Biomaterials 34 (2013) 8408–8415

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Click conjugated polymeric immuno-nanoparticles for targeted siRNA and antisense oligonucleotide delivery

Dianna P.Y. Chan [a,b,1], Glen F. Deleavey [d,1], Shawn C. Owen [a,b], Masad J. Damha [d,**], Molly S. Shoichet [a,b,c,*]

[a] Department of Chemical Engineering & Applied Chemistry, 200 College Street, Toronto, ON M5S 3E5, Canada
[b] Institute of Biomaterials & Biomedical Engineering, Room 514, 160 College Street, Toronto, ON M5S 3E1, Canada
[c] Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S 3H6, Canada
[d] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A 0B8, Canada

ARTICLE INFO

Article history:
Received 3 May 2013
Accepted 3 July 2013
Available online 7 August 2013

Keywords:
Gene therapy
Micelle
Self-assembly
Tumour-targeting
Copolymer
Antisense

ABSTRACT

Efficient and targeted cellular delivery of small interfering RNAs (siRNAs) and antisense oligonucleotides (AONs) is a major challenge facing oligonucleotide-based therapeutics. The majority of current delivery strategies employ either conjugated ligands or oligonucleotide encapsulation within delivery vehicles to facilitate cellular uptake. Chemical modification of the oligonucleotides (ONs) can improve potency and duration of activity, usually as a result of improved nuclease resistance. Here we take advantage of innovations in both polymeric delivery vehicles and ON stabilization to achieve receptor-mediated targeted delivery of siRNAs or AONs for gene silencing. Polymeric nanoparticles comprised of poly(lactide-*co*-2-methyl, 2-carboxytrimethylene carbonate)-*g*-polyethylene glycol-furan/azide are click-modified with both anti-HER2 antibodies and nucleic acids on the exterior PEG corona. Phosphorothioate (PS), 2′F-ANA, and 2′F-RNA backbone chemical modifications improve siRNA and AON potency and duration of activity. Importantly, delivery of these nucleic acids on the exterior of the polymeric immuno-nanoparticles are as efficient in gene silencing as lipofectamine transfection without the associated potential toxicity of the latter.

1. Introduction

RNA interference (RNAi) and antisense gene silencing strategies are oligonucleotide (ON)-based therapeutic approaches utilizing small interfering RNAs (siRNAs) or antisense ONs (AONs), respectively, to silence a target gene through mRNA knockdown [1–3]. siRNA and AON therapeutics are disadvantaged by poor nuclease stability, poor cellular uptake, unintended "off-target" effects [4] and immunostimulation [5]. New delivery technologies and ON chemical modifications, which reduce nuclease degradation and unwanted side effects [1,3,6,7], promise to facilitate ON therapeutic development, yet their delivery remains problematic.

Polymeric nanoparticle micelles are promising delivery vehicles for chemotherapeutics, as reflected by numerous clinical trials [8–11]. With Doxil and Genexol-PM already approved, greater accumulation in the tumour is achieved, thereby reducing systemic toxicity [12–14]. Polymeric nanoparticle micelles of poly(lactide-*co*-2-methyl-2-carboxytrimethylene carbonate)-*graft*-polyethylene glycol-furan/azide are particularly compelling for targeted delivery as Diels–Alder and Huisgen 1,3-dipolar cycloaddition click reactions are enabled at the nanoparticle PEG corona, resulting in biomolecule incorporation on the exterior of the polymeric particles [15].

Efforts to develop effective ON delivery agents are on-going, and typically employ nucleic acid conjugates [16–18] or delivery vehicles [19] to complex with ONs to facilitate uptake. For example, liposomes [20–22], cationic polymers [23–26], and polyvalent siRNA structures [27] have been studied. Delivery methods often encapsulate ONs to prevent degradation and facilitate uptake, and may utilize polycation block copolymers to complex with negatively charged siRNA [26,28,29]. ONs are usually encapsulated

* Corresponding author. Department of Chemical Engineering & Applied Chemistry, Donnelly Centre, University of Toronto, 160 College Street, Room 514, Toronto, ON M5S 3E1, Canada. Tel.: +1 416 978 1460; fax: +1 416 978 4317.
** Corresponding author. Department of Chemistry, McGill University, Otto Maass Chemistry Building - Room 413 A, 801 Sherbrooke St. West, Montreal, QC H3A 2K6, Canada. Tel.: +1 514 398 7552; fax: +1 514 398 3797.
E-mail addresses: masad.damha@mcgill.ca (M.J. Damha), molly.shoichet@utoronto.ca (M.S. Shoichet).
[1] Chan and Deleavey contributed equally to this manuscript and are joint first authors.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.07.019

within polymeric particles where they are protected from nuclease degradation, but have, on occasion, been delivered on the exterior of delivery vehicles such as gold nanoparticles [30], DNA-based nanoparticles [31], and cholesterol conjugates [32]. The challenge of the latter strategy is nuclease stability.

