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Efficient systemic delivery of siRNA to the mouse liver by pegylated lipopolymer

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ABSTRACT

Short interfering RNA (siRNA) drugs have entered clinical trials in various disease areas. However, systemic use of siRNA drugs faces a challenge of tissue in-specificity and membrane impenetrability. In this study, we hypothesized that the combined of lipidic molecules with a pegylated cationic polymer through random polymerization of Micheal reaction could enhance the hepatocyte's preferential uptake and improve membrane penetrability. We reported the efficacy of *in vitro* knockdown of apoB mRNA in HepG2 cell line and *in vivo* knockdown of the liver apoB mRNA using a pegylated lipopolymer–siapoB complex. Results show that apoB mRNA in the *nu*/*nu* and C57BL/6 black mice was knockdown to ~60–80%, up to 2 weeks, at low doses of 1.0–2.5 mg/kg of siRNA. The finding sets a new stage for further developments for apoB siRNA therapeutics.

1. Introduction

RNA interference (RNAi) is a specific, natural process of inhibiting endogenous gene expression that was first introduced in 1998 by Andrew Fire and Craig Mello in the nematode worm *Caenorhabditis elegans* (Fire et al., 1998). The discovery was later exploited for developing a new class of pharmaceutical drugs (Bumcrot et al., 2006). In 2006, Andrew Fire and Craig Mello both shared the Nobel Prize in physiology or medicine for that discovery (Daneholt, 2006). Short interfering RNA (siRNA) is one of the two vital molecules in RNAi process that binds to a specific messenger RNA (mRNA) and prevents the mRNA from producing a protein. The other vital molecule in RNAi process is micro-RNA. Preclinical studies of siRNA delivery have shown some success by inhalation (Aronin, 2006). A number of polycation-based nanoparticles complexed with siRNA have demonstrated the feasibility and capability of gene silencing in tumour, inflammatory, and other disease animal models (Howard, 2009). However, due to instability of siRNA molecules and their membrane impenetrability, it is a great challenge to successfully develop an efficient systemic carrier for a systemic application (Bumcrot et al., 2006; Vaishnaw et al., 2010).

In a previous study, we reported synthesis of a library of the water soluble pegylated lipopolymers that were the products of random cross-linking polymerization of low molecular weight branched poly(ethylenimine) (PEI, average molecular weight of 1200 Da), diacrylate lipid (lipid), and mono-acrylate poly(ethylene glycol) (PEG) (Liu et al., 2009). A library of 18 lipopolymers was generated with various ratios of lipid:PEI. From that library, lipopolymers with various lipid:PEI ratios from 11:1 to 16:1, formed a complex with anti-luciferase siRNA, and the complexes silenced *in vitro* luciferase gene expression. In addition, a number of the lipopolymer–siRNA complexes were able to knockdown siMDR1 mRNA and improved the chemosensitivity of human colon cancer stem cells to paclitaxel synergistically (Liu et al., 2009).

Two groups reported success of silencing of endogenous gene encoding apoB in mice using lipophilic compounds attached to siRNA (siRNA conjugated) to target hepatocytes (Soutschek et al., 2004; Nishina et al., 2008). Their approach to siRNA hepatocyte-targeted was focused on utilization of the hepatocyte's preferential uptake of lipophilic compounds. Since their siRNAs were covalently attached to lipophilic compounds, their siRNAs selectively ended up in hepatocytes. However, in order to achieve 60% knockdown of the apoB mRNA in mice, Soutschek et al. (2004) had to use a high dose of siRNA (50 mg/kg). In the present study, we hypothesized that lipopolymers could be utilized for siRNA hepatocytes-targeted delivery since the lipopolymers featured lipophilic properties and were able to efficiently deliver siRNA into a cell (Liu et al., 2009). We reported a pegylated lipopolymer–siRNA complex that efficiently targeted and silenced hepatocyte-apoB mRNA in mice at doses of 20–50 times less compared to that of Soutschek et al. (2004). Our results show that apoB mRNA was silenced in a range of ~60–80%, up to 2 weeks in the *nu*/*nu* and C57BL/6 mice, using a pegylated lipopolymer–siRNA complex at low doses of 1.0–2.5 mg/kg

