Targeted delivery of microRNA 146b mimic to hepatocytes by lactosylated PDMAEMA nanoparticles for the treatment of NAFLD

Shuying Hea, Weihong Guob, Feihong Denga, Kequan Chenc, Yonghong Jiangc, Minyu Dongc, Liang Pengc and Xueqing Chenč

aGuangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou, China; bNanfang Hospital, Southern Medical University, Guangzhou, China; cDepartment of Gastroenterology, First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide, and precision therapeutic will be a benefit for the NAFLD regression. In this study, we observed low microRNA 146b (miR-146b) expression in NAFLD mice model induced by methionine–choline-deficient diet (MCD) compared with control group. Furthermore, miR-146b−/− mice induced MCD exhibited severe liver steatosis and hepatitis. A bio-distribution study showed that novel Lactosylated PDMAEMA nanoparticles effectively targeted hepatocytes Lac-PDMAEMA. We coupled miR-146b mimic with Lac-PDMAEMA and then were administrated to NAFLD mice model, which could obviously alleviate the hepatic steatosis. Lac-PDMAEMA effectively delivered miR-146b mimic to hepatocytes with a ~8-fold upregulation of miR-146b mimic targeting MyD88 and IRAK1, and in turn suppressed the expression of PPARγ. Meanwhile, TNF-α and IL-6 mRNA levels were decreased after administration of Lac-PDMAEMA/miR-146b mimic. So, we made a conclusion that targeted delivering miR-146b mimic to the hepatocytes by coupling Lac-PDMAEMA nanoparticles could effectively alleviate the hepatic steatosis in NAFLD mice, which maybe bring a new and effective way to intervene and therapy the NAFLD.

Abbreviations: NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NCD: normal chow diet; MCD: methionine–choline-deficient diet; Lac-PDMAEMA: Lactosylated poly(2-(dimethylamine)ethyl methacrylate)nanoparticles; TLR: Toll-like receptor; RISC: RNA-induced silencing complex; MyD88: myeloid differentiation factor; IRAK1: IL-1 receptor-associated kinase 1; TRAF6: tumour necrosis factor receptor-associated factor 6; ASGR: asialoglycoprotein receptor; LA: lactobionic acid; PA: Palmitic acid; ACC: Acetyl-CoA carboxylase
Introduction

Currently, non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide, with worldwide prevalence has reached 20–40% [1,2]. Approximately 10–20% of NAFLD cases progress to non-alcoholic steatohepatitis (NASH), which characterized by hepato-cellular lipid accumulation (steatosis) along with inflammation and varying degrees of fibrosis [2,3]; however a partial of patients will progress to more severe and irreversible liver damage, including cirrhosis and hepatocellular carcinoma [4,5]. Furthermore, the development of NAFLD is typically accompanied by a series of metabolic disorders [5,6]. Therefore, controlling the progression of NAFLD may help to prevent the development of severe liver diseases and metabolic disorders. Diet and exercise are regarded as an important way to intervene the development of NAFLD, but not at all can keep the compliance of dietary or and exercise treatment. So certain drugs were administrated to help and improve the NAFLD development [7]. Unfortunately, there is no well-established pharmacological approach makes it imperative to develop novel therapeutic targets and exploring corresponding strategies.

The pathogenesis of NASH is highly heterogeneous and complex. Toll-like receptor (TLR)-4 has received much attention in the pathogenesis of NASH [8–10]. MicroRNAs (miRNAs) are small ~22 non-coding RNAs that regulate gene expression at a post-transcriptional level through the RNA-induced silencing complex (RISC) [11]. Recent discoveries have revealed that miRs play a key role in the regulation of metabolic gene expression. Further studies supported their critical roles in NAFLD by their ability to modulate hepatic lipid homeostasis [12,13]. miR-146b mimic was as a negative regulator of the TLR-4 signal pathway in human monocytes, where it was found to target three key adaptor molecules downstream of TLR4: myeloid differentiation factor (MyD88), IL-1 receptor-associated kinase 1 (IRAK1) and tumour necrosis factor receptor-associated factor 6 (TRAF6) [14]. Simultaneously, miR-146b mimic was an IL-10–responsive miR and candidate miR-146b mimic was a molecular effector of the IL-10 anti-inflammatory activity [14]. Our results suggest that IL-10 dependent miR-146b mimic plays an important role in the modulation of M1 macrophage orientation [15]. Interestingly, recent studies demonstrated that miR-146b mimic played inflammation suppressor activity in NASH by targeting IRAK1 and TRAF6 respectively [16,17]. Moreover, miR-146b mimic was downregulated in monocytes in obese patients [18]. In a conditional knockout mouse model, knockout MyD88 attenuated lipid accumulation and inflammation in high-fat diet (HFD)-induced NAFLD [8]. Despite these promising results, delivery of miRNAs into cells has been the major challenge for miRNA-based therapeutics so far.

