 2 wt% in water) was added to the copper grid to stain the samples, and then blotted with a piece of filter paper after 1 min. Finally, the grid was dried overnight at room temperature inside a desiccator before TEM observation. The drug loading content of Andro was determined by high performance liquid chromatography (1260 Infinity II, Agilent Technologies) as previously reported^[23].

Cell Culture and Treatment

The murine macrophage cell line RAW264.7 was provided by the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured at 37 °C in a 5% CO₂ incubator with Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin. Cells were grown to confluence and synchronized in serum-free DMEM for 3 h. The medium was then changed to (1) only serum-free DMEM for 24 h treatment as negative control, (2) serum-free DMEM containing 1 µg/mL LPS for 24 h treatment as positive control, (3) serum-free DMEM containing 1 µg/mL LPS for 24 h treatment with 100 nmol/L anti-Notch1 siRNA loaded by PALC (denoted as siR-NP), (4) serum-free DMEM containing 1 µg/mL LPS for 24 h treatment with 5 µmol/L andrographolide loaded by PALC (denoted as A-NP), (5) serum-free DMEM containing 1 µg/mL LPS for 24 h treatment with 100 nmol/L anti-Notch1 siRNA and 5 µmol/L andrographolide loaded by PEG-PAsp(DETA)-

Lys-CA₂ (denoted as A/siR-NP). Anti-Notch1 siRNA was purchased from Genpharma (Suchou, China) and LPS was provided from Sigma Aldrich (CA, USA).

Agarose Gel Electrophoresis

The scrambled siRNA (SCR, Genpharma) loaded polymer-based nanoparticles (denoted as SCR-NP or A/SCR-NP) were formed at various N/P ratios (0, 2, 4, 6, 8, 12, 16, 20) in a final volume of 10 μ L containing 30% of glycerol followed by loading onto the 1.5% agarose gels (Biowest, USA) with ethidium bromide (EB, 0.1 mg/mL). After ran with tris-acetate (TAE) buffer at 120 V for 15 min, siRNA motion retardation was visualized by irradiation with UV light of DNR Bio-Imaging System (NR Bio-Imaging System Ltd, Isreal).

MTT Assay

RAW264.7 cells were seeded at 4×10^3 cells per well in 96-well plate. After starvation for 3 h when reached 60%, the cells were incubated with SCR-NP at different polymer concentrations (0, 12.5, 25, 50, 100, 200, 400, 600 μ g/mL), or Andro, A-NP and A/SCR-NP at various Andro concentrations (0, 0.63, 1.25, 2.5, 5, 10, 20, 40 μ mol/L) in DMEM for 48 h. Then 10 μ L of 1% MTT (Promega, USA) was added into each well and the cells were incubated for another 4 h. Subsequently, the medium was removed carefully and 100 μ L of DMSO (Sangon, Shanghai, China) was added into each well, then the absorbance of solubilized blue formazan was read at the wavelength of 570 nm by using a microplate reader.

Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was utilized to detect the intracellular distribution of Cy3-labeled scramble siRNA (SCR) and Andro (replaced by fluorescent coumarin). Cells were cultured in petri dishes at a density of 2×10^4 cells per dish and incubated overnight. After treated with/without LPS for 36 h, cells were incubated with Cy3-labeled SCR-NP containing coumarin for 2, 4 and 6 h at a final SCR concentration of 100 nmol/L. Then cells were washed with PBS three times and stained with DAPI for 2 min after fixed by 4% paraformaldehyde for 5 min. To identify the location of coumarin and Cy3-labeled SCR inside cells, samples were observed on a Zeiss 710 confocal microscope (German).

RNA Extraction and Real-time PCR

TRIzol (Invitrogen) was utilized for cell RNA extraction according to the manufacturer's recommended protocol. A reverse transcription kit (Takara) and oligo (dT) primes were used for RNA samples reverse transcription, and real-time PCR was performed in a 20 μ L reaction mixture containing H₂O, primers, and Fast Start Universal SYBR Green master (ROX) reagents (Roche Applied Science, Mannheim, Germany). cDNA (2 μ L) was amplified and measured using Stepone Plus (Applied Biosystems). PCR prime sequences are listed as follows. IL-6 forward: TAGTCCTTCCTACCCCAATTTCC, reverse: TTGGTCCTTAGCCACTCCC; MCP-1 forward: TTAAAAACCTGGATCGGAACCAA, reverse: GCATTAGCTTCAGATTACGGGT; IL-10 forward: GCTCTTACTGACTGGCATGAG, reverse: CGC

AGCTCTAGGAGCATGTG; arginase-1 forward: CTCCAAGCCAAAGTCCTTAGAG, reverse: AGGAGCTGTCA TTAGGGACATC; β -actin forward: GGCTGTATCCCC TCCATCG, reverse: CCAGTTGGTAACAATGCCATGT; Quantitative measurement, were achieved using the $\Delta\Delta$ Ct method, and the expression of β -Actin was used as an internal control.