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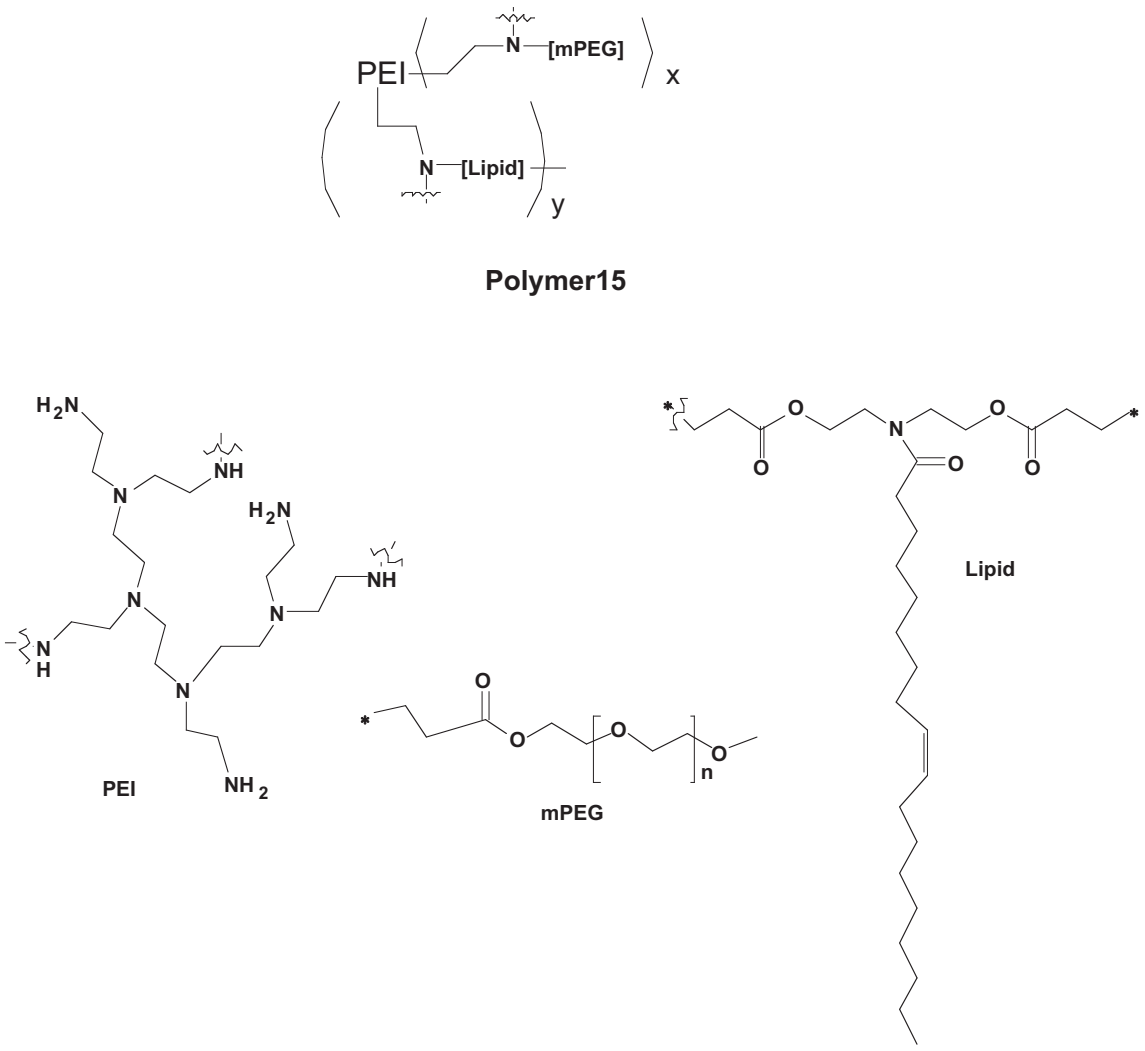

Fig. 1. Structure of polymer 15.

of siRNA. This finding shows that our pegylated lipopolymer–siRNA complex can deliver a substantial knockdown *in vivo* on apoB mRNA in mice at low siRNA doses.