Nanocarriers were promising carriers and frequently selected for miRNA delivery due to their low toxicity, clinical potential and the ease of production [19–21]. Moreover, nanocarriers also could accurately target the special cellular type, tissue and organization in combination with special ligand or antibody. Hepatic parenchymal was cells with expression of the asialoglycoprotein receptor (ASGR), it was useful as a cellular marker for targeted delivery to hepatocytes [22,23]. Lactosylated gramicidin-based lipid nanoparticles (Lac-GLN) were used to treat hepatocellular carcinoma, with high drug loading capability were investigated and showed excellent safety and anti-tumour efficacy in mice [24]. Furthermore, basing ROS responsive nanoparticles (mPEG-b-PPS-NP) coupled with molecule melatonin (Mel) could locate at the disease site, and be more biocompatible and much more efficacious than an equivalent amount of free drug in attenuating oxidative stress in sepsis-induced acute liver injury. These data indicated that limited control of the targeting and drug release profile was in favour of their therapeutic efficacy. Lac- were synthesized by a lipophilic ASGR targeting ligand, composed of lactobionic acid (LA), which beared a galactose moiety and linked to a phospholipid and incorporated into LNs for liver-specific delivery of miRs [24,25]. Some subjects verified that micro-RNAs were involved in the occurrence and development of NAFLD to NASH. It has recently reported that administration of miR-146b mimic obviously attenuated the hepatic lipid accumulation and alleviated the steatohepatitis in NAFLD mice model by targeting IRAK1 and TRAF6 [26]. However, systematically administered miRs based therapies to objective cells have been challenging due to its toxicity. MiRs selectively to the hepatic cells in NAFLD mice model may improve therapeutic outcomes and minimize systemic toxicity [27]. In this paper, we used Lac-PDMAEMA to target miR-146b mimic delivery to hepatic cells in NAFLD mice which there was a high expression of ASGR. We evaluated the inhibitory effects of miR-146b mimic in a NAFLD mouse model. Our results showed that Lac-PDMAEMA co-loaded with miR-146b, Lac-PDMAEMA/miR-146b mimic exhibited significant repression lipid droplets in hepatocytes in vitro and abrogated the progression of NAFLD in a MCD induced-mouse model.

Materials and methods

Chemicals and reagents

2′,2′-Azobisobutyronitrile, 5-bromo-4-chloro-3-indoly1-b-0-galactopyranoside (X-Gal, Gibco, Breda, Netherlands), sodium 3′-1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro (benzene sulphonic acid (XTT, Sigma, Bornem, Belgium), N-methyl dibenzopyrazine methylsulphate (NPS, Sigma), glutaraldehyde (Fluka), polyvinylpyrrolidone (PUP, molecular weight 40 KD, sigma). All other chemicals were of analytical grade. 2-(Dimethylamino)ethyl methacrylate (DMAEMA, Fluka), N-vinyl-pyrrolidone(NVP, Acros), ethoxytriethylene glycol methacrylate (triEGMA, polysciences, Inc. Warrington) and methyl methacrylate (MMA, Acros) were purified by distillation under reduced pressure just before use. Cell culture flasks and microtiter plates were obtained from Falcon. Dulbecco’s Modified Eagles’s Medium (DMEM) and RPMI 1640 and Fetal bovine serum were from Gibco. Lactobionic acid all purchased from Invitrogen (Grand Island, NY).
Preparation of Lac-PDMAEMA/miR-146b

Lac-PDMAEMA was prepared according to the literature [24]. The Cy3-labeled miR-146b mimic (Cy3-miR-146b), and FAM-labelled miR-146b mimic (FAM-miR-146b) were purchased in GenePharma (SuZhou, China). The miR-146b mimic sequences for mice were UGAGAACUGAAUUCCAUGGC; the sequences in mice and humans were the same. For negative control Cy3: UUCUCCGACGUGUCACGUTT.

To prepare Lac-PDMAEMA/miR-146b, Lac-PDMAEMA and miR-146b mimic were mixed at w/w (weight Lac-PDMAEMA/weight miR-146b) ratio of more than 10:1 in RNase free H2O by adding a stock solution of Lac-PDMAEMA into a miR-146b mimic solution. The samples were vortexed 5–10 s and then incubated at room temperature for 30 min to ensure the formation of Lac-PDMAEMA/miR-146b mimic nanoparticles.