2′-Deoxy-2′-fluoroarabino nucleic acid (2′F-ANA) is a particularly useful chemical modification in both siRNA and AON applications [28,33–36]. In AONs, when combined with the phosphorothioate (PS) backbone modification [37–39], 2′F-ANA enhances nuclease stability [40], is fully compatible with RNase H-mediated cleavage [41], and can improve binding stability, duration of activity, and potency [35,36]. 2′F-ANA modified PS-AONs can also function in gymnotic cellular delivery [42]. In siRNA, 2′F-ANA enhances nuclease stability [33,34], is readily tolerated in the passenger strand [33,34], and can be combined with 2′F-RNA to fully modify siRNAs for improved potency, reduced immunostimulation, and provides a thermodynamic bias for antisense strand RISC loading [34].

In this study, delivery of these stabilized ONs conjugated to the polymeric NP corona was achieved using dual functionalized poly(D,L-lactide-*co*-2-methyl-2-carboxytrimethylene carbonate)-*graft*-poly(ethylene glycol) (P(LA-*co*-TMCC)-*g*-PEG-X) micelle NPs, where X is furan or azide reactive groups on the NP PEG corona. This dual functionality enabled orthogonal click reactions - Diels–Alder and copper catalysed azide-alkyne cycloaddition reactions (CuAAC) [43] - to sequentially conjugate trastuzumab-maleimide antibodies and alkyne-functionalized ONs to the PEG corona. Controlling the composition of furan and azide NP components allowed the amount of targeting trastuzumab ligand and ON cargo to be tuned, an innovative feature of this approach. The efficacy of ON immunonanoparticle delivery was tested by gene knockdown relative to the gold standard of lipofectamine transfection.

2. Materials and methods

2.1. Polymer synthesis and characterization

5-methyl-5-benzyloxycarbonyl-1,3-trimethylene carbonate (benzyl-protected TMCC, TMCC-Bn) was synthesized as previously reported [44]. 3,6-dimethyl-1,4-dioxane-2,5-dione and 1-[3,5-bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(−)-2(dimethylamino) cyclohexyl] thiourea (Strem Chemicals, Newburyport, MA) was used according to previous protocols to synthesize P(LA-*co*-TMCC) [45]. Boc-NH-PEG(10K)-NHS (Rapp Polymere, Tübingen, Germany) was modified with furan and azide-functional groups as previously reported [44,46]. *N*,*N*′-diisopropyl carbodiimide, *N*,*N*′-diisopropylethylamine and hydroxybenzotriazole (TRC, Toronto, ON) were used as received. All solvents and reagents were purchased from Sigma–Aldrich and were used as received, unless otherwise noted.

The ^{1}H NMR spectra were recorded at 400 MHz at room temperature using a Varian Mercury 400 spectrometer. The chemical shifts are in ppm. The molecular weights and polydispersity of P(LA-*co*-TMCC) were measured by gel permeation chromatography (GPC) in THF relative to polystyrene standards at room temperature on a system with two-column sets (Viscotek GMHHR-H) and a triple detector array (TDA302) at a flow rate of 0.6 mL/min. The NH_2-PEG-azide was characterized using the Perkin–Elmer FT-IR Spectrum 1000. The Sepharose CL-4B column was prepared by soaking the beads in distilled water overnight and packing the beads in a column (5 × 15 cm). The column was washed with distilled water for 1 h before use and the flow rate was determined by gravity. The Sephadex G-25 column was prepared using the same method, except PBS buffer (1×, pH 7.4) was used as the eluent and the beads were packed in a column (1.5 × 15 cm). FPLC of the micelles used a Superdex 200 column. The column was washed with distilled water for 20 min and PBS buffer (1×, pH 7.4) for 20 min at a flow rate of 1.5 mL/min before use. Fluorescence measurements were completed with the Tecan Infinite M200 Pro fluorescent plate reader and absorbance was quantified using the NanoDrop Spectrophotometer. Luminescence was measured with the MicroLumat Plus LB96V (EG&G Berthold, Bad Wildbad, Germany).