2. Results and discussion

2.1. Pegylated lipopolymer

In this study, we utilized a mechanism of hepatocyte-uptake of lipidic molecules for their target-specific. We envisioned that lipopolymers had high lipid density and should be explored for hepatocyte-targeted siRNA delivery. Previously, we reported a synthesis of series of lipopolymers (Liu et al., 2009). In the present study, polymer 15 was selected from the series of the lipopolymers to explore *in vivo* hepatocyte-targeted siRNA delivery because of its high lipid content and might be preferentially uptaken by hepatocytes. Its average molecular weight was about 42,000 Da, determined by gel permeation chromatography (GPC) with Wyatt multi-angle light scattering detection. The proposed structure of polymer 15 is shown in Fig. 1, consisting of product of random polymerization of the lipid:PEI:PEG ratios of 16:1:2, respectively (Liu et al., 2009). Its polymerization chemistry was based on a random Michael addition reaction of amino groups of PEI 1200 Da, reacting with acrylate groups of the lipid moiety and PEG component. The Michael addition chemistry has been widely used for making amino-lipidic molecules (Akinc et al., 2008) and poly(beta-amino ester) polymers (Vandenbroucke et al., 2008; Jere et al., 2008) for siRNA delivery. Hybridizing a lipidic moiety with a cationic polymer and pegylated moiety using the versatile Michael addition chemistry can create a novel pegylated lipopolymer for siRNA delivery and hepatocyte-targeted.

2.2. Activity of in vitro siRNA knockdown in tissue culture

The goal was to assess siRNA delivery efficiency of polymer 15; we chose a known anti-luciferase siRNA sequence (Liu et al., 2009) (siLuc) with a negative control anti-enhanced green fluorescence protein siRNA sequence (siegfp) and a known anti-apoB siRNA sequence (Soutschek et al., 2004) (siapoB) to evaluate reporter gene expression and endogeneous gene expression, respectively. The siapoB sequence was used with a modification of removing a cholesterol moiety from the siRNA–cholesterol conjugate (Soutschek et al., 2004). All the siRNA sequences were obtained from a commercial vendor.

We explored *in vitro* silencing activity of gene reporter luciferase in PC-3M-Luc-C6 cell line and of endogeneous apoB mRNA in HepG2 cell line using pegylated lipopolymer–siRNA complex. Fig. 2 shows results of luciferase reporter gene expression knock-down using Lipofectamine 2000 (L2K)/siLuc complexes instructed by

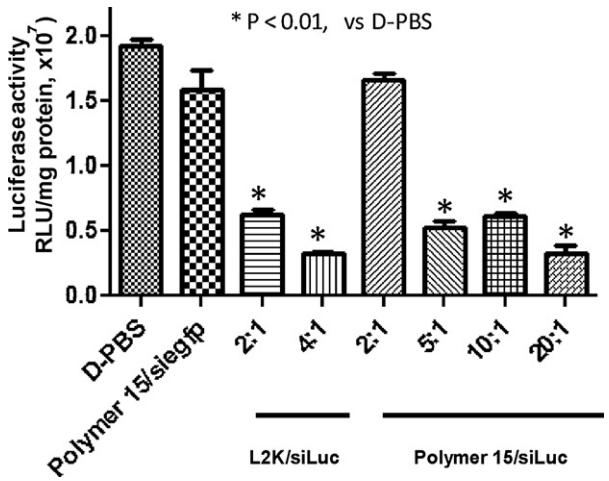

Fig. 2. *In vitro* siRNA transfection of pegylated lipopolymer–siRNA complexes in PC-3M-Luc-C6 cell line. The ratios of polymer 15/siLuciferase (siLuc) were expressed in weight to weight ratios. Lipofectamine 2000 (L2K)/siLuc complexes were used as a positive control. Their ratios were expressed in volume to volume. Polymer 15/siegfp (10:1, weight to weight) was used a negative control. Data are shown as mean ± SD.