Animals and treatment

Adult C57BL/6 female mice (6–8 weeks old) were obtained from and housed in the Southern Medical University Animal Experiment Center (Guangzhou, China). miR-146b deficient mice were maintained in the Southern Medical University Animal Experiment Center (gifted by Huabao Xiong, associated professor in Icahn Medicine of School at Mount Sinai [15]). All experimental procedures were approved by the Animal Ethics Committee of First Affiliated Hospital of Guangzhou Medical University. NASH was induced by fed chow or methionine choline-deficient diet (MCDD; Harlan-Tecklad, Indianapolis, IN) for 2, 3 or 6 consecutive weeks, at time which liver damage is already established [28]. For 2 and 6 weeks, mice were divided into three group: normal chow diet (NCD) (n = 11); mice that continued receiving MCD for 2 weeks (n = 9); mice that continued receiving MCD for 6 weeks (n = 9). C57BL/6 (WT) and miR-146b–/– mice were fed NCD or MCD for 3 weeks. After MCD 1 week, C57BL/6 mice were divided into different groups (n = 5 per group): mice that continued receiving mice fed MCD, mice that continued with MCD diet with tail vein injection of negative control (Lac-PDMAEMA/NC) and mice fed MCD diet and tail vein injection of Lac-PDMAEMA/miR-146b mimic (once a week, total 2 weeks). Liver tissues were collected at 3 weeks after initiation of MCD. Blood was collected just prior to sacrifice. Portions of liver were either frozen immediately in liquid nitrogen, frozen fixed in OCT mounting media or fixed in neutral-buffered formalin.

Cell culture, in vitro transfection studies and co-culture with fatty acids

HCC cell line HepG2 and normal mouse hepatocyte line AML12 were purchased from the Type Culture Collection of the Chinese Academy of Sciences in Shanghai, China, they were supplemented with 10% fetal bovine serum. At 50% of confluence, cells were grown arrested in serum-free medium for 12 h before the experiments.

For miR-146b mimic transfection, HepG2 and AML12 cells were plated at 2 x 10⁵ cells per well in six-well plates. Overnight cells were transfected with 100 nM miR-146b mimic using Lipofectamine 3000 or Lac-PDMAEMA and after 4 h incubation, the medium was replaced with fresh medium. Cells were then treated with Palmitic acid (PA, Sigma) (250 μM) for another 48 h, after which lipid accumulation and adiposeness related protein expression were determined by Oil-Red Staining (Sigma) and Western blotting, respectively. PA was dissolved in DMEM with 2% fatty-acid-free bovine serum albumin (BSA, Sigma) after the solvent was evaporated, then followed by two rounds of overtaxing and 30 s of sonication [29]. As a positive control, cells transfected with miR-146b mimic using Lipofectamine 3000 were performed following manufacturer’s protocol.

Characterization of Lac-PDMAEMA/miR-146b mimic

The particle size of miR-146b mimic containing Lac-PDMAEMA was determined by dynamic light scattering on ZetaPALS (Broken Haven company, Upton, NY). Particles were dispersed in cell culture medium. The morphology of Lac-PDMAEMA was examined by a Bruker AVANCE 400 MHz NMR spectrometer (Brucker Co., Switzerland). Briefly, samples were prepared as described above. A drop of the sample was negatively stained with uranyl acetate for 1 min on a perforated carbon grid for analysis. The zeta potential of miR-146b mimic containing Lac-PDMAEMA was examined in 20 mM HEPES buffer using ZetaPALS potential analyzer (Brookhaven Instruments Corp., Holtsville, NY).

Encapsulation efficiency of Lac-PDMAEMA was determined by Quant-iTTM RiboGreen RNA Kit (Invitrogen, Grand Island, NY) following the manufacturer’s protocol, and the fluorescent intensity (FI) was determined using a luminescence spectrometer (KS 54B, Perkin Elmer, UK) at an excitation of 480 nm and an emission of 520 nm. The encapsulation efficiency was calculated by the following equation.

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\text{Encapsulation efficiency} \% = \left(1 - \frac{F_{\text{Fl without Triton X-100}}}{F_{\text{Fl with Triton X-100}}} \right) \times 100
\]

Agarose gel electrophoresis

Agarose gel electrophoresis was used to evaluate miRNA loading in Lac-PDMAEMA. The miRNA or Lac-PDMAEMA/miR-146b mimic (the dose of miRNA was 100 pmol) was separated on 2% agarose gels containing ethidium bromide, and images were obtained using a UV transilluminator and a digital imaging system (Life Science Technologies, St. Petersburg, FL). Free miRNA and Lac-PDMAEMA/miR-146b mimic will migrate in the gels, but Lac-PDMAEMA/miR-146b mimic will travel to the opposite direction.

Cytotoxicity study

The cytotoxic effects of Lac-PDMAEMA/miR-146b mimic on HepG2 and AML12 cells were measured using Cell Counting
Kit-8 kit (CCK-8 kit, Dojindo Laboratories, Japan). The cells were seeded onto 96-well plates at a density of $1 \times 10^5$ cells/well. After overnight incubation cells were treated with free miR-146b, lipo3000/miR-146b mimic or Lac-PDMAEMA/miR-146b mimic for an additional 4 h, the portions of viable cells were measured using CCK-8 kits according to the user’s manual. Cell viability within each group was expressed as a percentage of the viability of untreated control cells.

