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molecular pharmaceutics

Controlling HBV Replication *in Vivo* by Intravenous Administration of Triggered PEGylated siRNA-Nanoparticles

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Received September 9, 2008; Revised Manuscript Received December 4, 2008; Accepted December 11, 2008

Abstract: Harnessing RNA interference (RNAi) to inhibit hepatitis B virus (HBV) gene expression has promising application to therapy. Here we describe a new hepatotropic nontoxic lipid-based vector system that is used to deliver chemically unmodified small interfering RNA (siRNA) sequences to the liver. Anti HBV formulations were generated by condensation of siRNA (A component) with cationic liposomes (B component) to form AB core particles. These core particles incorporate an aminoxy cholesteryl lipid for convenient surface postcoupling of polyethylene glycol (PEG; C component, stealth/biocompatibility polymer) to give triggered PEGylated siRNA-nanoparticles (also known as siRNA-ABC nanoparticles) with uniform small sizes of 80−100 nm in diameter. The oxime linkage that results from PEG coupling is pH sensitive and was included to facilitate acidic pH-triggered release of nucleic acids from endosomes. Nanoparticle-mediated siRNA delivery results in HBV replication knockdown in cell culture and in murine hydrodynamic injection models *in vivo*. Furthermore repeated systemic administration of triggered PEGylated siRNA-nanoparticles to HBV transgenic mice results in the suppression of markers of HBV replication by up to 3-fold relative to controls over a 28 day period. This compares favorably to silencing effects seen during lamivudine treatment. Collectively these observations indicate that our PEGylated siRNA-nanoparticles may have valuable applications in RNAi-based HBV therapy.

Keywords: Cationic lipids; nanoparticles; RNA interference; hepatitis B virus; lamivudine

Introduction

Approximately 6% of the world's population is chronically infected with HBV and between 25% and 40% of carriers of the virus will develop complicating hepatocellular carcinoma (HCC).[1] Licensed therapies for HBV, which include interferon-α (IFN-α), nucleoside (e.g., lamivudine) and nucleotide (e.g., adefovir) analogues, are only partially effective, and the response to treatment may not be durable.[2−4] Development of an effective antiviral therapy thus remains an important global priority.

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10.1021/mp800157x CCC: $40.75
Published on Web 01/21/2009

Recent demonstration that the RNAi pathway can be exploited to cause silencing of HBV genes has led to enthusiasm for new nucleic acid-based therapies.[5,6] However, the clinical use of therapeutic antiviral sequences is dependent on their incorporation into vectors which can be conveniently generated in large scale and are nontoxic, minimally immunogenic and efficiently hepatotropic. Although recombinant adenoviruses[7,8] and adeno-associated viruses (AAVs)[9,10] have been used successfully to deliver anti-HBV small hairpin RNA (shRNA) expression cassettes to liver cells, problems of toxicity, difficulty with large scale preparation and immunostimulation may limit application of these viral vectors in a clinical setting. As an alternative class of delivery agent nonviral vectors offer many advantages, and this has led to considerable interest in employing this class of vector to advance RNAi-based therapy.[11−14]

In studies carried out by others, liver-targeting siRNA formulations have been generated as cholesterol, α-tocopherol conjugates, apolipoprotein A1 complexes and in association with vitamin A-coupled liposomes.[15−18] "Stabilized nucleic acid−lipid particles" (SNALPs) have also been used to deliver functional siRNA *in vivo*. Stabilized nucleic acid−lipid particles mediate efficient delivery of anti-HBV synthetic siRNAs to liver in a murine hydrodynamic injection (MHI) model of transient HBV replication[19] and have also been used successfully to silence expression of an endogenous hepatic gene.[20] Previously we developed the siFECTamine cationic liposome system[13] to deliver siRNA to cells (siFection) and showed that this approach may be used for RNAi-based gene silencing. This cationic liposome system was formulated from the cationic cholesteryl polyamine N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) and the neutral colipid dioleoyl-L-α-phosphatidyl ethanolamine (DOPE) in a 45:55 molar ratio (Figure 1a). Highly efficient siRNA-mediated gene knockdown was routinely achievable without causing toxicity in cultured cells.[13] In order to advance use of nonviral vectors for therapeutic siRNA delivery, our main aim has been to devise a way to upgrade siFECTamine for *in vivo* use by means of a convenient, flexible modular molecular assembly process giving rise to biocompatible nanoparticles for siRNA delivery. Our approach has relied on the recently described synthetic, self-assembly ABCD nanoparticle paradigm that defines critical requirements for successful nonviral vector mediated nucleic acid delivery *in vivo*.[12] In addition, references were made to a number of different molecular conjugates and methodologies used to prepare other nonviral

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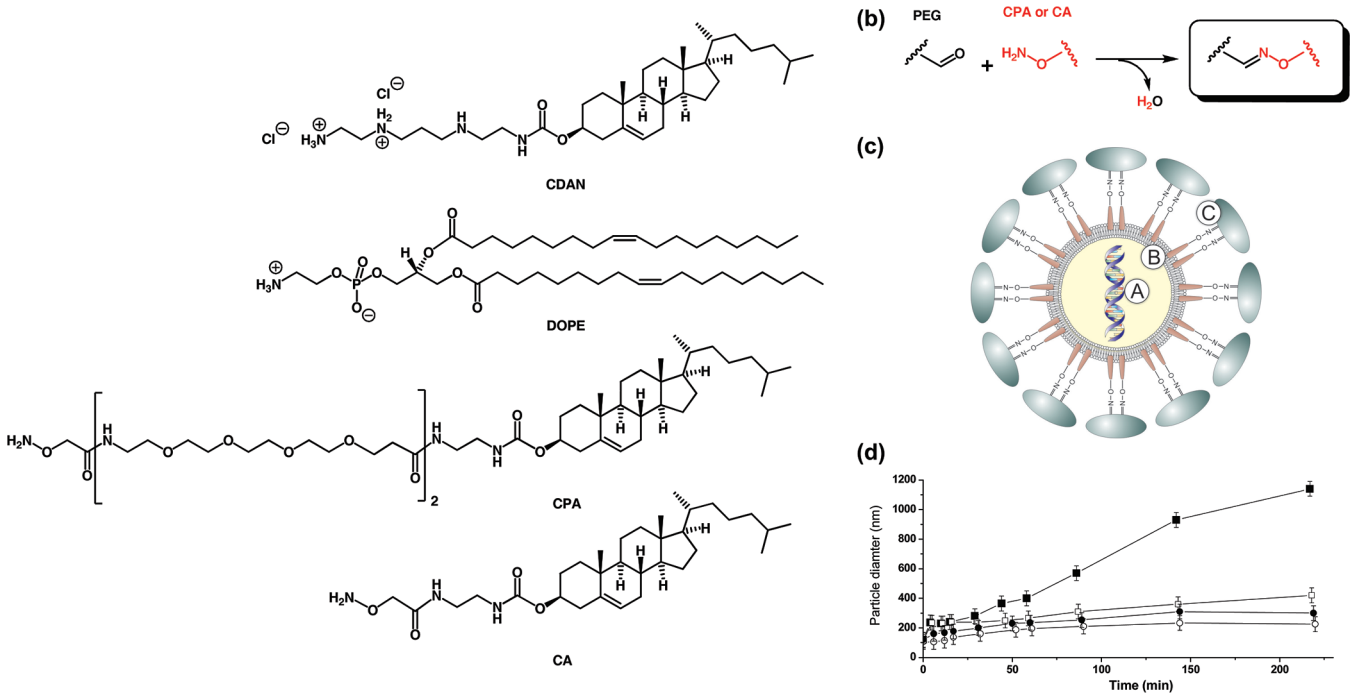

Figure 1. Chemical components and stability of siRNA nanoparticles. (a) Schematic illustration of structures of lipid components. (b) The aminoxy group of CPA or of CA allows for convenient addition of PEG^{2000}-$(CHO)_2$ in order to confer biocompatibility and stealth functions. (c) Schematic illustration of a triggered PEGylated siRNA nanoparticle (siRNA-ABC nanoparticle); siRNA (A component) is likely to be condensed as a multilammellar siRNA-lipoplex encapsulated within a lipid bilayer (B component) for cellular uptake and intracellular delivery of siRNA; PEG^{2000} is added by surface postcoupling to act as a stealth/biocompatibility polymer (C component). (d) Change in particle diameter of siRNA-nanoparticle formulations prepared from CDAN/DOPE/CPA (40:50:10, m/m/m) liposomes containing varying amounts of PEG (■ 0.1 mol %; □ 0.5 mol %; ● 1.0 mol %; ○ 5.0 mol %), as a function of time in the presence of 80% serum.

vector systems.[21−26] ABCD nanoparticles comprise nucleic acids such as plasmid DNA (pDNA) or siRNA (A component), which are condensed with cationic liposomes (B component) to form AB core nanoparticles. An important feature of our assembly approach described here is incorporation of an aminoxy cholesteryl lipid into these AB core nanoparticles to enable quantitative chemoselective postcoupling of biocompatibility polymers (C component) and optional tissue-targeting ligands (D component) to the core nanoparticles. In principle, ABCD nanoparticles thus prepared can be assembled from a variety of chemical components and represent a highly flexible nonviral vector system that is amenable to large-scale preparation. Here we describe the synthesis of novel hepatotropic anti-HBV triggered PEGylated siRNA-nanoparticle (also known as siRNA-ABC nanoparticle) vectors. Good efficacy of these formulations is demonstrated *in vivo*, which compares favorably to inhibition of HBV replication with lamivudine, and indicates that the vectors may have therapeutic use.