its manufacturer as positive controls, polymer 15/siegfp complex as a negative control, and polymer 15/siLuc complexes at a concentration of siRNA of 17.8 nM (0.25 μg/mL). The positive control (L2K/siLuc) complexes clearly demonstrated knock-down of luciferase reporter gene expression with statistical significance ($p < 0.01$, L2K/siLuc vs PBS). As expected, a negative control (polymer 15/siegfp) complex (10:1) did not show any significant knock-down of luciferase gene expression. Polymer 15/siLuc complex at the weight to weight (wt./wt.) ratio of 2:1, did not demonstrate any significant gene silencing of Luciferase reporter gene expression. However, with the ratios of 5:1, 10:1, and 20:1, polymer 15/siLuc complexes clearly and significantly inhibited luciferase mRNA ($p < 0.01$, polymer:siRNA vs PBS).

To assess silencing activity of endogeneous apoB mRNA, complexes of polymer 15/siapoB were used for transfection of HepG2 cell line. Fig. 3 shows RT-qPCR results of apoB mRNA knockdown in the HepG2 cell line using the polymer 15/siapoB complexes, with polymer 15 alone and siapoB alone as negative controls. The conditions for siRNA silencing assessment were optimized from previous report (Soutschek et al., 2004). The cells were transfected with the ratio of 2:1 of polymer 15 to the siapoB (weight to weight),

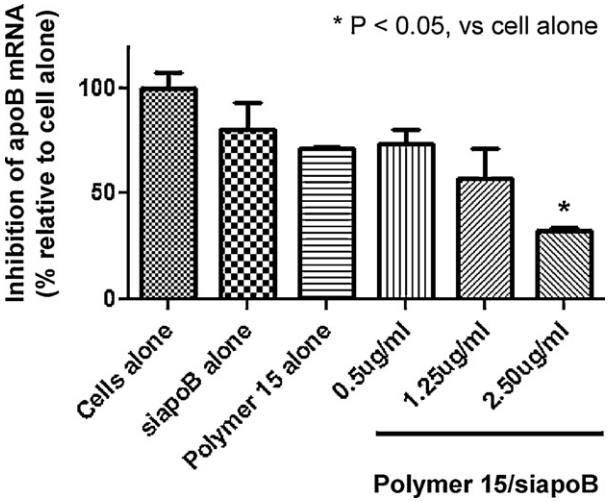

Fig. 3. *In vitro* siRNA transfection of pegylated lipopolymer–siRNA complexes in HepG2 cell line. Data are shown as mean ± SD.

at concentrations of the 0.5 μg/mL, 1.25 μg/mL, and 2.5 μg/mL of siapo≡ in a 6-well plate, in duplicate. After 48-h transfection, relative apoB mRNA levels were measured versus β-actin mRNA and then normalized to the values with untreated cells (cells alone). As expected, neither siapoB alone nor the lipopolymer (polymer 15) alone had any significant impact on expression of apoB in the HepG2 cell line. At the concentration of 0.5 μg/mL of siRNA, apoB mRNA expression of the HepG2 cell line did not seem to be silenced. This result was consistent with that of result of previous report of polymer in their HepG2 cell line (Nie et al., 2011). They had to use a concentration of 5 μg/mL of siRNA to silence *in vitro* mRNA with optimal ratios of polymer to siRNA. Using polymer 15, as the concentrations of siRNA increased from 1.25 μg/mL to 2.5 μg/mL, the apoB mRNA levels in the HepG2 cells decreased to 60%, that was statistically significant compared of that of cell alone ($p < 0.05$), showing that the pegylated lipopolymer transfection agent was effective for delivery of siRNA to HepG2 cell line.