**Uptake of Lac-PDMAEMA/miR-146b mimic**

Analysis of the cellular uptake of Lac-PDMAEMA/miR-146b mimic was performed by delivery of fluorescent FAM-miR-146b mimic or Cy3-miR-146b mimic into HepG2 and AML12 cells, evaluated by fluorescence inverted microscope and by flow cytometry. For fluorescence inverted microscope, $1 \times 10^5$ HepG2 and AML12 cells per well were seeded in six-well plates and allowed to grow overnight. Cells were then treated with 100 nM Lac-PDMAEMA-Cy3-miR-146b mimic or Lipofectamine/miR-146b mimic for 4 h at 37°C. Cells were then replaced with fresh medium and were viewed after 24 h using an Olympus BX-53 fluorescence inverted microscope (Olympus, Tokyo, Japan).

For the flow cytometric analysis, $1 \times 10^5$ HepG2 and AML12 cells were treated with 100 nM Lac-PDMAEMA-Cy3-miR-146b mimic or Lipofectamine/miR-146b mimic for 4 h at 37°C. Cells were suspended using 0.25% trypsin, washed with PBS twice times. The fluorescent intensity was measured on a FACS (BD Airl III Flow Cytometer, San Jose, CA).

**In vivo biodistribution studies by IVIS imaging**

For in vivo imaging, the C57/6 mice were injected via tail vein a single dose of PBS or Lac-PDMAEMA/miR-146b mimic (1 mg/kg miRNA). Fluorescent Cy3-miR-146b mimic containing Lac-PDMAEMA was used for measuring in vivo uptake in different tissues by IVIS imaging. Whole tissues were harvested and then whole tissue Cy3 fluorescence signals were measured using Xenogeny IVIS-200 Optical in Vivo Imaging system (Caliper Life Sciences, Hopkinton, MA). Liver was harvested and the Lac-PDMAEMA/miR-146b mimic was examined by a transmission electron microscope (FEI Company, Hillsboro, OR).

**Serum biochemistry**

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using standard enzymatic procedures according to the manufacturers’ instruction (Thermos Fisher, Pittsburgh, PA).

**Oil red O staining**

Liver tissues were embedded in OCT compound and then frozen in −20°C. Frozen sections (10 μm) were stained with Oil Red O (O0625, Sigma, St. Louis, MO) and counterstained with Mayer’s haematoxylin to visualize intracellular lipid droplets.

All digital images were obtained with an Olympus IX73 light microscope (Olympus, Tokyo, Japan).

**Immunohistochemistry**

De-paraffinized liver sections were immunostained for PPARγ, ACC (Cell Signaling Technology, Danvers, MA), MyD88 (Proteintech, Rosemont, IL) and IRAK-1 (Proteintech). The immunostaining was examined with an Olympus IX73 microscope (Olympus, Tokyo, Japan). Positive staining for MyD88, ACC and IRAK-1 were semi-quantified using 100× magnification fields as a percentage of the total surface area and graded into five categories: grade 1 (<10%), grade 2 (10–29%), grade 3 (30–49%), grade 4 (50–79%) and grade 5 (80%). For the PPARγ stain, the number of positive cells was counted in five 400× objective high-power fields (HPFs; 400× magnification) to determine the average number of positively stained cells.

**Sirius-red stain**

Formalin-fixed paraffin-embedded sections of liver samples were either stained with haematoxylin and eosin, PicroSirius red. Sirius-red stains were performed on formalin-fixed paraffin-embedded sections as previously described [30]. Computer-aided quantification of fibrotic areas was performed on Sirius red stained liver sections from different groups (n=5) indicated in respective figure legends. Eight microscopic fields from each animal were randomly chosen and photographed under 100× magnification on an Olympus IX73 light microscope (Olympus). Fibrotic areas were calculated as per cent of the whole image area after subtraction of vessel lumen area.