Experimental Section

General Synthesis. Boc-amino-oxyacetic acid and HBTU were obtained from Novabiochem (Merck Chemicals Ltd., Nottingham, U.K.). *N*-Fmoc-amido-$dPEG_4$-acid was purchased from Quanta BioDesign Ltd. (Powell, OH). PS-chlorotrityl-Cl resins were obtained from Argonaut Technologies, Inc. (Foster City, CA). All other chemicals were purchased from Sigma Aldrich (Dorset, U.K.) unless otherwise stated. Dried dichloromethane was distilled with phosphorus pentoxide; other solvents were purchased predried or as required from Sigma-Aldrich or BDH Laboratory Supplies (Poole, U.K.). HPLC-grade acetonitrile was pur-

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chased from Fisher Chemicals (Leicester, U.K.) and other HPLC-grade solvents from BDH Laboratory Supplies. Thin layer chromatography (TLC) was performed on precoated Merck-Kieselgel 60 F_{254} aluminum backed plated and revealed with ultraviolet light, iodine, acidic ammonium molybdate(IV), acidic ethanolic vanillin, or other agents as appropriate. Flash column chromatography was accomplished on Merck-Kieselgel 60 (230−400 mesh). Mass spectra were recorded using Bruker (Coventry, U.K.) Esquire 3000, VG-7070B or JEOL SX-102 instruments. ^{1}H- and ^{13}C- NMR were recorded on Advance Bruker 400 Ultrashield machine using residual isotopic solvent as an internal reference (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, br = broad singlet). Analytical HPLC (Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories (Varian Ltd, Oxford, U.K.) PL-ELS 1000 evaporative light scattering detector) was conducted with a Vydac C4 peptide column with gradient 0.1% aqueous trifluoroacetic acid (TFA) to 100% acetonitrile (0.1% TFA) [0−15 min], followed by 100% acetonitrile (0.1% TFA) [15−25 min], followed by 100% methanol [25−45 min].

Synthesis of N-Capped PEG^{350} Linker. Chlorotrityl chloride resin (1.27 mmol/g loading, 55 mg, 0.070 mmol) was swollen in dichloromethane for 16 h. The resin was loaded with *N*-Fmoc-amido-dPEG$_4$-acid (102 mg, 0.209 mmol) and assisted by Hünig base (60 μL, 0.349 mmol) in dimethyl formamide (DMF) (15 mL) for 1 h. Fluorenylmethyloxycarbonyl (Fmoc) removal was achieved with piperidine (20% v/v) in DMF (2 × 5 min) followed by extensive washing with DMF. The resulting resin-bound free amine was then reacted again with *N*-Fmoc-amido-dPEG$_4$-acid (102 mg, 0.209 mmol) that was necessarily preactivated with the coupling reagent 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (132.5 mg, 0.209 mmol) and Hünig base (60 μL, 0.349 mmol) in DMF (15 mL). After 1 h a capping by acetic anhydride (10% v/v) in DMF was performed in the presence of Hünig base (3 equiv). Finally, Fmoc was removed once more (as above) yielding resin-bound free amine that was immediately capped by *N*-Boc-amino oxyacetic acid (40 mg, 3 equiv) with the assistance of HBTU (3 equiv) and Hünig base (5 equiv) in DMF (15 mL) to yield the desired resin-bound *N*-capped PEG^{350} linker. This was cleaved in a solution consisting of trifluoroethanol 50% (v/v) in dichloromethane (3 mL) over 4 h to yield the desired *N*-capped PEG^{350} linker (40 mg, 0.058 mmol): ^{1}H NMR (400 MHz, $CDCl_3$) 1.48 (9H, Boc), 2.51 (2H, t, J = 6.1 Hz, −CH_2CO$_2$H), 2.59 (2H, t, J = 6.05, −CH_2CONHCH$_2$−), 3.45 and 3.52 (2H and 2H, m, CONHCH_2CH$_2$), 3.55−3.7 (28H, m, CH_2OCH$_2$ and CH$_2$OCH_2), 3.77 (4H, m, NHCH$_2$CH_2O), 4.34 (2H, s, BocHNOCH_2CONH), 7.0 (1H, m, BocN*H*O), 7.9 (1H, m, CH$_2$N*H*COCH$_2$) and 8.3 (1H, m, CH$_2$N*H*COCH$_2$); ESI-MS 684.30 $(M - H)^+$.

Synthesis of Cholesterylamine. Cholesteryl chloroformate (7.5 g, 0.0167 mol) was dissolved in ethylene-1,2-diamine (180 mL) and the mixture stirred for 18 h. The reaction mixture was then quenched with water and extracted with dichloromethane. The organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to afford a residue, which was purified by flash column chromatography [CH_2Cl_2:MeOH:NH_3 192:7:1 → CH_2Cl_2:MeOH:NH_3 92:7:1 (v/v)] giving the pure cholesterylamine (5.5 g, 0.0116 mol, 73%) as a white solid (mp 175−177 °C): FTIR (Nujol mull) ν_{max} 3338 (amine), 2977 (alkane), 2830 (alkane), 1692 (carbamate) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) 0.66 (3 H, s, H-18), 0.838−0.854 (3 H, d, H-27, J = 6.4 Hz), 0.842−0.858 (3 H, d, H-26, J = 6.4 Hz), 0.890−0.906 (3 H, d, H-21, J = 6.4 Hz), 0.922 (3 H, s, H-19), 1.02−1.63 (21 H, m, H-1, H-9, H-11, H-12, H-14, H-15, H-16, H-17, H-20, H-22, H-23, H-24, H-25), 1.76−2.1 (5 H, m, H-2, H-7, H-8), 2.22−2.36 (2 H, m, H-4), 2.79−2.81 (2 H, m, H$_2$NCH_2), 3.197−3.210 (2 H, m, H$_2$NCH$_2$CH_2), 4.52 (1 H, m, H-3), 5.31 (1 H, s, H-6); MS (ESI +ve) 473 (M + H); HRMS (FAB +ve) calcd for $C_{30}H_{53}N_2O_2$ (M + H) 473.4119, found 473.4107.

Synthesis of Boc-Protected CPA. The *N*-capped PEG^{350} linker (40 mg, 0.058 mmol) in anhydrous dichloromethane was combined with 4-dimethylaminopyridine (DMAP) (22 mg, 0.18 mmol), HBTU (24 mg, 0.063 mmol) and cholesterylamine (28 mg, 0.06 mmol) and the mixture stirred at ambient temperature under a nitrogen atmosphere for 15 h. The reaction was quenched with 7% aqueous citric acid and extracted with dichloromethane. The dried ($MgSO_4$) extracts were concentrated *in vacuo* to afford a residue which was purified by flash column chromatography (gradient CH_2Cl_2:MeOH:H_2O) affording *N*-Boc-protected CPA (47 mg, 0.0411 mmol, 71%): ^{1}H NMR (400 MHz, $CDCl_3$:MeOD) 5.32 (m, 1H, Chol C6), 4.35 (m, 1H, Chol C-3), 4.28 (s, 2H, (CO)CH_2ONH), 3.67 (4H, m, NHCH$_2$CH_2O), 3.56−3.61 (24H, m, CH_2OCH$_2$ and CH$_2$OCH_2), 3.56 (2H, m, CH_2CH$_2$CO), 3.50 (2H, m, CH_2CH$_2$CO), 3.35 and 3.43 (2H and 2H, m, CONHCH_2CH$_2$), 3.24 (m, 2H, CholO(CO)-NHCH_2CH$_2$), 3.18 (m, 2H, CholO(CO)NHCH$_2$CH_2), 2.42 (4H, m, −CH_2CONHCH$_2$−), 2.27 (m, 2 H, Chol C-24), 1.46 (s, 3 H, Boc), 0.94 −2.10 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 1.0 (s, 3 H, Chol C-19), 0.89 (d, 3 H, J = 6.4, Chol C-21), 0.83, 0.82 (2 × d, 6 H, J = 6.5 and 2.0 Hz), 0.68 (s, 3 H, Chol C-18); ESI-MS 1162.40 [M + K].