Western Blot Analysis

Total cell protein was extracted from RAW 264.7 cells after treated by RIPM lysis buffer (Beyotime, Shanghai, China) according to the recommended protocol. Protein samples (30 μ g) were separated on 10% sodium dodecyl sulfate polacrylamine gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene difluoride) (PVDF) membranes. After incubation with the blocking buffer (5% non-fat milk) for 1 h, the membranes were incubated with rabbit antibody against (p)-p65 (Santa Cruz, USA) as well as mouse antibody against p65 (Santa Cruz, USA) overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated donkey anti-mouse or donkey anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA) were used to amplify the signal. Protein signals were detected by using chemiluminescence kit (KeyGEN, Nanjing, China).

Statistics

Significant differences between the two groups were analyzed by unpaired Student's t test, and differences between multiple groups of data were analyzed using one-way ANOVA with Bonferroni correction (Graphpad Prism 5.0, USA). All experiments were performed at least three times with similar results. The data are expressed as the means $\pm$ SD. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Polymer

The successful synthesis of polymers *via* multi-step reactions was confirmed by ¹H-NMR analyses. As shown in Fig. 1(A), the major resonance peaks of the copolymer PEG-PBLA in the ¹H spectrum well fit into the expected chemical structure. 3.35 ppm (s, $\text{CH}_3\text{O}-$ of PEG, a), 3.50 ppm (m, $-\text{OCH}_2\text{CH}_2-$ of PEG, b), 4.60 ppm (m, $-\text{NH}(\text{CH}_2)\text{CHCO}-$ of PBLA, c), 2.54–2.90 ppm (m, $-\text{CHCH}_2\text{CON}-$ of PBLA, d), 5.00 ppm (m, $-\text{CH}_2\text{C}_6\text{H}_5$ of PBLA, e), 7.25 ppm (m, $-\text{CH}_2\text{C}_6\text{H}_5$ of PBLA, f), 8.16 ppm (m, $-\text{CHCH}_2\text{CONH}-$ of PBLA, g). After Boc-Lys was coupled onto the PEG-PBLA polymer, characteristic resonances of lysine and Boc appeared: 1.18–1.78 ppm (m, $-\text{CH}_2-$ of Lys, i, j and h), 1.40 ppm (s, $-\text{CH}_3$ of Boc, p). The Boc group was then removed, resulting in the disappearance of the signal with chemical shifts in 1.40 ppm (Figs. 1B and 1C).

After CA molecules were coupled onto the polymer, its characteristic resonance signals appeared at 0.59, 0.80 and 1.21 ppm. Finally, the disappearing characteristic proton signals in benzene group and the appearing proton signals of DETA (m, 2.50–2.72, q) evidenced the successful and complete ammonolysis of PBLA. These results indicated the successful synthesis of polymer (Figs. 2A and 2B).

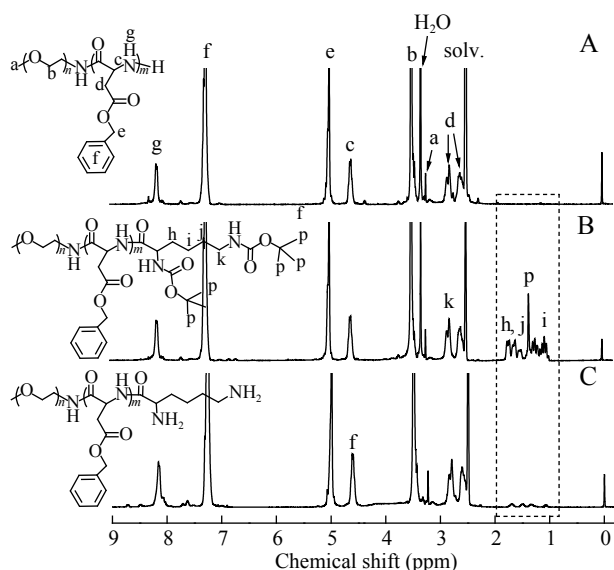


Fig. 1 ^1H -NMR spectra of (A) PEG-PBLA, (B) PEG-PBLA-Lys(Boc) $_2$ and (C) PEG-PBLA-Lys in DMSO- d_6