**RNA isolation and quantitative real-time RT-PCR (qPCR)**

Total RNA was isolated by using Trizol reagent (Takara, Japan) and then reversely transcribed into cDNA through PrimeScript™ RT reagent Kit or an oligo (dT) primer (QIAGEN, Valencia, CA). Real-time PCR analyses were performed with SYBR Green mix (Takara). Primer sequences: mouse miR-146b, F:5'-TCAGAGCACTGAATTCCATAAGGC-3’, R:5'-GCAGGGTCCGAGGTATTC-3’. mouse U6, F: 5'-GCTTCGGCAGCGACATATCATAAAT-3’, R: 5'-GGATGGTGCTTCAGTC-3’. mouse IL-6, F:5'-CCCAATTCTCAATCTTCC-3’, R:5'-GGATGTTCTTGTSCTTTCAGC-3’. mouse TNF-a, F:5'-GCCACGCGCTCTTATGTC-3’, R: 5'-GGCTGGGGGCATAGAAGATG-3’. mouse β-actin, F:5'-GGCCAGGCAGCTCTTATGTC-3’, R: 5'-CACAGGTTTCATACCCAAGAG-3’.
anti-MyD88 and anti-IRAK-1 were obtained from Proteintech. GAPDH and Secondary HRP-conjugated antibodies were come from Zhonshanjinqiao (Wuhan, China). The image acquisition of blots was performed using a Gene5 image acquisition system (Syngene, Frederick, MD).

**Statistical analysis**

Results are reported as mean ± standard deviation (SD). SPSS 21.0 software (SPSS Inc., Chicago, IL) was used for analysis. Differences groups were compared using analysis of variance (Student's t-test OR one way ANOVA post hocs) when applicable or the non-parametric test. *p < .05, **p < .01, ***p < .001 was considered statistically significant.

**Results**

**Expression of miR-146b mimic was decreased in MCD induced-NAFLD mice model**

MCD diets will quickly induce measurable hepatic steatosis by 2–4 weeks and this progress to inflammation and fibrosis shortly thereafter [31,32]. Recently have reported that miR-146b mimic ameliorated HFD-induced NASH [26]. However, the expression level of miR-146b mimic in NAFLD was still unclear.

To identify expression of miR-146b mimic in livers, we performed qPCR of hepatic RNA in mice fed an MCD diet for different periods. As shown in Figure 1(A), the MCD induced murine NAFLD model exhibited more severe inflammation, steatosis and fibrosis at week 6 than week 2, which were assessed by H&E, oil-red O and Sirius-red staining respectively. Scale bar, 100 μm. (B) Histological score in liver tissue from the three groups as described above. (C–D) Serum levels of ALT and AST by ELISA. (E) The expression of Tnfα, Il6 and Il1β in liver tissue either from WT mice that were fed a NCD or MCD by qPCR. Gene expression was normalized to β-actin. (F) qPCR analysis for miR-146b expression in liver tissues from the three groups as described above. Gene expression was normalized to the U6. Data represent as means ± SD; *p < .05, **p < .01, ***p < .001 versus NCD (2 weeks); #p < .05, ##p < .01, ###p < .001 versus MCD (6 weeks).

**MiR-146b deficiency enhanced steatosis, steatohepatitis and fibrosis in vivo**

To investigate the effect of miR-146b on steatosis and steatohepatitis, we generated miR-146b-knockout mice by CRISP/CAS9 and fed them either NCD or an MCD for 3 weeks. First, we developed miR-146b−/− mice [15]. miR-146b−/- mice showed that inflammation progressively advanced in intensity and distribution and included portal areas compared with WT mice induced by MCD diet (Figure 2(A)). In terms of hepatic steatosis, the deletion of miR-146b significantly increased the lipid accumulation in liver (Figure 2(A)). The fibrotic index was significantly increased in miR-146b−/- mice fed with MCD (Figure 2(A)). The histological score also demonstrated that miR-146b deficiency was effective in promoting inflammation and fibrosis (Figure 2B), which were associated with a significant increase in serum level of ALT and AST in the MCD-treated miR-146b−/- mice compared to MCD-treated WT mice (Figure 2(C,D)). These observations also were
correlated with the high expression of mRNA of TNF-α, IL-6 and IL-1β in miR-146b−/− mice as determined by qPCR (Figure 2(E)). These results suggested that miR-146b attended in the development of NALFD to NASH.