Synthesis of P(LA-*co*-TMCC), modification of NH_2-PEG-NHS with furan and azide functionalities, and grafting of the PEG onto the polymer backbone was accomplished according to previously reported methods (Supp. Fig. S1) [15,45]. ^{1}H NMR characterization can be found in the Supplementary Data.

2.2. Oligonucleotide synthesis and characterization

All oligonucleotides (ONs) were synthesized on an Applied Biosystems (ABI) 3400 DNA synthesizer at 1 μmol scale. Unylink CPG (ChemGenes) was used for the syntheses of all ONs, except those modified with 3′ alkyne functionality, which was introduced using a 3′ alkyne-modifier serinol CPG, which is commercially available from Glen Research (20-2992-41). 2′F-ANA, 2′F-RNA, Cyanine 5 (Glen Research) and RNA phosphoramidites were prepared as 0.15 M solutions in dry acetonitrile (ACN), DNA as 0.1 M. RNA amidites were 5′-DMT, 2′-TBDMS protected, and base protection was benzoyl (A), *i*-Bu (G) or acetyl (C). 5-Ethylthiotetrazole (0.25 M in ACN, ChemGenes) was used to activate the phosphoramidites from coupling. Detritylations were accomplished with 3% trichloroacetic acid in dichloromethane for 110 s. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran, and 16% *N*-methylimidazole in tetrahydrofuran. Oxidations were done using 0.1 M I_2 in 1:2:10 pyridine:water:tetrahydrofuran, except for those following Cyanine 5 addition to ON 5′ termini, which was accomplished with 0.02 M I_2 instead. AONs containing phosphorothioate linkages were sulfurized using a 0.1 M solution of Xanthane Hydride (TCI) in 1:1 vol/vol pyridine/ACN (anhydrous). The sulfurization step was allowed to proceed for 2.5 min, with new sulfurization reagent added after 1.25 min. Phosphoramidite coupling times were 600 s for 2′F-ANA, 2′F-RNA, and RNA, with the exception of the guanosine phosphoramidites, which were allowed to couple for 900 s. DNA coupling times were 110 s, and 270 s for guanosine. Cy5 coupling times were extended to 20 min.

Base deprotection and cleavage from the solid support was accomplished with 1 mL of 3:1 aqueous NH_4OH:EtOH for 48 h at room temperature (for modified sequences), after which samples were chilled on dry ice for 15 min and subsequently lyophilized to dryness in a speedvac concentrator (Savant). Standard RNA sequences were base deprotected with 1 mL of 40% (w/v) aqueous methylamine at 65 °C for 10 min, chilled on dry ice, and lyophilized to dryness. 2′-TBDMS protecting groups were removed with 250 μL neat triethylamine trihydrofluoride (TreatHF) for 48 h at room temperature (modified sequences), or with 300 μL of TreatHF/N-methyl pyrollidinone (NMP)/triethylamine solution (prepared by adding 0.75 mL NMP, 1 mL TEA, and 1.5 mL TreatHF together at 65 °C) at 65 °C for 3 h. Following desilylation, ONs were precipitated by the addition of 25 μL 3 M NaOAc and 1 mL of *n*-butanol followed by cooling on dry ice. The ON pellets were lyophilized to dryness.

Oligonucleotides were desalted on NAP-25 Sephadex size exclusion columns (GE Healthcare) according to manufacturer protocol to prepare for HPLC purification. ONs were purified by either anion exchange or reverse phase HPLC, on either a Waters 1525 or Agilent 1200 HPLC, using a Varian Pursuit 5 semipreparative reverse phase C18 column, or a Waters Protein Pak DEAE 5 PW semipreparative anion exchange column. For reverse phase purifications, a stationary phase of 100 mM triethylammonium acetate in water with 5% ACN (pH7) and a mobile phase of HPLC-grade ACN (Sigma) were used (with a gradient of 0–35% over 30 min). Purified ONs were lyophilized to dryness, which also served to remove excess triethylammonium acetate salts. For anion exchange purifications, a stationary phase of milliQ H_2O and a mobile phase of 1 M $LiClO_4$ in milliQ water was used (with a gradient of 0–38% over 38 min). Following anion exchange purification, excess $LiClO_4$ salt was removed using a second desalting with NAP-25 sephadex size exclusion columns (GE Healthcare) according to manufacturer protocol.