The Preparation and Characterization of Nanoparticles

The drug loading content and encapsulation efficiency of the Andro-loaded micelle were $(7.6 \pm 1.1)\%$ and $(69 \pm 5.4)\%$, respectively. Then, the agarose gel electrophoresis assay was utilized to evaluate the siRNA complexing ability of nanocarrier since insufficiently complexed siRNA at low N/P values migrated towards the anode. As shown in Fig. 3(a), complete retardation of siRNA migration was achieved at N/P ratio of 6 in both SCR-NP and A/SCR-NP, indicating that the siRNA was fully complexed. In addition, whether loading Andro or not did not affect the complexing ability of nanocarrier. Aiming at obtaining a better siRNA encapsulation, N/P ratio of 8 was chosen for subsequent tests.

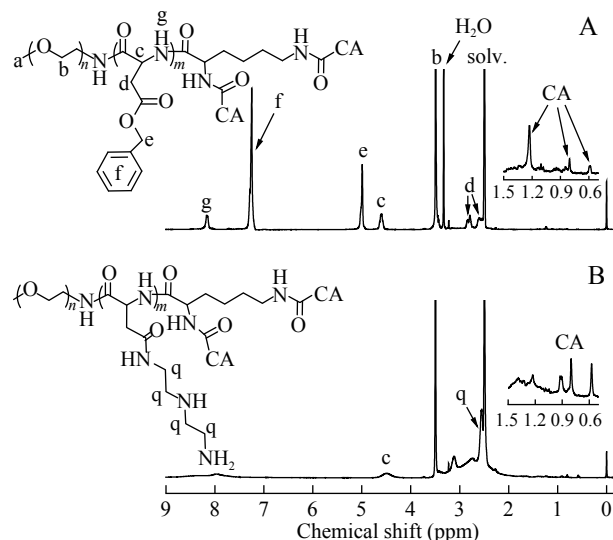


Fig. 2 ^1H -NMR spectra of (A) PEG-PBLA-Lys-CA $_2$, (B) PEG-PAsp(DETA)-Lys-CA $_2$ in DMSO- d_6

The Characterization of Nanoparticles

The sizes and zeta potentials of the nanoparticles were detected *via* dynamic light scattering (DLS) measurement. As shown in Fig. 3(b), the nanoparticles showed appropriate sizes of sub 100 nm ((87.8 ± 18.9) nm for A-NP and (107.6 ± 16) nm for A/SCR-NP) for circulation and vascular injury accumulation^[24,25]. The zeta potential of A-NP was $(+32.52 \pm 2.74)$ mV, which showed great potential in complexing gene. After loading siRNA, the zeta potential decreased to $(+14.11 \pm 1.28)$ mV. TEM observation showed that the nanoparticles were spherical and possessed uniform size distribution around 40–60 nm. The nanoparticle observed by TEM was smaller than the result detected by DLS, which could be attributed to the hydration shell unobservable under TEM

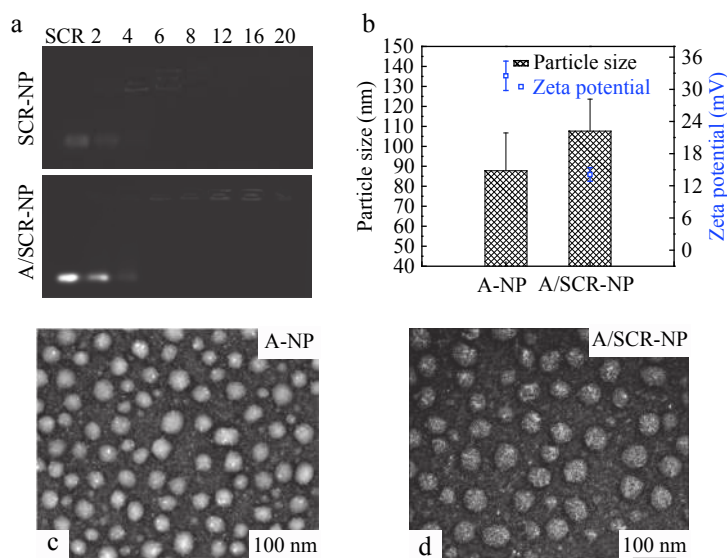


Fig. 3 (a) Electrophoretic mobility of scramble RNA in agarose gel after complexing with SCR-NP and A/SCR-NP at various N/P ratios; (b) Particle sizes and zeta potentials of A-NP and A/SCR-NP detected by DLS ($n = 3$); TEM images of (c) A-NP and (d) A/SCR-NP

observation (Figs. 3c and 3d).