Preparation and characterization of Lac-PDMAEMA/miR-146b mimic nanoparticles

Lac-PDMAEMA and Lac-PDMAEMA/miR-146b mimic were synthesized as previously described and were characterized by TEM, AFM and DLS [24]. The average particle sizes of Lac-PDMAEMA and Lac-PDMAEMA/miR-146b mimic were about 150–350 nm respectively as measured by DLS. The PDI of Lac-PDMAEMA/miR-146b mimic was 1.21, and GPC curves exhibited the single-peak curve, which showed the characteristics of the complex narrow distributions (Figure 1(A–C)). The zeta potentials of Lac-PDMAEMA/miR-146b mimic was 10.3 mV, The reduced zeta potential of Lac-PDMAEMA/miR-146b mimic was due to the incorporation of the miRNA with a negative potential. TEM images showed that the Lac-PDMAEMA/miR-146b mimic nanoparticles were dispersed in the solution and particle shape was uniform. DLS determination also indicated that the particle size had a normal distribution (Figure 1(D,E)). The average particle size of the Lac-PDMAEMA/miR-146b mimic determined by TEM was about 168.9 nm. A similar result was obtained by AFM (Figure 1(F)). Furthermore, we also investigate the cellular uptake and cytotoxicity of Lac-PDMAEMA/miR-146b mimic nanoparticle. Because HCC HepG2 cells, which has high ASGR expression, HepG2 and AML12 cells were used as in vitro model [24]. No significant change in cell viability was observed between treated cells (the ratio of Lac-PDMAEMA/miRNA was 5:1 or 10:1) and untreated cells, but the cell viability decreased when the ratio increased (Figure 3(G)). The weight ratio for the best loading efficiency of miRNA was carefully optimized using the agarose gel electrophoresis analysis. The miRNA was fully entrapped in the loading wells with Lac-PDMAEMA when the ratio of Lac-PDMAEMA and miRNA was 10:1 (w/w).
Suggesting maximum loading of the miRNA into the Lac-PDMAEMA at this ratio. To determine whether Lac-PDMAEMA-FAM-miR-146b mimic nanoparticles can be taken up by HepG2 and AML12 cells, we treated HepG2 and AML12 cells with Lac-PDMAEMA-FAM-miR-146b mimic at a miR-146b mimic concentration of 100 nM. As shown in Figure 3(E) that Lac-PDMAEMA-FAM-miR-146b mimic was taken up by the HepG2 and AML12 cells. These data suggest that Lac-PDMAEMA/miR-146b mimic can enhance efficiently deliver miR-146b mimic to the cells. These results suggested a low cytotoxicity and high efficiently deliver of Lac-PDMAEMA (10:1) in HepG2 and AML12 cells.

**Lac-PDMAEMA/miR-146b mimic inhibited the lipid accumulation and adipogenesis**

To further define the role of miR-146b mimic on lipid accumulation, the well-established in vitro model of lipid accumulation (PA administration model) was applied. HepG2 and AML12 cells respectively were treated with Lac-PDMAEMA/NC or Lac-PDMAEMA/miR-146b mimic and then incubation with PA for 48 h. The Oil-Red results showed that Lac-PDMAEMA/miR-146b mimic significantly inhibited the lipid accumulation in HepG2 and AML12 cells compared with untreated control cells (Figure 4(A)). The mass of adipose tissue could be reduced by increasing lipolysis, or/and inhibiting adipogenesis. Further, we examined whether adipogenic markers were affected by Lac-PDMAEMA/miR-146b mimic in hepatocytes. It has been reported that the PPARγ is essential for the formation lipid droplets [34], we observed a significant reduction in PPARγ expression in the Lac-PDMAEMA/miR-146b mimic treatment group (Figure 4(B)). These results implied that Lac-PDMAEMA/miR-146b mimic suppressed the formation of lipid droplets.

**Downregulation of MyD88 and IRAK1 by Lac-PDMAEMA/miR-146b mimic in vitro**

The miR-146b mimic was as a negative regulator of the TLR4-signalling pathway [14,17,26]. We wondered whether the Lac-Lac-PDMAEMA/miR-146b mimic affected the hepatic steatosis and steatohepatitis by targeting MyD88 and IRAK1

**Figure 3.** Synthesis of Lac-PDMAEMA coupling miR-146b mimic. (A) GPC curve; (B) Process of synthesis of Lac-PDMAEMA; (C) 1H NMR spectra of (a) Lac, (b) PDMAEMA and (c) Lac-PDMAEMA; (D,E) Determination of diameter distribution of Lac-PDMAEMA/miR-146b mimic nanoparticles using dynamic light scattering (DLS). (F) TEM images of the Lac-PDMAEMA/miRNA complexes: a (N/P = 5), b (N/P = 10), c (N/P = 20), d (N/P = 30). (G) The evaluation of cytotoxicity for Lac-PDMAEMA/miR-146b mimic complex in different ratio: (H) Uptake effect of Lac-PDMAEMA/miR-146b mimic complex in HepG2 and AML12 cell lines. Cells were then treated with 100 nM Lac-PDMAEMA/FAM-miR-146b mimic and then were viewed after 24 h using a fluorescence inverted microscope.
expression in hepatic parenchymal cells after PA administration. As shown in Figure 4(C), PA significantly increased the expression of MyD88 and IRAK1 protein in a time-dependent manner. MyD88 and IRAK1 were significantly decreased in LAC-PDMAEMA/miR-146b mimic group compared with LAC-PDMAEMA/NC group (Figure 4(D)). These results implied that miR-146b mimic was able to decrease the MyD88 and IRAK1 expression in PA-administered hepatic parenchymal cells.