2.3. In vivo knockdown of target gene expression in livers of nu/nu mice

We and others (Chapman, 2006; Liszewski, 2008) believe that a general siRNA carrier that is one-size-fits-all *in vitro* delivery would be unsuitable for an *in vivo* specific application. Each siRNA drug aims at one particular mRNA target, and that target is uniquely located in a particular tissue that shields with distinct physical tissue barriers. It must accompany with a designed siRNA carrier for that target tissue in order to reach its destination and transform itself into a potential drug candidate. Apolipoprotein B (apoB) is a primary lipoprotein for the formation and secretion of low-density proteins. It is expressed in hepatocytes and may be responsible to transporting cholesterol to tissues. It is also a diagnosis marker for monitoring a risk of vascular disease (Sniderman et al., 2004). Inhibition of apoB expression may reduce risk of heart disease. ApoB is a promising target for siRNA drugs due to therapeutic value and hepatocyte-targeted carrier efficiency (Watanabe et al., 2009). A number of groups utilized inhibition of apoB to elucidate their siRNA delivery systems. In a mouse model, Soutschek et al. (2004) demonstrated that they could target hepatocytes using siRNA–cholesterol conjugate with resultant of ~60% knockdown of apoB mRNA in the mouse liver. Polymer–siRNA conjugate attaching N-acetyl-galactosamine ligand (Dynamic PolyConjugate) (Rozema et al., 2007) and siRNA–α-tocopherol (vitamin E) conjugate (Nishina et al., 2008) for hepatocytes-targeting were also shown, independently, a ~80% knockdown of apoB mRNA in the mouse liver. Watanabe et al. (2009) showed that non-conjugated siRNA complexed with dendritic poly(L-lysine) could be used to target hepatocytes with ~50% reduction in apoB mRNA. The most advanced stable acid lipid particle (SNALP) siRNA delivery system to target apoB mRNA in terms of drug developments was reported by Zimmerman et al. (2006). Using SNALP to encapsulate siRNA, Zimmerman et al. were able to deliver maximal silencing of >90% after 48 h intravenous administration in monkey at 1–2.5 mg/kg of siRNA. However, a therapeutic dose of 2.5 mg/kg of siRNA in monkeys caused an increase in liver enzymes (Zimmerman et al., 2006).

Knockdown of target gene expression in livers of *nu*/*nu* mice was demonstrated via intravenous administration of a pegylated lipopolymer–siRNA complex. To obtain the maximum effect of the lipopolymer–siRNA complex to silence apoB mRNA, experiments were first designed to find an optimal ratio of transfection agent to siRNA in *nu*/*nu* mice. The complexes with a volume of 0.2 mL were injected intravenously via tail vein of the mice using 5/1, 7.5/1, and 10/1 (by weight to weight) lipopolymer:siRNA ratios. The amount of the siRNA injected was maintained at 1.0 mg/kg for three different experimental groups ($n = 3$). RT-qPCR analyses of the liver

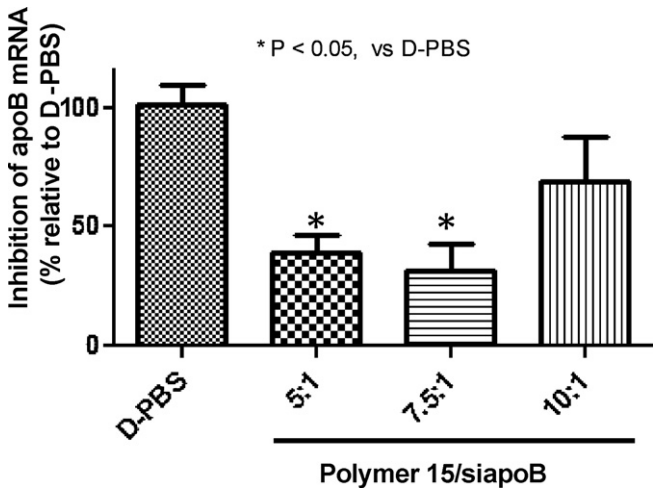

Fig. 4. *In vivo* knockdown of apoB mRNA in the liver of *nu*/*nu* mice with 1 mg/kg of siapoB in different polymer/siRNA ratios after 48 h post injection. Data are shown as mean ± SD.

apoB mRNA levels versus β-actin mRNA levels were performed 48 h after the siRNA administrations, which were normalized to the group of PBS as a control. Results are shown in Fig. 4, that all the ratios inhibited expression of apoB mRNA. The strongest inhibition was observed at the ratios of 5/1 and 7.5/1, which was statistically significant compared with that of PBS control. The polymer:siRNA ratio of 10:1 did not provide maximal inhibition, even though it was the highest ratio of polymer:siRNA. The low inhibition might be due to side-effects of excess of the polymer. The polymer:siRNA ratio of 5:1 was chosen for further *in vivo* experiments.