The Cytotoxicity of Nanoparticles

The cytotoxicity of nanoparticles was evaluated in RAW264.7 cells by MTT assay. The results in Fig. 4(a) showed that SCR-NP exerted low toxicity when the concentration of polyplexes reached 600 $\mu\text{g/mL}$ with a cell survival rate of 86.1%. To evaluate the cytotoxicity of A-NP and A/siR-NP at the same Andro concentrations, further studies were conducted as shown in Fig. 4(b). Both A-NP and A/siR-NP exhibited low cytotoxicity compared with Andro treatment at the same concentration. The results demonstrated the low toxic side effects of A-NP and A/siR-NP to RAW264.7 cells.

Cellular Distribution of Drugs

To verify the cellular uptake and distribution of Andro and siRNA in macrophages, fluorescent coumarin instead of Andro and Cy-3 labeled scramble RNA (Cy3-siRNA) were encapsulated into the nanoparticles and investigated by confocal laser scanning microscopy. As shown in Fig. 5, the green fluorescence of coumarin and red fluorescence of Cy-3 labeled SCR were observed in the cytoplasm within 2 h, indicating a rapid cellular uptake of nanoparticles. In addition, an overlaid yellow fluorescence was obtained, indicating the co-delivery of two drugs. After 6 h, both the green and red

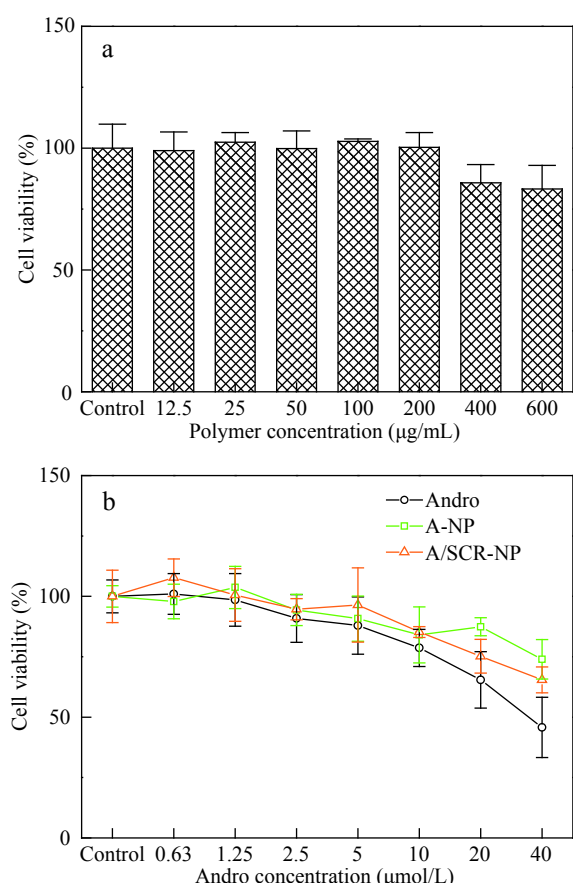


Fig. 4 (a) MTT assay detecting the cell toxicity of polyplexes SCR-NP with different polymer concentrations for 48 h treatment; (b) The cell toxicity of A-NP and A/SCR-NP at various andrographolide concentrations for 48 h treatment

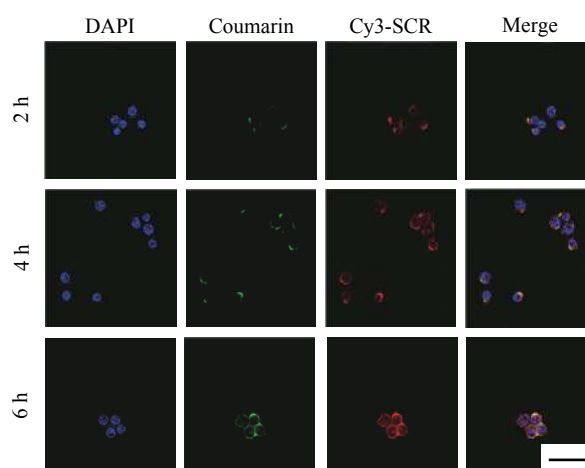


Fig. 5 Confocal laser microscopic images of RAW264.7 cell incubated with coumarin and Cy-3 labeled siRNA co-loaded nanoparticles at different time points. Green fluorescence: coumarin; red fluorescence: Cy3; blue fluorescence: nuclei stained with DAPI. Bar: 50 μm .

fluorescence became much stronger in the cells, suggesting an effective drug accumulation into the cells.