Lac-PDMAEMA/miR-146b could directly target the hepatocytes and attenuated the hepatic steatosis in vivo

In order to assess the in vivo delivery efficiency and tissue specificity of Lac-PDMAEMA, Cy3-miR-146b mimic coupling with Lac-PDMAEMA particles was injected in C57BL/6 tail at a dose of 1.5 mg/kg. As shown in Figure 5(A), maximal fluorescence signals accumulated in the liver when mice were treated with Lac-PDMAEMA/146b with no detectable signal in lung, spleen and kidney. These results suggested that the delivery of Cy3-miR-146b mimic by Lac-PDMAEMA was specific to liver. Furthermore, TEM was performed to investigate the nanoparticle location of liver. TEM images showed a spherical shape and a relatively uniform size distribution of Lac-PDMAEMA, which located the hepatocytes (Figure 5(B)).

Next, Lac-PDMAEMA/miR-146b mimic function was evaluated in miR-146b−/− NAFLD mouse model. Lac-PDMAEMA/NC or Lac-PDMAEMA/miR-146b mimic was administered via tail vein at doses 170 μg/kg miR-146b mimic (once a week, total 2 weeks) in 0.1 ml saline under anaesthesia at 1 week after the first MCD feed. Mice were sacrificed until 3 weeks. MiR-146b level increased in Lac-PDMAEMA/miR-146b mimic group by qPCR (Figure 5(C)), these data confirmed that miR-146b could be accurately delivered to hepatocytes by Lac-PDMAEMA. Next, we analyse the change of pathological of liver. As shown in Figure 5(D), H&E staining and Oil-Red staining showed inflammation and lipid droplets formation was significantly decreased, respectively. Sirius Red staining showed significantly decreased fibrosis in livers over-expression Lac-PDMAEMA/miR-146b mimic compared to the control and Lac-PDMAEMA/NC consistently. The histological score also demonstrated that Lac-PDMAEMA/miR-146b mimic given was effective in promoting inflammation and steatosis (Figure 5(E)). Consistently with these results, the mRNA levels of TNF-α, IL-6 and IL-1β were significantly decreased in MCD mice treated with Lac-PDMAEMA/miR-146b (Figure 5(F)). Serum ALT and AST levels were also significantly decreased (Figure 5(G,H)).

In vivo, we investigated the effects of Lac-PDMAEMA/miR-146b mimic on the MyD88, IRAK1 and adipogenic markers, such as PPARγ, ACC (Acetyl-CoA Carboxylase) and C/EBPα, etc.
IHC showed less hepatic expression of PPARγ, MyD88 and IRAK1 were found in the MCD-treated Lac-PDMAEMA/miR-146b mimic mice than in the control and Lac-PDMAEMA/NC mice, and ACC significantly increased (Figure 6(A)). Consistently, Western blot analysis revealed that PPARγ, C/EBPα, MyD88 and IRAK1 were significantly decreased in liver and ACC was significantly increased after administration of miR-146b mimic (Figure 6(B)). So, these data stated that Lac-PDMAEMA could directly target the hepatocyte, and effectively delivered the miR-146b mimic, which obviously suppressed the hepatic steatosis in vivo.

**Discussion**

It is widely considered that rising saturated liver fat content is the first “hit” of the pathogenesis of NAFLD, followed by multiple additional factors that trigger the inflammatory activity [35,36]. However, no specific anti-steatosis medical therapy exists. In the present study, we revisited the NAFLD mice induced by MCD with decreased miR-146b. MiR-146b deficient mice exhibit susceptible steatosis and inflammation. Furthermore, a novel hepatocyte-targeted Lac-PDMAEMA exhibited more delivery efficacy and target to hepatocytes in vitro and in vivo. Finally, treatment with Lac-PDMAEMA/miR-146b mimic significantly suppressed lipid droplets formation in vivo and ameliorated NAFLD development. Collectively, the results suggest miR-146b expressed in liver may play an important role in the process of lipid droplets formation, and Lac-PDMAEMA/miR-146b mimic maybe as the potential therapeutic approaches for NAFLD and possibly for liver fibrosis.