Synthesis of CPA. *N*-Boc-protected CPA (40 mg, 0.035 mmol) in propan-2-ol (2 mL) was treated with 4 M HCl in dioxane (2 mL) and the mixture stirred at room temperature for 3 h. The solvents were removed *in vacuo* affording CPA (37 mg, 98%): ^{1}H NMR (400 MHz, d_4-MeOD) 5.31 (m, 1H, Chol C6), 4.57 (s, 2H, (CO)CH_2ONH$_2$), 4.38 (m, 1H, Chol C-3), 3.69 (4H, m, NHCH$_2$CH_2O), 3.53−3.62 (28H, m, CH_2OCH$_2$ and CH$_2$OCH_2), 3.37 and 3.43 (2H and 2H, m, CONHCH_2CH$_2$), 3.26 (m, 2H, CholO(CO)NHCH_2CH$_2$), 3.19 (m, 2H, CholO(CO)NHCH$_2$CH_2), 2.45 (4H, m, −CH_2CONH-CH$_2$−), 2.27 (m, 2 H, Chol C-24), 0.94−1.99 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 0.97 (s, 3 H, Chol C-19), 0.87 (d, 3 H, J = 6.4, Chol C-21), 0.80, 0.82 (2 × d, 6 H, J = 6.5 and 2.0 Hz), 0.64 (s, 3 H, Chol C-18); ^{13}C NMR (100 MHz, $CDCl_3$) 173.3, 172.8 and 170.5 (NH(*C*O)CH$_2$ONH$_2$), 157.6 (O*C*ONH), 140.16 (C-5), 122.94 (C-6), 75.03 (C-3), 71.90 ((CO)*C*H$_2$ONH$_2$), 70.4−70.83 (*C*H$_2$OCH$_2$ and CH$_2$O*C*H$_2$), 69.54 and 70.14 (NHCH$_2$*C*H$_2$O), 67.62 (2 × *C*H$_2$CH$_2$CO overlapping), 57.12 (C-14), 56.56 (C-17), 50.50 (C-9), 42.7 (C-13), 40.54 and 39.91 (CholO(CO)-

NH*C*H$_2$*C*H$_2$) 40.14 (C-4), 39.88 (C-12), 39.38 and 39.65 (CONH*C*H$_2$CH$_2$), 38.94 (C-24), 37.3 (C-1), 36.95 (C-10), 36.87 (−*C*H$_2$CONHCH$_2$−), 36.55 (C-22), 36.17 (C-20), 32.28 (C-8), 32.26 (C-7), 28.5 (C-16 and C-2 overlapping), 28.36 (C-25), 24.6 (C-15), 24.17 (C-23), 22.98 (C-26), 22.73 (C-27), 21.42 (C-11), 19.6 (C-19), 18.96 (C-21) and 12.11 (C-18). ESI-MS 1102.50 $[M + K + Na]^+$. Analytical HPLC: 1 peak, rt 31 min.

Synthesis of CA. *N*-Boc-amino oxyacetic acid (145 mg, 1.5 equiv) in anhydrous CH_2Cl_2 was treated successively with DMAP (292 mg, 4 equiv), HBTU (2 equiv) followed by cholesterylamine (272 mg, 0.576 mmol). The mixture was then stirred at ambient temperature under an inert (N_2) atmosphere for 15 h. The reaction was quenched with citric acid 7% (w/v) and extracted with CH_2Cl_2. The dried ($MgSO_4$) extracts were concentrated *in vacuo* to give a residue, which was purified by flash chromatography, affording white solid *N*-Boc-protected CA (302 mg, 81%): ^{1}H NMR (400 MHz, $CDCl_3$) 8.56 (s, 1H, BocN*H*OCH$_2$), 8.2 (br, CH$_2$CON*H*CH$_2$), 5.5 (m, 1H, Chol C6), 5.4 (m, 1H, Chol-O(CO)N*H*), 4.5 (m, 1H, Chol C-3), 4.3 (s, 2H, (CO)C*H*$_2$ONH), 3.4 (m, 2H, O(CO)NHC*H*$_2$CH$_2$), 3.3 (m, 2H, O(CO)NHCH$_2$C*H*$_2$), 2.32 (m, 2 H, Chol C-24), 1.46 (s, 3 H, Boc), 0.94 −2.10 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 1.0 (s, 3 H, Chol C-19), 0.89 (d, 3 H, $J = 6.4$, Chol C-21), 0.83, 0.82 (2 × d, 6 H, $J = 6.5$ and 2.0 Hz), 0.68 (s, 3 H, Chol C-18); ESI-MS 646 $[M + H]^+$; HRMS calcd for $C_{37}H_{64}N_3O_6$ 646.479512, found 646.479874.

N-Boc-protected CA (86 mg, 0.067 mmol) in propan-2-ol (3 mL) was treated with 4 M HCl in dioxane (3 mL) after which the mixture was stirred at ambient temperature for 4 h. Solvents were removed *in vacuo* and the residue was redissolved in a minimum of propan-2-ol:dioxane 1:5 (v/v). Thereafter the desired CA was precipitated by the slow addition of diethyl ether and collected as a white solid (28 mg, 84%): ^{1}H NMR (400 MHz, d_4-MeOD) 5.35 (m, 1H, Chol C6), 4.8 (m, 1H, Chol-O(CO)N*H*), 4.5 (s, 2H, (CO)C*H*$_2$ONH$_2$), 4.4 (m, 1H, Chol C-3), 3.3 (m, 2H, O(CO)NHC*H*$_2$CH$_2$), 3.1 (m, 2H, O(CO)NHCH$_2$C*H*$_2$), 2.32 (m, 2 H, Chol C-24), 0.94 −2.10 (Chol C-1, 2, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 20, 22, 23, 25), 1.0 (s, 3 H, Chol C-19), 0.89 (d, 3 H, $J = 6.4$, Chol C-21), 0.83, 0.82 (2 × d, 6 H, $J = 6.5$ and 2.0 Hz), 0.68 (s, 3 H, Chol C-18); ^{13}C NMR (100 MHz, $CDCl_3$) 171.4 (NH(*C*O)CH$_2$ONH$_2$), 158.3 (O*C*ONH), 140.55 (C-5), 132.2 (C-6), 75.4 ((CO)*C*H$_2$ONH$_2$), 71.9 (C-3), 57.5 (C-14), 57.0 (C-17), 51.0 (C-9), 43.0 (C-13), 40.2 (C-4), 40.0−40.6 (C-12, C-4 and O(CO)-NH*C*H$_2$*C*H$_2$ overlapping), 39.2 (C-24), 37.8 (C-1), 37.3 (C-10), 36.9 (C-22), 36.6 (C-20), 32.7 (C-8), 32.6 (C-7), 28.9 (C-16), 28.8 (C-2), 28.7 (C-25), 24.9 (C-15), 24.5 (C-23), 23.2 (C-26), 22.9 (C-27), 21.8 (C-11), 19.7 (C-19), 19.2 (C-21) and 12.3 (C-18); ESI-MS 546 $[M + H]^+$.