Down-regulated Expression of Pro-inflammation Cytokines

The increasing expression of pro-inflammatory cytokines such as MCP-1 and IL-6 is an important character in the progression of atherosclerosis^[26]. Andrographolide, as a traditional Chinese medicine, has been considered to be effective in blocking IL-6 and MCP-1 expression^[27], which further mitigated the progression of atherosclerosis. The data in Figs. 6(a) and 6(b) proved that IL-6 and MCP-1 mRNA levels were significantly increased by LPS compared with negative control. In contrast, both A-NP and A/siR-NP treatment decreased IL-6 and MCP-1 levels. Interestingly, A/siR-NP treatment had a significant effect on inhibiting IL-6 and MCP-1 expression in macrophages stimulated by LPS, which indicated the collaborative anti-inflammatory effect of andrographolide and Notch1 gene targeting siRNA treatment *in vitro*. NF- κB signal pathway is a classic signal pathway for inflammatory response regulation. It has been reported that andrographolide participated in inhibition of NF- κB signal pathway activation *via* blocking p65 phosphorylation^[28]. In this subject, the phosphorylation of NF- κB subunit p65 expression was measured by western blot assay to evaluate the activation of NF- κB signal pathway in macrophages. As shown in Fig. 6(c), both A-NP and A/siR-NP co-cultured with LPS-induced macrophages significantly impeded the phosphorylation of p65 with the similar effects, indicating the blocking effect on NF- κB signal pathway activation of A/siR-NP *via* andrographolide delivery in macrophages.

Up-regulated Expression of Anti-inflammatory Factors

Down-regulated expression of Notch1 has been demonstrated to inhibit the inflammatory response in macrophages, which most likely because blocked Notch1 expression increased anti-inflammatory factors such as IL-10 and arginase-1 expression^[29]. As shown in Fig. 7(a), CLSM

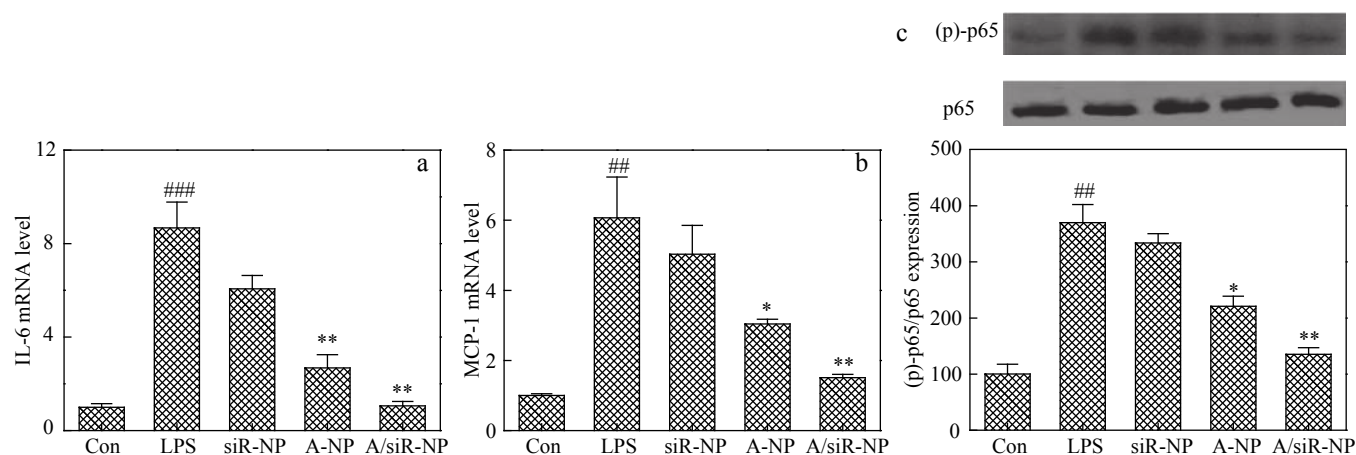


Fig. 6 The (a) IL-6 and (b) MCP-6 transcription in RAW264.7 cells after treated with nanomedicines evaluated by real-time PCR; (c) Western blot for evaluating p65 phosphorylation levels in macrophages after nanomedicines treatment (The control group was normalized as 100%. LPS was positive control, * and ** stand for $p < 0.05$ and $p < 0.01$ compared with LPS treated group. ## and ### stand for $p < 0.01$ and $p < 0.001$ compared with control group.)