As previously reported, aberrant miRs expression was observed in NAFLD and NASH-induced hepato-carcinogenesis [13,22,37,38]. MiRs were able to modify gene expression at the post-transcriptional level by binding to the 3’UTRs of gene transcripts and thereby repressing their translation or inducing their degradation [39]. They often regulated multiple target genes within the same biological pathway. Largely of studies further supported their critical role in metabolism, differentiation, cell growth, stress response,
tissue remodelling and safeguards the stability of biological systems [12,39]. Post-transcriptional gene silencing by miR-146b mimic was known to play important role in regulating inflammatory response and tumour development [40–42]. The expression of miR-146b mimic was greatly increased in human monocytes by LPS, TNF-α and IL-1β [16]. Meanwhile, miR-146b mimic function as negative regulators of the inflammatory process due to their ability to target IRAK1 and TRAF6, known modulators of the TLR4 pathway, for translational repression and thereby inhibiting pro-inflammatory cytokine signalling [16,43]. In addition, some study provided evidence that miR-146b induction depended on the activity of IL-10 produced after LPS [14]. We had reported that IL-10 induces the expression of miR-146b in the modulation of M1 macrophage differentiation, and we also generated the miR-146b−/− mice by CRISP/CAS9, which spontaneously developed the colitis the same as IL-10−/− mice [15,44]. So miR-146b mimic was thought to play a critical role in the process of inflammatory disease. NASH was characterized by steatosis with inflammation. The previous study showed that upregulation of miR-146b mimic could significantly inhibit the lipid accumulation and inflammation both in vitro and in vivo [26,45]. However, despite its important roles in anti-steatosis and anti-inflammation, the potential therapeutic of miR-146b mimic has not been adequately evaluated in a translational research setting. In our study, we firstly demonstrated that miR-146b mimic was frequently downregulated in NAFLD, and functions as a lipid accumulation and inflammation suppressor in NAFLD.

Some miRNA-targeted therapeutics has been considered applied in clinical, but the major challenge is identifying the most efficacious delivery candidates. Traditional methods for miRs delivery included electroporation, a lipid-based delivery agent such as Lipofectamine 3000 and virus transfection. However, these methods often exhibited limited delivery efficacy in application due to various barriers such as lysosomal degradation and off-target uptake in vivo. It has already been demonstrated that free miR-146b mimic exhibit anti-steatosis and anti-inflammation activity [26]. However, despite the encouraging anti-inflammation efficacy of the mono-delivering miR-146b, limited delivery efficacy and degradation eventually contributes to treatment failure. The thrilling news was that hepatocyte-targeted LN with the capability of overcoming in vivo delivery barriers was designed and synthesized [25]. Furthermore, Lac-PDMAEMA was synthesized that favoured localization to the liver and diminished the off-target uptake from other tissues to a great extent [24]. In this study, Lac-PDMAEMA/miR-146b mimic was successfully synthesized using the method originally developed by Zhang et al. [24]. As shown in this study, Lac-PDMAEMA/miR-146b mimic system was able to efficiently deliver to hepatocytes.

We uncovered that miR-146b acted as a lipid accumulation and inflammation suppressor in NAFLD model with a series of in vitro and in vivo assays. Although miR-146b attenuates high-fat diet-induced non-alcoholic steatohepatitis (NASH), but miR-146b couldn’t discriminate the cell type [26], and the lipid accumulation maybe be triggered by the certain cytokines releasing by Kupffer cells in the treatment of miR-146b. It was unclear whether miR-146b could directly regulate the hepatocyte metabolism. So, Lac-PDMAEMA particles were employed to delivery miR-146b mimic to the hepatocyte. Our data confirmed that miR-146b was downregulated in NAFLD and significantly associated with lipid accumulation degree. Furthermore, we also have shown that miR-146b−/− deficient

![Figure 6.](image-url) Downregulation of IRAK1, MyD88 and PPARγ, MyD88 and IRAK1, as well increased ACC by miR-146b in mice fed a MCD diet. (A) Immunohistochemically staining for IRAK1, MyD88, PPARγ and ACC in mice. Scale bar represents 50 μ. (B) Representative western blots showed the protein levels of C-EBP, ACC, PPARγ, MyD88 and IRAK1 in mice. These results were normalized with GAPDH.
mice were easier to develop NAFLD. And then, Lac-PDMAEMA particles coupled miR-146b mimic could obviously inhibit the hepatic steatosis with only aiming at the hepatocyte. After validating miR-146b mimic acted as a NAFLD-associated miRNA in vitro and in vivo, the previous study also revealed that TLR4 related pathway plays a key role in the anti-inflammation process of miR-146b [14]. Other research reported that mTOR signalling pathway played a vital function and inactivating the mTOR signalling could change the metabolic process, which was involved in the development of NAFLD and NASH, and inhibit the expression of IL-6, IL-1β, and IL-10 expression [46,47]. We also confirm that Lac-PDMAEMA–miR-146b mimic particle could attenuate the proinflammation cytokines including TNFα, IL-6 and IL-1β, which may be mediate by myD88 and IRAK-1. Thus, by facilitating the degradation of TLR4 related markers in liver, miR-146b mimic attenuated both NAFLD pathologies and its complications.

To the best of our knowledge, this was the first report on the therapeutic value of miR-146b mimic in a NAFLD model when it was delivered with targeted nanoparticles (Graphical Abstract). We have provided evidence that nanoparticles delivering miRs modulating specific molecular targets in hepatocytes represent a potential therapeutic agent for NAFLD.

Disclosure statement

The authors declare that they have no competing interests.

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