All oligonucleotides were quantitated by UV (extinction coefficients were calculated using the online IDT OligoAnalyzer tool (www.idtdna.com/analyzer/Applications/OligoAnalyzer); 2′F-ANA extinction coefficients were calculated using DNA values). Oligonucleotides were characterized by LC-MS on a Waters Q-TOF2 using an ESI NanoSpray source. A CapLC (Waters) with a C18 trap column was used for LC prior to injections. The sequences of all oligonucleotides used in this work are given in Table 1. Thermal denaturation measurements were performed for select siRNA sequences on a Cary 300 UV/Vis spectrophotometer, by ramping from 15 °C to 95 °C at a rate of 1 °C/min using common ON buffer (140 mM KCl, 1 mM $MgCl_2$, 5 mM $NaHPO_4$, pH 7.2). The T_m for the unmodified 21mer siRNA targeting sequence is 63.8 °C, well above the temperatures encountered during the click reaction to attach siRNAs to the nanoparticles. To determine if ON backbone damage could be expected following the copper-mediated click reaction to attach ONs to the nanoparticles, a Cy5-labelled 21mer poly-dT ON was treated with Sodium L-ascorbate, $CuSO_4 \cdot 5H_2O$, and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), and followed at regular time points over a 30hr period. Strand integrity was observed using 24% PAGE, and bands were visualized by both UV shadowing and using Stains-all reagent (Sigma) in isopropanol (50 mL formamide, 125 mL isopropanol, 325 mL water, soak gel overnight). No strand cleavage was detected.

2.3. Annealing of oligonucleotides

Equimolar amounts of the sense and antisense strands (or AON and RNA complement strands for antisense gene silencing) of each oligonucleotide duplex were combined in annealing buffer (140 mM KCl, 1 mM $MgCl_2$, 5 mM $NaHPO_4$, pH 7.2) for a final concentration of 28 μM for each strand. The vial was heated at 90 °C for 1 min, then cooled to room temperature over an hour. The UV visible light spectrophotometer was used to confirm annealing of the two strands by measuring absorbance at 260 nm.

Table 1
Sequences of Di-siRNAs, siRNAs and AONs used for targeting Firefly luciferase.

Duplex	Sequence	MW (calc.)	MW (exp.)
Di-siRNA	5' Cy5 – CAG AAG CUA UGA AAC GAU AUG GGC U – alkyne 3'	8934.85	8934.9
	3' AC GUC UUC GAU ACU UUG CUA UAC CCG A 5'	8491.1	8490.6
Mod-Di-siRNA	5' Cy5 – **CAG** *AAG* **CTA** *UGA* **A***A***C** *G***A***U* **ATG** GGC U – alkyne 3'	9004.86	9005.4
	3' *AC GUC UUC GAU ACU UUG CUA U*AC CCG A 5'	8533.1	8532.2
NT-Di-siRNA	5' Cy5 – GCU UGA UUU CUG AAA UUA AUU CUG C – alkyne 3'	8748.56	8749.5
	3' AC CGA ACU AAA GAC UUU AAU UAA GAC G 5'	8632.3	8631.1
NT-Mod-Di-siRNA	5' Cy5 – **GCT** *UGA* **TTT** *CUG* **A***A***A** *UT***A** **ATT** CUG C – alkyne 3'	8888.76	8889.5
	3' *AC CGA ACU AAA GAC UUU AAU U*AA GAC G 5'	8674.3	8681.5 (Li^+)
siRNA	5' CAG AAG CUA UGA AAC GAU Att 3'	6724.2	6724.0
	3' tt GUC UUC GAU ACU UUG CUA U 5'	6545.9	6545.5
Mod-siRNA	5' **CAG** *AAG* **CTA** *UG*A **A***A***C** *G***A***U* **ATG** 3'	6837.2	6837.1
	3' *AC GUC UUC GAU ACU UUG CUA U* p 5'	6693.9	6693.3
NT-siRNA	5' GCU UGA UUU CUG AAA UUA AUU 3'	6621.0	6619.9
	3' AC CGA ACU AAA GAC UUU AAU U 5'	6649.1	6648.1
NT-Mod-siRNA	5' **GCT** *UGA* **TTT** *CUG* **A***A***A** *UT***A** **ATT** 3'	6761.2	6760.6
	3' *AC CGA ACU AAA GAC UUU AAU U* p 5'	6771.1	6771

AONs:

Duplex	Sequence	MW (calc.)	MW (exp.)
D-AON	5' Cy5 – AGA CAA AGG CUA UCA GGU GGC CU – alkyne 3'	8275.36	8277
	3' t* g*t* t* t*c* c*g*a*t* a*g*t* c*c*a* c*c*g 5'	6043.9	6042.8
Mod-D-AON	5' Cy5 – AGA CAA AGG CUA UCA GGU GGC CU – alkyne 3'	8275.36	8277
	3' **T*G*T*T*T*C*C*** g*a*t*a*g*t* **C*C*A*C*C*G** 5'	6259.9	6259.7
NT-D-AON	5' Cy5 – GCU UGA UUU CUG AAA UUA AUU CUG C – alkyne 3'	8748.56	8749.3
	3' a*a*c* t*a*a* a*g*a*c* t*t*t* a*a*t* t*a*a 5'	6092.0	6090.6
NT-Mod-D-AON	5' Cy5 – GCU UGA UUU CUG AAA UUA AUU CUG C – alkyne 3'	8748.56	8749.3
	3' **A*A*C*T*A*A*** a*g*a*c*t*t*t* **A*A*T*T*A*A** 5'	6308.0	6307.0

Non-targeting controls are indicated with (NT). Legend for sequence modifications: RNA (uppercase), **2′F-ANA** (bold), *2′F-RNA* (italic underlined), dna (lowercase); * = phosphorothioate linkage; Cy5 = Cyanine 5, alkyne = 3′ alkyne at terminus, p = 5′ phosphate.

2.4. Nanoparticle formation

The nanoparticle micelles were prepared by co-self-assembly of P(LA-*co*-TMCC)-*g*-PEG-furan and P(LA-*co*-TMCC)-*g*-PEG-azide by membrane dialysis, as previously shown for single functionalized chains [47]. P(LA-*co*-TMCC)-*g*-PEG-furan (2.5 mg, 18.2 kDa) and P(LA-*co*-TMCC)-*g*-PEG-azide (1.3 mg, 20.2 kDa) were combined to form the dual-functional micelles with an average ratio of 1:2 furan to azide-functional groups available. Polymeric micelle size and distribution were measured by dynamic light scattering (DLS) using the Zetasizer Nano ZS system (Malvern, UK). DLS measurements of the micellar nanoparticles determined a

hydrodynamic diameter of 62.85 ± 6.95 nm and a polydispersity distribution of 0.202 ± 0.030.

2.5. Double-click reaction

P(LA-*co*-TMCC)-*g*-PEG micelles with azide and furan functional groups (680 nmol, 200 μM) were sequentially reacted with maleimide and alkyne-functional moieties. First, trastuzumab-SMCC (250 nmol, 80 mg/mL) was added at room temperature with MES buffer (1 mL, 100 mM, pH 5.5) for 24 h. The solution was dialysed overnight against PBS buffer (1×, pH 7.4) using 2 kDa MWCO dialysis tubing. The buffer was changed every 2 h for the first 6 h. The trastuzumab-nanoparticles were concentrated to 100 μM using centrifuge concentrators at 1600 rpm.

The trastuzumab-nanoparticles (50 nmol, 40 μM) were transferred to a glass vial to react with the alkyne-modified oligonucleotide (35 nmol, 30 μM), copper sulphate ($CuSO_4$, 40 μM), sodium ascorbate (NaAsc, 113 μM) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 60 μM) in 3% methanol at room temperature for 24 h. The alkyne-modified siRNA and AONs were conjugated to the nanoparticles as preformed duplexes. Micelles were purified by FPLC (GE Healthcare AKTA Purifier) monitoring absorbance at 215 and 280 nm. Free oligonucleotides were eluted at 7–10 mL and purified nanoparticles were eluted at 15–20 mL. Fluorescence of the Cy5 (ex. 640 nm, em. 675 nm) was used to quantify the conjugation of the oligonucleotides. Absorbance at 260 nm was used to determine the micelle concentration after subtracting absorbance contributions from the oligonucleotides. The aggregation number of the micelles has previously been calculated to be 3500. There was approximately 30 trastuzumab and 120 siRNA molecules per micelle.

2.6. Cell culture with oligonucleotide-nanoparticles

The human ovary cancer cell line SKOV-3luc was purchased from Cell Biolabs, Inc (San Diego, CA). This cell line features a pGL3 firefly luciferase construct from Promega. The Lipofectamine LTX® and Plus Reagent were purchased from Invitrogen (Burlington, ON) and the Luciferase Assay System was from Promega (Madison, WI). All statistics were completed with a one-way ANOVA, unless otherwise noted.