SiRNA Nanoparticle Formulations. siRNAs were synthesized using standard procedures (Dharmacon, Fisher Scientific, Loughborough, U.K.). Formulation of triggered PEGylated siRNA-nanoparticles (also known as siRNA-ABC nanoparticles) was performed as follows. Appropriate volumes of CDAN·3HCl ([MW 680]; 4 mg/mL in $CHCl_3$), DOPE ([MW 744]; 10 mg/mL in $CHCl_3$) and CPA·HCl ([MW 1075] 5 mg/mL in $CHCl_3$), or CA ([MW 545.8] 10 mg/mL in $CHCl_3$) were combined together in a round-bottomed flask (5 mL) (presilanized by treatment with nitric acid, 10 min, followed by dimethyldichlorosilane, 10 min). Lipid molar ratios were 40:50:10 for CDAN/DOPE/CPA or 40:50:10 for CDAN/DOPE/CA. In each case, organic solvent was evaporated to dryness (rotary evaporator) producing a thin, lipid-film that was rehydrated by addition of water (18 MΩ) (5 mL) and vortex mixing. The multilamellar liposome formulation (pH 3.5−4) was then sonicated at 40 °C for 30 min (Sonomatic water bath, Langford (Coventry, U.K.) Ultrasonics, 33 kHz ultrasound frequency, 150 W electric power output) to produce very small unilamellar vesicles (SUVs) (30−50 nm), giving a final lipid concentration of 3 mg/mL (approximately 4 μmol/mL). Thereafter, an aliquot was removed and an aqueous solution of siRNA (0.28 mg/mL) was added slowly with constant vortex mixing, until a final lipid:siRNA ratio of 10:1 (w/w) was achieved. An appropriate volume of polyethylene glycol 2000-dialdehyde [PEG^{2000}-(CHO)$_2$, SunBio Ltd., Anyang City, South Korea] ([MW 2000] 10 mg/mL in water) was then introduced for postcoupling such that final composition of PEG^{2000}-modified lipid was between 0.1 and 5.0 mol % of total lipid. Mixtures were then incubated for 16 h at ambient temperature, and pH was maintained constant throughout. Thereafter, pH was corrected to 7 and volumes were adjusted with PBS, to give a final siRNA concentration of 0.1 mg/mL, ready for immediate use. Final triggered PEGylated siRNA-nanoparticles were essentially monodisperse with particle sizes that ranged from 80 to 100 nm in diameter (photon correlation spectroscopy, N4 plus Coulter Electronics, Beckman Coulter UK Ltd, High Wycombe, U.K.). For long-term storage, triggered PEGylated siRNA-nanoparticle suspensions were supplemented with trehalose solution (100%, w/v) to give a final trehalose concentration of 5−10% (w/v) and these samples were then flash-frozen in liquid nitrogen and lyophilized for storage. Prior to further use, nanoparticles were resuspended in a 50% (v/v) PBS solution such that the final siRNA concentration was also 0.1 mg/mL (as above). In order to assess the possibility of nanoparticle aggregation, sizes of triggered PEGylated siRNA-nanoparticle formulations were studied as a function of time by means of photon correlation spectroscopy (N4 plus Coulter Electronics), in the presence of 80% fetal calf serum (FCS).

Nanoparticle Biodistribution. These animal experiments were carried out in accordance with protocols approved by the Imperial College, London, U.K., animal ethics committee. The biodistribution of our triggered PEGylated siRNA-nanoparticles was investigated by substituting 0.05 mol % of CDAN with 4-[^{14}C] cholesterol. Labeled triggered PEGylated siRNA-nanoparticles were then administered via the tail vein to mice (3 animals per group and approximately 0.04 μCi per mouse). After 1 h, mice were anesthetized and blood was obtained by cardiac puncture and immediately mixed with heparin (15U). The blood concentration of the

nanoparticles was calculated assuming that the total blood weight is 6% body weight. After cervical dislocation, liver, spleen, kidney, lung and heart were harvested and weighed. Organs were homogenized in PBS and aliquots (200 μL) solubilized with a minimum volume of Solvable (Dupont, Boston, MA) at 60 °C for 1 h. Samples were then treated with EDTA (0.1 M, 0.1 mL), followed by aqueous hydrogen peroxide. Each treated sample was allowed to stand for 15−30 min at ambient temperature, then 1 h at 60 °C, Ultima Gold (10 mL; Perkin-Elmer, Waltham, MA) was added and radioactivity determined using liquid scintillation counting. Detected radioactivity was expressed as a percentage of injected dose per organ (% ID). To determine distribution of Cy3-labeled synthetic oligonucleotides (Dharmacon), mice were treated as described above and the labeled siRNA was detected in frozen sections (5 μm) using fluorescence microscopy.

Transient Transfection of Huh7 Cells. Huh7 liver-derived cells were maintained under standard conditions of cell culture. siFECTamine was used to codeliver either pCH-9/3091 HBV replication competent target plasmid,[27] or a vector constitutively expressing enhanced green fluorescent protein (eGFP),[28] together with relevant siRNA sequences. Briefly, to prepare the complexes, nucleic acid (0.25 pmol of pDNA and 20 pmol of siRNA) (1 μg total) was added to Opti-MEM (100 μL) (Invitrogen, Carlsbad, CA) and in a separate tube siFECTamine (12 μg) as added to 100 μL of Opti-MEM. The two mixtures were then combined and incubated at room temperature for up to 20 min and then added to the Huh7 culture medium. Controls included mock-treated or untransfected cells. Equivalent transfection efficiencies were confirmed by detection of eGFP using fluorescence microscopy.[28] HBsAg secretion into the culture supernatants was measured daily using the Monolisa (ELISA) immunoassay kit (BioRad, Hemel Hempstead, U.K.).

***In Vivo* Assessment of Efficacy of Anti HBV siRNAs.** The MHI method was initially employed to determine the effects of siRNAs on the expression of HBV genes *in vivo*. These experiments were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A saline solution comprising 10% of the mouse's body mass was injected via the tail vein over 5−10 s. The saline solution (NaCl 0.9%) included a combination of 1 μg of target DNA (pCH-9/3091) or 10 μg of pLTR β-gal[29] (a control for hepatic DNA delivery, which constitutively expresses β-galactosidase marker gene). Eight hours after MHI, naked siRNAs as indicated or triggered 5 mol % PEGylated siRNA-nanoparticles (known as siFECTplus nanoparticles) were injected iv. Each group of mice comprised 8 animals, and siRNA was administered at a dose of 1 mg/kg body weight. Blood was collected from the tail vein, and animals were sacrificed after 4 days.

HBV transgenic mice[30] were also used to assess anti viral efficacy of formulations. For these experiments, procedures were approved by the Animal Care Committee at Stanford University, CA. Nanoparticle formulations were prepared as described above, and the siRNA dose for each injection was 1 mg/kg animal body weight every 3 days. Groups initially comprised 6 animals. In mice receiving lamivudine, the drug was administered ip daily at a dose of 200 mg/kg mouse body weight.

Markers of HBV Replication *in Vivo*. Measurement of circulating VPEs and intrahepatic HBV mRNA concentrations was carried out using quantitative real time PCR according to previously described methods.[7,31,32] Fixed and unfixed frozen liver sections were processed respectively for immunohistochemical HBV core antigen (HBcAg) detection or for β-galactosidase staining.[7,33] Serum HBsAg was measured using a quantitative sandwich ELISA from Abbott Laboratories (Maidenhead, U.K.), and HBeAg was determined using the electrochemiluminescence assay (ECLIA) from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions.

Experimental Toxicity. NMR1 mice (5 animals per group) were injected with saline, naked siRNAs as indicated or triggered 5 mol % PEGylated siRNA-nanoparticles as previously described above (siRNA was administered as single dose 1 mg/kg per animal). Four days after the injection, the mice were anesthetized, and blood samples were collected by cardiac puncture before sacrifice. The blood samples were submitted for hematological analysis, urea and electrolyte concentration measurement, alanine transaminase (ALT) and lactate dehydrogenase (LDH) activity determination. Assays were performed in the accredited Haematology and Chemical Pathology Department laboratories of the South African National Health Laboratory Services (NHLS) in Johannesburg.

Statistical Analysis. Data are expressed as the mean ± standard error of the mean. Statistical difference was considered significant * when $P < 0.05$ and was determined

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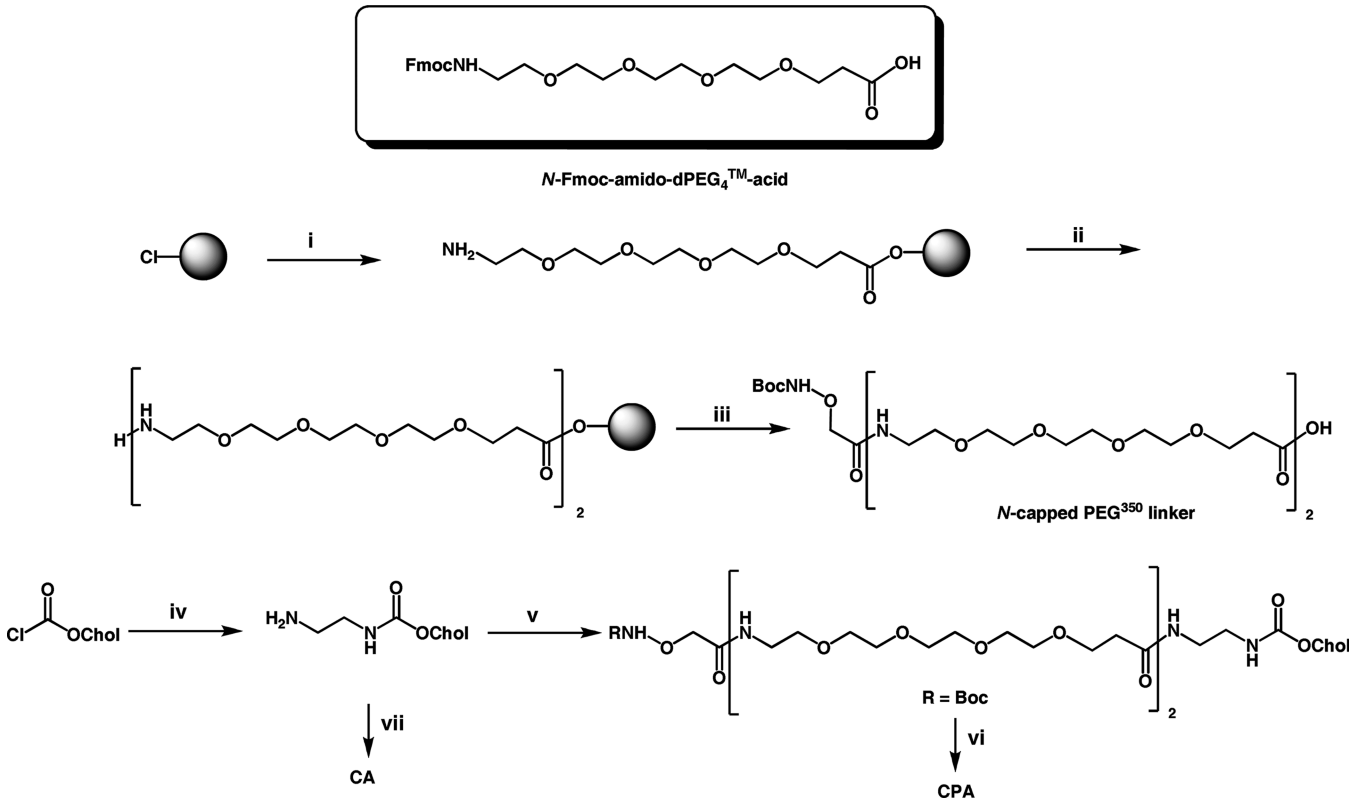
Scheme 1. Reagents and Appropriate Reaction Conditions[a]