In a next set of experiment, an optimal dose of siRNA and duration for siRNA efficacy were determined. With the polymer:siRNA ratio of 5:1, different doses of 1.0 mg/kg and 2.5 mg/kg of the siRNA were investigated with the duration of 2 days, 1 week, and two weeks, along with the controls of PBS, the siRNA alone (2.5 mg/kg) for 2-week duration, polymer 15 alone (12.5 mg/kg) for 2-week duration, and a scrambled siRNA with polymer at the ratio of 5:1, weight to weight of polymer:siRNA, for 2-day duration. As seen in Fig. 5, administrations of 1.0 mg/kg of the siRNA with 2-day post injection showed significant knockdown of apoB mRNA level, compared of that of the PBS in *nu*/*nu* mice. Similar inhibition of apoB mRNA was observed with 2.5 mg/kg of the siRNA for 1-week and 2-week post injections, whereas all the controls did not show any significant inhibition of apoB gene expression. The inhibitory effect of the pegylated lipopolymer–siRNA complex in the mice persisted for 2 weeks showing that the lipopolymer may be viable siRNA carrier for further developments.

2.4. *In vivo knockdown of target gene expression in livers of C57BL/6 mice*

To demonstrate the versatility and applicability of the lipopolymer–siRNA complex in a number of different mice, C57/BL black mice were chosen. Knock-down of apoB mRNA in the black mice was demonstrated. With the optimal dose of 1.0 mg/kg of the siRNA and 5:1 (weight by weight) polymer:siRNA ratio, the pegylated lipopolymer–siRNA complex was administered intravenously via tail vein of the black mice. The efficacy of apoB mRNA inhibition was determined after 2-day, 1-week, 2-week, and 3-week post injections. Fig. 6 shows that inhibition of apoB mRNA in the black mice was statistically significant. Inhibition of ~80% of liver apoB mRNA in the black mice was observed for up to 2 weeks. However, in the third week, the levels of apoB mRNA returned to control levels.

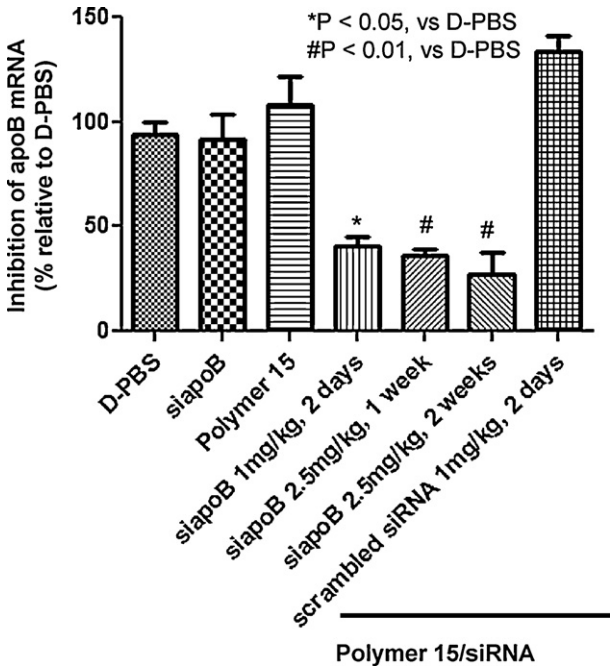

Fig. 5. *In vivo* knockdown of apoB mRNA in the liver of *nu*/*nu* mice with different siapoB doses in the ratio of 5:1 (by weight to weight) of polymer:siRNA after an indicated period of post-injection time. Data are shown as mean ± SD.

Taking together of all the data of inhibition of apoB mRNA *in vivo* in mice, the hybrid of lipid and pegylated cationic polymer (pegylated lipopolymer) for siRNA apoB hepatocyte-targeted delivery at very low doses of 1.0–2.5 mg/kg of siRNA to achieve 60–80% silencing of apoB mRNA in mice was comparable to silencing of apoB mRNA at 50 mg/kg of siRNA of the gold standards for SNALP siRNA delivery. Further studies of different siRNA doses, lipopolymer:siRNA ratios, and toxicology in different animal models would be required for the future development of systemic siRNA delivery to achieve more potency and effectiveness. Nonetheless, the finding sets a new stage for further developments for siRNA therapeutics of apoB targeting.