SKOV-3luc cells were cultured in McCoy's 5A media containing 10% FBS and 1% penicillin/streptomycin under standard culture conditions (37 °C, 5% CO_2, 100% humidity). Cells were seeded in 96 well plates at 7000 cells/well and allowed to adhere overnight. The cells were washed three times with serum free media, then dual functionalized micelles having both trastuzumab and oligonucleotides were added. The cells were treated with three concentrations of AON or siRNA designed for luciferase gene knockdown. As described above, after the NP coupling reaction with the oligonucleotides, the number of AON or siRNA per nanoparticle was calculated. Using this information, the nanoparticles were diluted with unmodified immuno-nanoparticles for a final nanoparticle concentration of 1.7 μM and AON or siRNA concentrations of 56 nM, 14 nM or 2.8 nM (100 μL final volume in each well). Concentrations of siRNA were based on protocols targeting firefly luciferase in HeLa X1/5 cells [34].

The cells were incubated with the nanoparticles for 24 h to ensure gene knockdown of SKOV3-luc cells, which have a similar doubling time. Each well was treated with 50 μL of nanoparticles and an additional 50 μL of serum free McCoy's 5A media was added. For each type of nanoparticle, 8 replicates were prepared. Untreated cells were prepared simultaneously as a baseline for comparison. Controls of unmodified nanoparticles and trastuzumab-nanoparticles were used to observe the effect of the nanoparticles on luciferase activity.

For the positive controls, the AON and siRNA were delivered using a cationic lipid delivery system, Lipofectamine, to confirm the effectiveness of the AON and siRNA sequences against luciferase. The AON or siRNA (5 μL) were complexed with the Lipofectamine (10 μL) and Plus reagent (10 μL) according to manufacturer protocol for 30 min, then added to the cells. β-galactosidase was co-transfected with the free siRNA so the luminescence readings could be normalized to account for transfection efficiency. The final volume of cell media was adjusted to 100 μL using serum free media. Non-targeting sequences of oligonucleotides were also included as a negative control.

2.7. Persistence of gene silencing duration

Following the procedure outlined above, the cells were treated with AON- or siRNA-nanoparticles and then silencing was monitored for 1, 3, 4 and 7 days. Briefly, 7×10^5 cells were seeded into T-25 flasks and allowed to adhere overnight. The cells were treated with 5 mL of serum free media and 5 mL of nanoparticles (final concentrations of 1.7 μM nanoparticles, 56 nM AON or siRNA) for 5 h of transfection, which allows HER2-mediated endocytosis. Unlike the previous study measuring luciferase knockdown after 24 h, these cells were monitored for up to 7 days. To ensure healthy cell growth, the cell media was replaced with complete media. At each of the time points, the luciferase activity was measured by the luciferase cell assay. The cells were split when confluent (i.e. at 4 days) and cell media was replaced every 2 days so the luciferase levels of untreated cells were within the optimal detection range of the luminometer. 5 repeats of each sample were prepared.

2.8. Luciferase cell assay

The cell media was removed and each well was washed three times using PBS buffer (1×, pH 7.4) before adding 20 μL of lysis buffer. After 20 min, the 10 μL of lysate was transferred to a white bottom 96 well plate. Following the Promega protocol, the luciferase assay reagent was prepared. The auto-injector was used to add 25 μL of luciferase reagent to each well and measure luminescence.

For each sample where β-galactosidase was co-transfected, another 10 μL of lysate was transferred to a clear 96 well plate. A fresh mixture of $Na_2HPO_4 \cdot 7H_2O$ (81 mM), $NaH_2PO_4 \cdot H_2O$ (18 mM), $MgCl_2$ (2.3 mM), β-mercaptoethanol (49 mM) and *ortho*-nitrophenyl-β-galactoside (ONPG, 3.3 mM) was prepared. 90 μL of the ONPG mixture was added to the lysate and incubated for 30 min. The absorbance of the *o*-nitrophenol formed was measured by the Tecan plate reader at 420 nm.

3. Results

3.1. Nanoparticle modification with siRNA and antibodies

For siRNA delivery, NPs were prepared using Dicer substrate [48] 27nt versions of both native unmodified siRNAs (Di-siRNA) and 2′F-ANA/2′F-RNA-modified dicer-substrate siRNAs (Mod-Di-siRNA) corresponding to potent designs observed in Deleavey et al. (sequences and modifications are outlined in Fig. 1 and Table 1). Sense strands were synthesized with an alkyne functionality at the 3′ terminus for reaction with the azide groups on the micelle via CuAAC reactions [49]. A Cy5 label was added to the 5′ end of the sense strands for quantification, revealing a mean (±standard deviation) of 120 ± 40 siRNA molecules and 30 ± 10 antibodies per polymeric nanoparticle.