[a] (i) (a) *N*-Fmoc-amido-dPEG$_4$-acid (3 equiv), Hünig base (5 equiv) in DMF, 1 h, rt; (b) 20% (v/v) piperidine in DMF (2 × 5 min), rt; (ii) (a) *N*-Fmoc-amido-dPEG$_4$-acid (3 equiv), HBTU (3 equiv), Hünig base (5 equiv) in DMF, 1 h, rt; (b) 20% (v/v) piperidine in DMF (2 × 5 min), rt; (iii) (a) *N*-Boc-amino-oxyacetic acid (3 equiv), HBTU (3 equiv), Hünig base (5 equiv) in DMF, 1 h, rt; (b) 50% (v/v) 1,1,1-trifluoroethanol in dichloromethane, 4 h, rt; (iv) ethylene diamine (large excess), rt, 18 h, 73%; (v) *N*-capped PEG^{350} linker, HBTU (1 equiv), DMAP (3 equiv), dichloromethane, rt, 15 h, 71%; (vi) 4 M HCl, dioxane/propan-2-ol, 3 h, 98%; (vii) (a) *N*-Boc-amino-oxyacetic acid (1.5 equiv), HBTU (2 equiv), DMAP (4 equiv) in dichloromethane, 15 h, rt, 81%; (b) 4 M HCl, dioxane/propan-2-ol, 4 h, rt, 84%.

according to the Dunnett's multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software Inc.).

Results

Lipid Synthesis. Two new cationic lipids were synthesized according to the procedures outlined in Scheme 1. These cholesterol derivatives were cholesteryl-PEG^{350} aminoxy lipid (CPA) and cholesteryl-aminoxy lipid (CA). CPA contains a short polyethylene glycol 350 (PEG^{350}) moiety with a remote aminoxy functional group. CA is the same as CPA but lacks the PEG^{350}-moiety intervening between the cholesteryl and aminoxy groups (Figure 1a). The two lipids differ according to the length of their spacer arm between the cholesterol and reactive aminoxy functional groups. In brief the synthesis of CPA required the following steps. A short PEG^{175} linker *N*-Fmoc-amido-dPEG$_4$-acid (Quanta BioDesign, Inc.) was loaded onto 2-chlorotrityl polystyrene resin (Argonaut) and then coupled to a second PEG^{175} linker *N*-Fmoc-amido-dPEG$_4$-acid using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU). The resulting PEG^{350} linker was then *N*-capped with *N*-Boc-amino-oxyacetic acid (Novabiochem, U.K.) and subsequently released from resin for coupling to cholesterylamine to yield Boc-protected CPA (71% yield). Finally, this was treated with 4 M HCl to yield CPA. Cholesterylamine was prepared previously by reaction of cholesterol chloroformate with excess ethylene diamine. The synthesis of CA was much simpler. In brief, cholesterylamine was *N*-capped with *N*-Boc-amino-oxyacetic acid (Novabiochem, U.K.) and then treated with 4 M HCl to yield CA in good yield. CDAN was prepared as indicated previously.[12,13] All other lipids were purchased from Sigma Aldrich (Dorset, U.K.) unless otherwise stated.

siRNA Formulations. In order to prepare siRNA-containing vectors, core AB nanoparticles (70−80 nm in diameter) were initially formulated in aqueous solution by combining selected siRNAs with cationic liposomes comprising CDAN/DOPE/CPA (40:50:10 molar ratio) or CDAN/DOPE/CA (40:50:10 molar ratio). Polyethylene glycol^{2000}-dialdehyde (PEG^{2000}-(CHO)$_2$) (C component), in a range of 0.1−5.0 mol %, was then coupled to AB particles under aqueous acidic conditions (pH 4). This surface postcoupling was enabled by rapid, quantitative chemoselective aminoxy−aldehyde conjugation between the aminoxy functional group of aminoxy lipids CA or CPA (as appropriate) and the aldehyde functional groups of PEG^{2000}-(CHO)$_2$. At pH 4, only aminoxy functional groups remain unprotonated (pK_a approx 4) to combine with free

aldehyde functional groups, hence the amine functional groups of the CDAN cytofectin head groups provide negligible competition in terms of Schiff's base formation through conjugation with PEG^{2000}-$(CHO)_2$. Moreover, the resulting oxime linkages (Figure 1b) are robust at pH 7, but prone to decomposition at pH 5.5 and below. This behavior might be expected to enhance pH-triggered endosome release of the C component polymer and improve efficiency of nucleic acid delivery.[12] Immediately after formulation, our triggered PEGylated siRNA-nanoparticles (schematically illustrated in Figure 1c) had an average diameter of 80−100 nm and narrow poly dispersity index (<0.3), which is suitable for use *in vivo*. By allowing functional group conjugation to be carried out in aqueous solution or organic solvent, the methodology allows for simple, controlled, modular and highly flexible formulations of triggered PEGylated siRNA-nanoparticles.[34] The surface postcoupling to aminoxy groups has the added advantage of enabling the incorporation of ligands to create targeted, triggered PEGylated siRNA-nanoparticles.

In order to assess propensity to aggregate, particle sizes of our triggered PEGylated siRNA-nanoparticles were determined in the presence of 80% serum over a 4 h time period (Figure 1d). With formulations containing 0.1 mol % polyethylene glycol 2000 (PEG^{2000}), average particle diameter rapidly rose above 1000 nm. However, there was little evidence for aggregation when PEG^{2000} content was increased to 5 mol %, which indicates that PEG diminishes siRNA nanoparticle aggregation. Since aggregation is likely to lead to particle susceptibility to clearance by the reticuloendothelial system (RES)[12] and compromise hepatocyte delivery of anti HBV siRNAs, we selected triggered PEGylated siRNA-nanoparticles containing PEG^{2000} at 5 mol % for further analysis.

Vector Hepatotrophism and Experimental Toxicology Assessment. In order to assess hepatotrophism of the vectors, 4-[^{14}C]-labeled cholesterol was included in CDAN/DOPE/CPA cationic liposomes. Thereafter, triggered PEGylated siRNA-nanoparticles were prepared from these cationic liposomes and injected at a dose of 1 mg/kg (siRNA/mouse body weight) via the tail vein of mice. Mice were sacrificed 1 h postinjection and extracts prepared from various tissues. Labeled cholesterol accumulated preferentially in the liver in all cases, and increasing PEG^{2000} content had little effect on nanoparticle hepatotrophism (Figure 2a). Equivalent results were obtained with triggered PEGylated siRNA-nanoparticles prepared from CDAN/DOPE/CA liposomes (not shown). This indicated that the PEG^{350} spacer arm between the cholesteryl and conjugated PEG^{2000} was not significantly affecting either the efficiency of PEG coupling or the extent of nanoparticle biodistribution. We therefore chose to focus only on nanoparticles prepared from CDAN/DOPE/CA cationic liposomes, because CA-containing formulations could be prepared with a higher level of reproducibility and uniformity than was possible with CPA-containing formulations (not shown). However, we also noted that CPA was less prone to compound degradation on storage than CA, therefore we anticipate that some benefit is likely to result should the use of CPA be studied in future formulations.