3. Materials and methods

Polymer 15 was synthesized in our laboratory, as described previously (Liu et al., 2009). Polymer 15 is a crosslinked pegylated

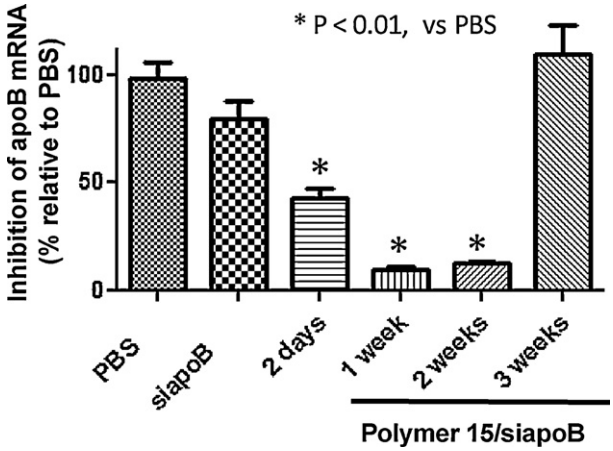

Fig. 6. *In vivo* knockdown of apoB mRNA in the liver of C57BL/6 mice with 1 mg/kg of siapoB doses at 5:1 polymer:siRNA by weight to weight ratio after an indicated period of post-injection time. Data are shown as mean ± SD.

lipopolymer with molar ratios of 1:16:2, corresponding to PEI 1200 Da:diacrylate lipid crosslinker:PEG454 acrylate, respectively. An average molecular weight of polymer 15 was 42 kDa, determined by gel permeation chromatography with Wyatt multi-angle light scattering detection. The sequences of siapoB (sense: 5′-GUCAUCACACUGAAUACCAAUUU-3′ and antisense: 5′-AUUGGUAUUCAGUGUGAUGACUU-3′) and the scrambled siRNA (as a negative a control) used in this *in vivo* study were reported previously elsewhere (Soutschek et al., 2004). All the siRNA sequences were purchased from Dharmacon (Lafayette, CO).

3.1. In vitro siRNA transfection

3.1.1. Luciferase reporter gene knock-down

Polymer 15/siRNA complex was evaluated in firefly luciferase stable transfected cell line PC-3M-Luc-C6 (Xenogen Corp, now part of Taconic). The transfection was conducted in a 96-well plate at initial seeding density at 1×10^5/mL in the medium that was advised accordingly to manufacturer's instructions. Different weight to weight ratios of polymer 15:anti-luciferase siRNA (siLuc) (2:1, 5:1, 10:1, 20:1) with a concentration of siLuc at 17.8 nM (0.25 μg/mL) were used. Lipofectamine 2000 (L2K) (Invitrogen, Carlsbad, CA) was used as positive control. Anti-enhanced green fluorescence protein siRNA (siegfp) sequence was employed as unrelated negative siRNA control. Luciferase knockdown efficiency was measured with luminescent spectrometer after 48 h of transfection. Data are expressed as mean with a standard deviation of luciferase activity (RLU/mg protein).

3.1.2. Endogeneous mRNA knock down

Hepatoma (HepaG2) cells were purchased from ATCC (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 (g/mL streptomycin, and incubated at 37 °C at 100% humidity atmosphere containing 7.5% CO_2. Hep G2 cells were seeded in 6-well plate at a density of 3×10^5 cells/2 mL/well in MEM medium containing 10% FBS, 1% NEAA, 1% Na pyruvate and were cultured overnight. Polymer 15 was dissolved in Milli-Q water at a concentration of 5 μg/μL as a stock solution. The polymer stock solution of 0.4 μL, 1 μL, and 2 μL, was further diluted with Opti-MEM buffer of 119.6 μL, 119 μL, and 118 μL, respectively. The siapoB was dissolved in RNase-free water at a concentration of 0.25 μg/μL, as a stock solution. The siapoB stock solution of 4 μL, 10 μL, and 20 μL, was further diluted with Opti-MEM buffer of 116 μL, 110 μL, 100 μL, to yield 1 μg siapoB/well, 2.5 μg siapoB/well, and 5 μg siapoB/well, respectively, when combined with the polymer diluted solutions at the ratio of 2:1 of the polymer to the siapoB. The mixture of the siapoB and the polymer solutions was incubated at room temperature for 15 min before applied them onto the designated well. The control treatments were (1) buffer (cells alone), (2) siapoB (5 μg) alone as a negative control, and (3) polymer 15 (10 μg) alone as another negative control.