3.2. Cell studies of gene knockdown by siRNA nanoparticles

SKOV3-luc cells, expressing HER2 and firefly luciferase, were treated with dual functionalized nanoparticles having both Di-siRNA and trastuzumab (Her-NPs, for Herceptin®-Nanoparticles) (Fig. 2). Her-NP delivery of Mod-Di-siRNA (containing 2′F-ANA and 2′F-RNA) was equally effective at silencing luciferase when compared with LipA delivery of the corresponding Mod-siRNA. LipA delivery of unmodified siRNA was more effective than Her-NP delivery of unmodified Di-siRNA ($p < 0.05$). Her-NP delivery of modified vs. unmodified Di-siRNA showed greater knockdown with the modified version ($p < 0.05$), whereas LipA transfection with modified vs. unmodified siRNA showed no statistical difference in activity ($p > 0.05$).

3.3. Gene knockdown by AON nanoparticles

AON-functionalized immuno-NPs were also evaluated (Fig. 1 and Fig. S2), utilizing phosphorothioate (PS) DNA and PS-2′F-ANA gapmer AONs. Her-NP delivery of PS-2′F-ANA gapmers elicited gene silencing, and was significantly more potent than PS-DNAs ($p < 0.001$), which had very little activity, underscoring the importance of chemical modification. It may be that with Her-NP delivery, the PS-2′F-ANA gapmer AON is better able to localize in the nucleus than the PS-DNA AON; however, elucidating this exact mechanism will require further study. Using LipA, PS-DNA and PS-2′F-ANA gapmer AONs had similar potency.

3.4. Persistence of gene silencing

The ON modifications (PS, 2′F-ANA, 2′F-RNA) are expected to not only stabilize AONs and siRNAs during cellular delivery, but should also extend the duration of activity. To monitor this effect, silencing by Her-NP-delivered Di-siRNAs and AONs was monitored over 7 days. Di-siRNA, Mod-Di-siRNA, and the PS-2′F-ANA gapmer all showed persistent gene knockdown for 4 days (Fig. 3 and Fig. S3). Moreover, Mod-Di-siRNA and the PS-2′F-ANA gapmer showed greater knockdown than RNA or DNA controls at all time points