Hepatotrophic delivery of Cy3-labeled siRNA was examined after systemic iv administration of triggered 5 mol % PEGylated siRNA-nanoparticles to mice. Forty five minutes after injection, livers were removed from mice and frozen sections examined using fluorescence microscopy (Figure 2b). Labeled siRNA was detected primarily in the liver with trace amounts observed in other organs such as the kidneys (data not shown). Cy3-labeled siRNA was predominantly localized in the cytoplasm of 4′,6-diamidino-2-phenylindole-(DAPI-) positive hepatocytes. Large aggregates of Cy3 label, consistent with nanoparticle sequestration by Kupffer cells, were occasionally observed (not shown). We estimated that delivery to approx 60−70% of liver cells had occurred after a single bolus administration.

Potential toxic side effects of triggered 5 mol % PEGylated siRNA-nanoparticles were investigated experimentally *in vivo* using markers of hepatic, renal and hematological damage. In particular, we examined serum activity of lactate dehydrogenase (LDH) a general marker of cell lysis (Figure 2c), and alanine transaminase (ALT) which is a specific indicator of hepatocyte damage (Figure 2d). Activities of these enzymes were not elevated significantly in the sera of any mice 4 days after receiving the these triggered PEGylated siRNA-nanoparticles. Renal function appeared unaffected by the nanoparticles since blood concentrations of urea, creatinine and electrolytes did not differ significantly from those of the control group (Supporting Information Figure 1). Furthermore, morphological assessments of liver sections and peripheral blood smears from animals that received regular doses of triggered 5 mol % PEGylated siRNA-nanoparticles over a 4-week period did not reveal evidence for unintended harmful effects (Supporting Information Figures 2 and 3). Collectively these data indicated that our nanoparticles are passively hepatotrophic without obvious toxic side effects. These properties were considered sufficiently significant to warrant further investigation by using the vector system to silence HBV replication. These triggered 5 mol % PEGylated siRNA-nanoparticles were subsequently renamed siFECTplus nanoparticles.

Antiviral Efficacy of siRNA Nanoparticles in Cell Culture and in Murine Hydrodynamic Injection Models of HBV Replication. Initially anti-HBV efficacy of a panel of 7 different siRNAs was assessed. These siRNAs targeted conserved regions of the HBV genome (Figure 3a and Supporting Information Figure 4)[35,36] and were selected to avoid homologous targets found within the human or mouse genomes. Their design was according to the predicted susceptibility of HBV targets to RNAi-mediated silencing.[35,36] siRNAs (Table 1) were delivered using by means of siFECTamine and gene knock-down effects determined in cultured liver-derived Huh7 cells that had been transfected with a HBV replication competent plasmid. Of the panel, siRNA 1407 and siRNA 1794 were reliably capable of achieving inhibition of HBV s-antigen

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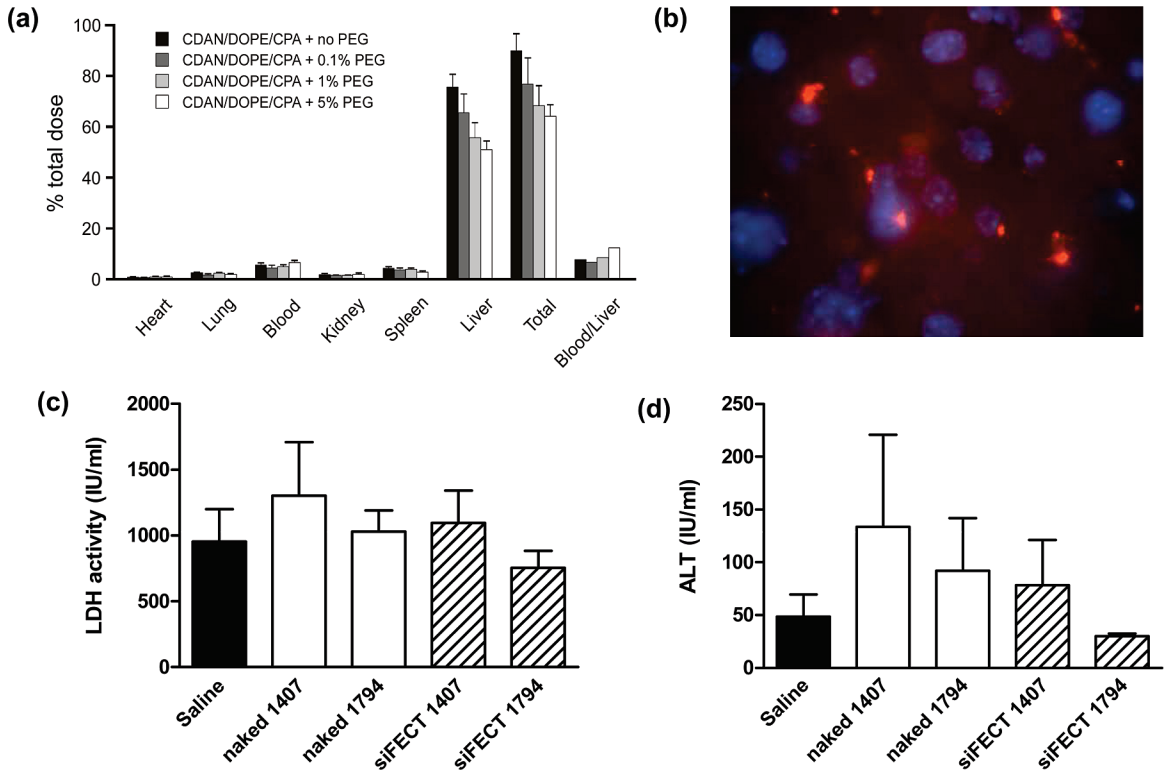

Figure 2. Biodistribution and experimental toxicity assessment of siRNA nanoparticle formulations. (a) Triggered PEGylated siRNA nanoparticle formulations (0.1−5.0 mol % PEG) containing 4-[^{14}C]-labeled cholesterol were administered iv to mice. Animals were sacrificed after 1 h, and radioactivity present in heart, lung, blood, liver, spleen, kidney and blood in the liver (blood/liver) was determined. (b) Frozen section of liver examined using microscopy (100× magnification). Labeled Cy3-siRNA was incorporated into triggered 5 mol % PEGylated siRNA nanoparticles (siFECTplus nanoparticles) and administered intravenously. Cy3 labeled siRNA (red) is noted in the liver parenchyma 45 min after injection. For histological assessment, liver sections have been counter stained with DAPI (blue) to delineate the nuclei. Serum concentrations of LDH (c) and ALT (d) at 4 d after iv administration of saline, naked siRNA-1407 (naked 1407) and naked siRNA-1794 (naked 1794) or siFECTplus nanoparticles delivering functional siRNA-1407 and siRNA-1794 (siFECT 1407 and siFECT 1794 respectively).

(HBsAg) by 40% to 50% (Figure 3b), and were selected for further studies *in vivo*.

Initially, to assess HBV gene knockdown *in vivo*, a murine hydrodynamic injection (MHI) model of viral replication was employed. A saline solution comprising 10% of mouse body weight, which contained a HBV replication-competent plasmid, was injected over 5−10 s via the tail vein. Equivalent efficiency of hepatotropic delivery using this method was confirmed by staining for activity of β-galactosidase, which was expressed from a coinjected reporter plasmid.[7] At 8 h post MHI, siFECTplus nanoparticles were administered iv at a dose of 1 mg/kg (siRNA/mouse body weight). Serum concentrations of HBsAg and VPEs were measured thereafter. Compared to controls, siFECTplus nanoparticle-mediated administration of siRNA1407 or siRNA1794 resulted in significant knockdown of HBsAg at time points of 2 and 4 days (Figure 3c). Our observations were similar to those reported when an equivalent dose of chemically modified siRNA was delivered by means of stabilized nucleic acid−lipid particles.[19] Morrissey et al. were also able to demonstrate a more efficient knockdown of HBsAg with daily iv administrations of stabilized nucleic acid−lipid particles using a higher siRNA dose of 2.5 mg/kg (siRNA/mouse body weight) for 6 days after MHI. In our case, functional siRNAs were administered earlier after hydrodynamic injection when HBV replication may have been higher and more difficult to silence. Based on these positive observations, and in order to assess HBV gene knockdown in a setting that simulates the condition of chronic HBV infection, further investigations with siFECTplus nanoparticles were carried out on HBV transgenic mice.