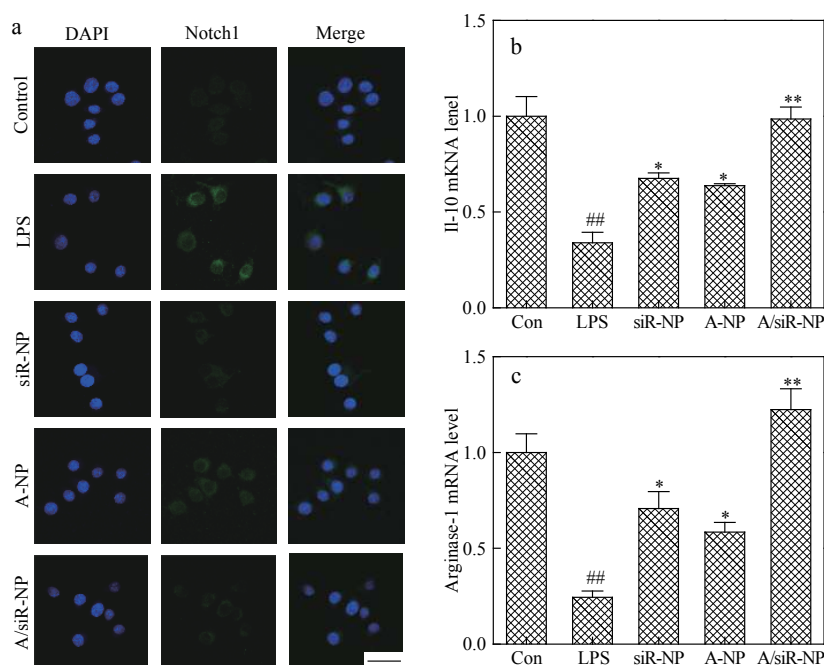


Fig. 7 (a) The expression of Notch1 in RAW264.7 cells after treated with nanomedicines (The scale bars represent 25 μ m); (b) The IL-10 transcription in RAW264.7 cells after treated with nanomedicines evaluated by real-time PCR; (c) The arginase-1 transcription in RAW264.7 cells after treated with nanomedicines evaluated by real-time PCR (Group LPS was positive control, * and ** stand for $p < 0.05$ and $p < 0.01$ compared with LPS treated group. ## stand for $p < 0.01$ compared with control group.)

was used to evaluate the effect of Notch1 gene silence by A/siR-NP. After treated with LPS, the expression of Notch1 was obviously increased in macrophages. On the contrary, the siR-NP and A/siR-NP significantly inhibited the Notch1 gene expression induced by LPS in macrophages. These results indicated that the up-regulation of Notch1 gene in macrophages of atherosclerosis was successively suppressed by A/siR-NP.

Further studies were conducted to verify the amelioration

of LPS-stimulated IL-10 and arginase-1 down-regulation by nanoparticles in macrophages. As shown in Fig. 7(b), the A/siR-NP produced prior effects to increase the expression of anti-inflammatory factors of IL-10, which was down-regulated by LPS in macrophages compared with both siR-NP and A-NP treatment. In addition, the same tendency for arginase-1 transcription was obtained after treated with nanoparticles (Fig. 7c). These results indicated that A/siR-NP exerted collaborative effect on ameliorating the

expression of anti-inflammatory factors in macrophages.

CONCLUSIONS

In this subject, multifunctional polymer PEG-PAsp(DETA)-Lys-CA₂ was synthesized to co-deliver andrographolide and Notch1 siRNA into macrophages. The *in vitro* studies demonstrated that the drug-loaded nanoparticle could successfully block the expression of pro-inflammatory cytokines such as IL-6 and MCP-1, and meanwhile ameliorate the LPS-induced decreasing of anti-inflammatory factors of IL-10 and arginase-1 in macrophages. Consequently, the nanomedicine revealed excellent therapeutic effect by enhancing anti-inflammatory action, which proved to be a potential strategy for atherosclerosis treatment.

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