After 48 h incubation, mRNA expression was determined by quantitative RT-PCR with the primer for apoB mRNA, forwarded as 5′-TTTGCCCTCAACCTACCAAC-3′ and reversed as 5′-TGCGATCTTGTTGGCTACTG-3′, as previously reported (Watanabe et al., 2009). The relative *apoB* mRNA levels were measured versus β-actin mRNA, in duplicate, and then normalized to the values in untreated cells (cells alone). The data are shown as mean with a standard deviation.

3.2. In vivo knockdown of target gene expression in the liver of mice

BALB/C (*nu*/*nu*) mice and C57BL/6 (black) mice were purchased from Charles River (Wilmington, MA) and were housed in the approved animal care facility. All the mice were acclimated for at least 1 week before experimentation. All the studies were performed in accordance with the approved animal protocol. RNA extraction from siapoB treated the mouse liver was carried out using RNeasy Mini Kit (Qiagen). qRT-PCR of apoB mRNA from siapoB treated mouse liver samples was carried out accordingly as described in a manufacture's protocols (SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and Brilliant SYBR Green QPCR Master Mix Kit (Stratagen)).

3.2.1. Knock-down of apoB mRNA in the liver of nu/nu mice

3.2.1.1. Optimization of lipopolymer:siRNA ratios. Prior to injection, formulations were diluted in PBS at siRNA concentration such that each group of the *nu*/*nu* mice was administered a dose of 1 mg/kg with the lipopolymer concentrations of 5 mg/kg, 7.5 mg/kg, and 10 mg/kg in PBS as a control. 3 groups ($n = 3$) of the mice were injected with the indicated lipopolymer–siRNA complexes and 1 group ($n = 3$) of the mice was injected with PBS as a control group. The mice were administered intravenously approximately 0.2 mL via tail vein injection. After 48 h, the mice were sacrificed, and their liver was harvested and frozen. Measurements of apoB mRNA were performed by qRT-PCR.

3.2.1.2. Optimization of siRNA doses and duration of efficacy. Prior to injection, formulations were diluted in PBS of the ratio of 5:1 (weight to weight) of the lipopolymer:siRNA and siRNA concentrations of 1.0 mg/kg for 2 days post injection, 2.5 mg/kg for 1 week post injection, 2.5 mg/kg mg/kg for 2 weeks post inject, and 3 groups (PBS, the siRNA (2.5 mg/kg), and polymer 15 (12.5 mg/kg) of the controls for 2 weeks post injection). The mice ($n = 3$, each group) were administered intravenously approximately 0.2 mL via tail vein injection. After the indicated period of time, the mice were sacrificed, and their liver was harvested and frozen. Measurements of apoB mRNA were performed by qRT-PCR.

3.2.2. Knock-down of apoB mRNA in the liver of C57BL/6 black mice

Prior to injection, formulations were diluted in PBS of the ratio of 5:1 (weight to weight) of the lipopolymer:siRNA and siRNA concentrations of 1.0 mg/kg and 2 groups (PBS and the siRNA (2.5 mg/kg), of the controls). The mice ($n = 3$, each group) were administered intravenously approximately 0.2 mL via tail vein injection. After the indicated period of time, the mice were sacrificed, and their liver was harvested and frozen. For the controls, the mice were sacrificed after 3 weeks post injection. Measurements of apoB mRNA were performed by qRT-PCR as described in the previous section.

3.3. Statistical analysis

The statistical significance of the data was evaluated by Student's *t* test with $p < 0.05$ or $p < 0.01$ was considered significant.

Competing interests

The authors declare that they have no competing interests.

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