Antiviral Efficacy of siRNA Nanoparticle Formulations in HBV Transgenic Mice. The HBV transgenic mouse line used here was propagated after stable integration of greater than genome length HBV sequences.[30] In this model of chronic

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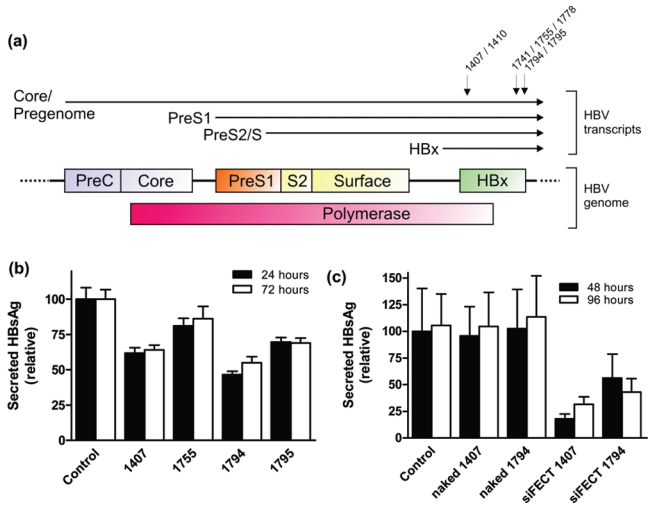

Figure 3. Effect of siFECTplus nanoparticles on markers of HBV replication in cell culture and MHI models. (a) Schematic illustration of HBV genome to indicate the siRNA target sites. (b) HBsAg was determined in the cell culture supernatant at times of 24 and 72 h after siFECTamine-mediated delivery of the indicated siRNAs (see Table 1) to Huh7 liver-derived cells, including an irrelevant, nonfunctional siRNA (control).[13] (c) Serum concentrations of HBsAg measured in MHI-treated mice at 48 and 96 h after the iv administration of naked siRNA-1407 (naked 1407) and naked siRNA-1794 (naked 1794), or siFECTplus nanoparticles delivering either an irrelevant, nonfunctional siRNA (control)[13] [equivalent result obtained with saline injection], or functional siRNAs 1407 and 1794 (siFECT 1407 and siFECT 1794 respectively).

Table 1. Designed Anti-HBV siRNAs and Their HBV Target Sequences[a]

siRNA	HBV target sequence	siRNA sequence[b]
1407	1407−1429	5′-GCGGGACGUCCUUUGUUUACG **AGCGCCCUGCAGGAAACAAAU**-5′
1410	1410−1432	5′-GGACGUCCUUUGUUUACGUCC **GCCCUGCAGGAAACAAAUGCA**-5′
1741	1741−1783	5′-GGGGAGGAGAUUAGGUUAAAG **ACCCCCUCCUCUAAUCCAAUU**-5′
1755	1755−1777	5′-GUUAAAGGUCUUUGUAUUAGG **UCCAAUUUCCAGAAACAUAAU**-5′
1778	1778−1800	5′-GCUGUAGGCAUAAAUUGGUCU **UCCGACAUCCGUAUUUAACCA**-5′
1794	1794−1816	5′-GGUCUGCGCACCAUCAUCAUG **AACCAGACGCGUGGUAGUAGU**-5′
1795	1795−1817	5′-GUCUGCGCACCAUCAUCAUGC **ACCAGACGCGUGGUAGUAGUA**-5′

[a] See *Supporting Information* Figure 1 for sequence alignments. [b] The siRNA antisense strands are in bold font.

infection, HBV particles are constitutively produced and the number of circulating VPEs is typically in the range from 0.5 to 1.0 × 10^7 per mL. siFECTplus nanoparticle formulations were administered iv at a dose of 1 mg/kg (siRNA/mouse body weight) every third day over a 4 week period. Nanoparticles effected a statistically significant decrease in HBsAg blood concentrations at day 28 when compared to controls. This inhibition was equivalent to the effect produced by daily intraperitoneal (ip) administration of lamivudine at a dose of 200 mg/kg mouse body weight (Figure 4a). Similar but diminished trends were also observed when measuring hepatitis B e-antigen (HBeAg) (Figure 4b). In mice receiving siRNA 1407 or siRNA 1794 containing nanoparticles, we also observed that circulating VPEs were diminished significantly by approximately 3-fold relative to controls at 28 days (Figure 4c). Furthermore, compared to lamivudine administration, siFECTplus nanoparticle mediated delivery of siRNA 1407 or siRNA 1794 appeared to have up to twice the inhibitory effect on VPEs (Figure 4c). A similar decrease in HBsAg mRNA was observed relative to controls in livers of mice receiving nanoparticles (Figure 4d).

Interferon Response *in Vivo.* Oligoadenylate synthase-1 (*OAS-1*) and interferon-β (*IFN-*β) mRNA concentrations were measured to determine whether induction of the interferon (IFN) response caused by siRNA administration had occurred (Figure 4e). Comparisons with controls revealed that siFECTplus nanoparticles delivering siRNA 1407 or siRNA 1794 were not inducing significant activation of the IFN response genes. These data support an interpretation that inhibitory effects on markers of HBV replication were unlikely to be due to any unintended toxic immunostimulatory mechanisms. Silencing of HBV replication in a stringent mouse model of chronic HBV infection without obvious signs of IFN stimulations indicate that our triggered PEGylated siRNA-nanoparticles have potential RNAi therapeutic use.

Discussion

The application of RNAi to developing new HBV treatment is very promising. Replication of the virus is susceptible to RNAi-mediated inhibition and unlike HIV-1 or hepatitis C virus, HBV is not prone to mutation with escape from silencing by antiviral siRNAs. This is primarily because HBV has a very compact genome with overlapping reading frames (Figure 3a) that limit its sequence plasticity. Accordingly, the HBV genome is a good target for anti-HBV RNAi-based therapies and HBV is also an excellent disease model to assess the feasibility of therapy that is based on systemic administration of siRNA. One of the most important challenges facing development of RNAi-based HBV therapy is achieving safe, convenient and efficient delivery of siRNAs to hepatocytes *in vivo*. In this study, we report on the use of nonviral vectors that can be used to deliver anti HBV sequences efficiently. Although viral vectors have been used successfully to deliver expressed anti HBV sequences, convenience of large-scale chemical synthesis and modifications to alter biological properties seem likely to make nonviral vectors the preferred class of vector for therapeutic use.

Thus far, a number of nonviral vector approaches have been employed to deliver siRNAs to the liver after systemic injections. These include use of cholesterol-conjugation, apolipoprotein AI complexes, α-tocopherol conjugates, and vitamin A coupled liposomes.[15−18] Silencing of markers of HBV replication in the MHI model[19] and also the endogenous ApoB gene in monkeys[20] were achieved with stabilized nucleic acid−lipid particle vector systems. In the study reported here, we have extended the range of synthetic,

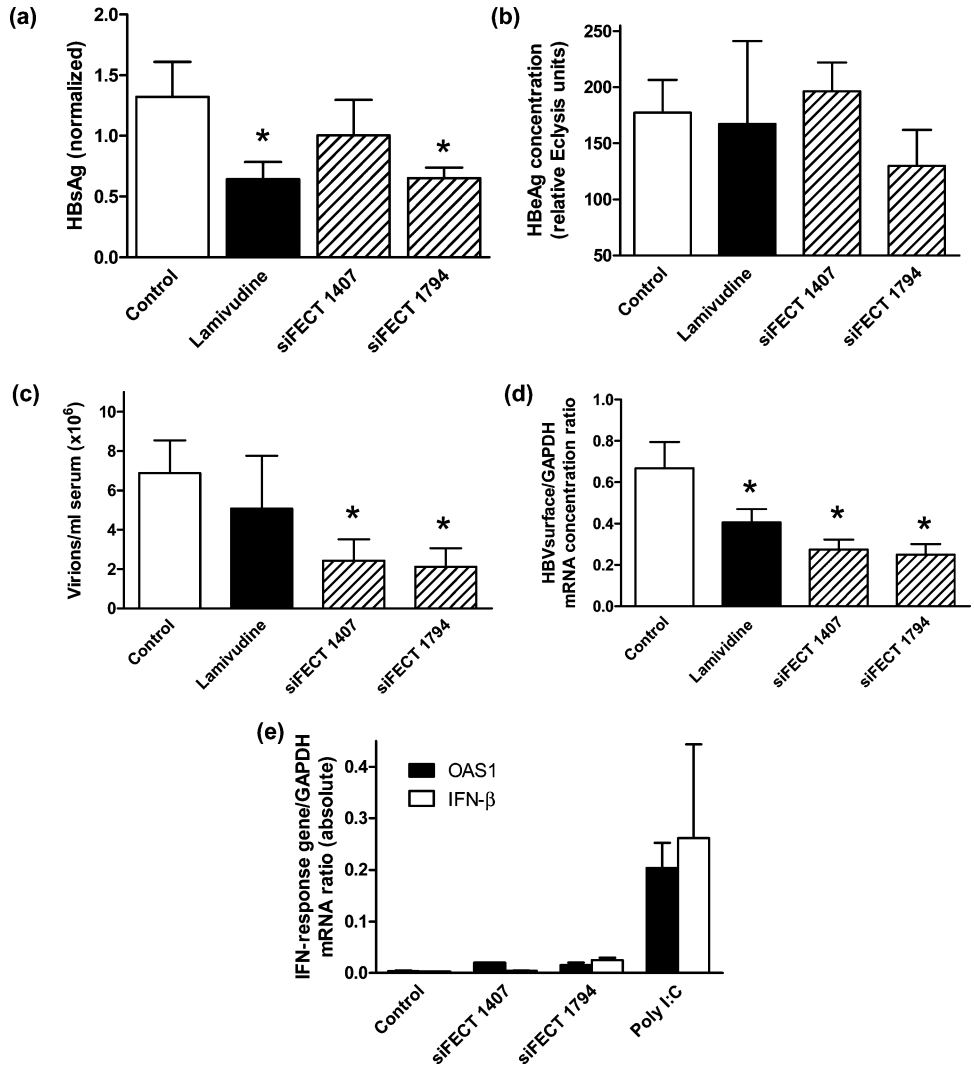

Figure 4. Markers of viral replication and immunostimulation in HBV transgenic mice. (a) HBsAg concentrations measured at 28 days in HBV transgenic mice treated every 3 days by iv administration of siFECTplus nanoparticles delivering either an irrelevant, nonfunctional control siRNA (control)[13] [equivalent result obtained with saline injection], or functional siRNAs 1407 and 1794 (siFECT 1407 and siFECT 1794 respectively), Positive control lamivudine was administered daily. (b) HBeAg concentrations measured at 28 d in HBV transgenic mice treated as described in (a). (c) Circulating VPEs (d) HBV mRNA and (e) *OAS-1* and *IFN-β* mRNA measured at 28 days in HBV transgenic mice treated as described in (a). As positive control for induction of the IFN response, mice were treated with poly I:C using MHI at 6 h before sacrifice.

nonviral hepatotropic vectors to include novel triggered 5 mol % PEGylated siRNA-nanoparticles (also known as siFECTplus nanoparticles). After peripheral iv administration, these vectors appear capable of safely delivering chemically unmodified siRNAs to murine livers to achieve inhibition of markers of HBV replication. Importantly siRNA-mediated silencing of HBV replication with siRNA 1407 or siRNA 1794 was more effective than the inhibition of viral proliferation achieved with lamivudine, which is a licensed HBV drug. The observed suppression of HBV (Figures 3 and 4) is significant and our data compare well with reported results obtained using higher doses of chemically modified siRNA or expressed RNAi effectors.[37,38]

Our triggered PEGylated siRNA-nanoparticles (siRNA-ABC nanoparticles) are prepared using a rapid, quantitative chemoselective reaction for the postcoupling of PEG stealth/biocompatibility polymer to the outer surface of core siRNA-nanoparticles (siRNA-lipoplexes)[12,13] (Figure 1c). Bioconjugation gives rise to oxime bonds that are themselves stable at pH 7 but can be expected to be labile at pH 5.5 and below. To the best of our knowledge the data described here provide a first demonstration of applying a modular surface post-coupling approach to produce simultaneously a pH-triggerable vector system.[12] Moreover this methodology should

(37) Morrissey, D. V.; Blanchard, K.; Shaw, L.; Jensen, K.; Lockridge, J. A.; Dickinson, B.; McSwiggen, J. A.; Vargeese, C.; Bowman, K.; Shaffer, C. S.; Polisky, B. A.; Zinnen, S. Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus infection. *Hepatology* **2005**, *41*, 1349–1356.

enable large-scale preparation, which would be appropriate for pharmaceutical applications.

The potential advantage of such a pH triggerable vector system should be as follows. Successful functional delivery of siRNA to liver by passive (biophysical) targeting, without the requirement for a targeting ligand, has also been demonstrated by others using stabilized nucleic acid−lipid particles.[19,20] In fact passive targeting to hepatocytes occurs because of a natural trophism of nanoparticles for the liver post iv administration where capillary fenestrations (approximately 100 nm in diameter) allow nanoparticles to leave the main circulation and enter the hepatic space of Disse. Once there, Kupffer cells (of the reticuloendothelial system) would be expected to engulf nanoparticles unless protected by PEGylation.[12] Consequently, triggered PEGylated nanoparticles (<100 nm in diameter) would appear to remain largely free to enter hepatocytes. We have noted previously that PEGylation does not necessarily impair uptake into cells by endocytosis.[12] However, we have also noted previously that release of nucleic acids after endocytosis may be substantially limited unless there is some form of in-built triggereability (such as a pH-dependent trigger) in the nanoparticle. This pH-triggerability in particular is needed to enable PEG release, overall particle destablization and associated escape from early endosomes of functional nucleic acids once the internal endosome pH has dropped from an initial pH 7 to approximately pH 5.5.[12]

In this study we report on a partial analysis of immune stimulation by our triggered PEGylated siRNA-nanoparticles. The rationale behind this focused study was to determine whether nanoparticle-mediated siRNA delivery provoked an innate immune response. This nonspecific innate response could be a direct result of effects of siRNA sequence, concentration and method of delivery. *OAS-1* and *IFN-β* gene expression are useful and well-characterized indicators of the activation of this pathway. The analysis of these markers (Figure 4e) indicates that observed inhibitory effects on HBV replication were unlikely to be a result of any unintended toxic immunostimulatory mechanisms, but rather the direct consequence of RNAi mediated effects.

In summary, the major distinguishing features of our triggered PEGylated siRNA-nanoparticles compared with other lipid-nanoparticle systems described elsewhere are the modular assembly process, the use of a postcoupling methodology to include PEG, and the simultaneous incorporation of pH-triggerability by through the formation of a pH-labile oxime bond. Going forward in order to develop our triggered PEGylated siRNA-nanoparticles for therapeutic applications, comprehensive characterization and further refinement is being carried out. Dose optimization, detailed evaluation of non specific effects, assessment of stability, pharmacokinetic analysis and metabolite profiling are being undertaken. We are also carrying out studies to improve silencing efficiency of the formulations by introducing stabilizing modifications to the siRNAs. Although challenges remain, we believe that our data together with those reported by others[19,20,37,38] are encouraging and support the notion that synthetic nonviral siRNA delivery technologies can be used to advance HBV therapy as well as for treatment of other hepatic diseases where silencing of pathology-causing diseases is indicated.

Abbreviations Used

RNAi, RNA interference; HBV, hepatitis B virus; siRNA, small interfering RNA; VPEs, viral particle equivalents; HCC, hepatocellular carcinoma; IFN-α, interferon-α; AAVs, adeno-associated viruses; shRNA, small hairpin RNA sequences; shRNAi, small hairpin interfering RNA; pDNA, plasmid DNA; SNALP, stabilized nucleic acid−lipid particle; MHI, murine hydrodynamic injection; CDAN, N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; DOPE, dioleoyl-L-α-phosphatidylethanolamine; CPA, cholesteryl-PEG^{350}-aminoxy lipid; CA, cholesteryl-aminoxy lipid; PEG^{175}, polyethylene glycol 175; PEG^{350}, polyethylene glycol 350; PEG^{2000}, polyethylene glycol 2000; PEG^{2000}-$(CHO)_2$, polyethylene glycol 2000-dialdehyde; Fmoc, 9-fluorenylmethyloxycarbonyl; Boc, *t*-butyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DAPI, 4′,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; ALT, alanine transaminase; HBsAg, hepatitis B s-antigen; HBeAg, hepatitis B e-antigen; OAS-1, oligoadenylate synthase-1; IFN-β, interferon-β.

Acknowledgment. Financial support from ImuThes Ltd., Mitsubishi Chemical Corporation, the South African Innovation Fund, National Research Foundation and the South African Poliomyelitis Research Foundation is gratefully acknowledged. The pCH-9/3091 plasmid was generously provided by Michael Nassal (The University Hospital Freiberg, Germany).

Note Added after ASAP Publication. Michael Keller has been removed as an author on this paper. The article was reposted on January 30, 2009.

Supporting Information Available: Figures depicting urea, creatinine and electrolyte levels in blood post iv administration to NMR-1 mice of saline, naked siRNAs 1794 and 1407, or siFECTplus nanoparticles formulated with siRNAs 1794 and 1407; representative smears of murine blood post iv administration to NMR-1 mice of saline, naked siRNAs 1794 and 1407, or siFECTplus nanoparticles formulated with siRNAs 1794 and 1407; histological examination of NMR-1 murine liver post iv administration of saline control and siFECTplus nanoparticles; and alignment of siRNAs with representative HBV genotype sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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