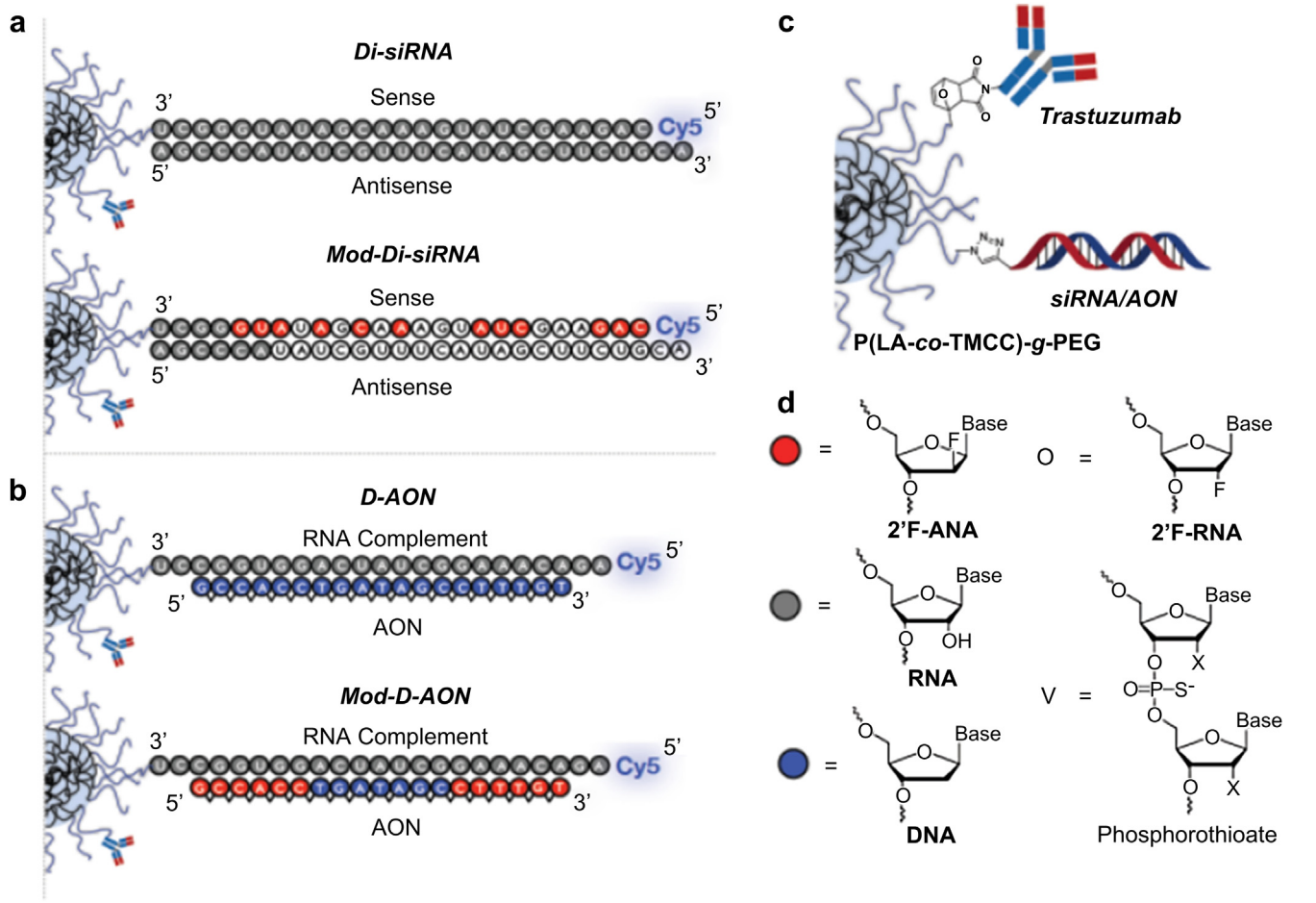

Fig. 1. siRNA and AON Delivery Strategies. (a) siRNA delivery. Immuno-NPs carry dicer-substrate siRNAs. Unmodified dicer-substrate siRNAs (Di-siRNA) are shown on top; 2′F-ANA/2′F-RNA modified Di-siRNAs (Mod-Di-siRNA) are shown below. (b) AON delivery. Immuno-NPs carry AONs annealed to RNA complement strands. Both a PS-DNA AON (D-AON, top) and a PS-2′F-ANA gapmer AON (Mod-D-AON, bottom) were utilized. (c) Dual functionalized NPs allowed antibody attachment through Diels Alder cycloaddition, and ON attachment via a CuAAC reaction. (d) Chemically modified ON backbones used in these siRNAs and AONs.

until rebound at day 7 ($p \leq 0.02$). It is likely that gene silencing persisted beyond 4 days; however cells were split at day 4 due to confluency, likely diluting knockdown effects.

3.5. Only targeted nanoparticles result in gene silencing

Individually, the components of the immuno-NP system do not have a gene silencing effect in SKOV-3luc cells. Treatment with NPs, Her-NPs (lacking Di-siRNA), Di-siRNA-NPs (lacking trastuzumab), or Her-NPs mixed with Di-siRNA (without covalent attachment of the Di-siRNAs) fail to produce an observable gene silencing effect (Fig. 4). Likewise, AONs fail to produce gene silencing without a delivery vehicle (Fig. S5). Silencing requires a combination of all 3 components covalently bound: NP, Di-siRNA, and trastuzumab.

4. Discussion

The cell studies demonstrate that Her-NP delivery on ONs provides an efficient alternative to standard Lipofectamine (LipA) transfection of 21-mer siRNAs (siRNA) and modified siRNAs (Mod-siRNA). LipA, a cationic lipid-based liposome system, is a widely used transfection reagent in cell culture. Despite some observed dose dependent toxicity [20], and a non-specific delivery mechanism that does not address endosomal escape, LipA has been very useful for siRNA transfections in cell-based assays. Importantly, Her-NPs provide advantages of targeted delivery and reduced cytotoxicity relative to LipA without sacrificing potency.

Dicer-substrate siRNA and 21-mer siRNA duplexes with the same active sequence can have similar target knockdown in vitro and in vivo [50]. Notwithstanding, to ensure that, in Fig. 2, an unfair comparison was not made between Di-siRNA-Her-NPs and siRNA + LipA, Di-siRNA and siRNA were directly compared with LipA delivery. In our experiments, there was no statistical difference observed between Di-siRNA and 21-mer siRNA delivered by LipA (Fig. S4). Thus, Di-siRNA-Her-NP and siRNA-LipA methods have comparable potencies. Her-NPs are as effective as LipA in these studies.

The mechanism for gene knockdown is likely receptor-mediated endocytosis. This is supported by the data in Fig. 4 and by previous studies where Her-NPs functionalized with a peptide colocalized both trastuzumab and the peptide inside the cell and on its surface. When treated with nonfunctionalized micelles, there was no uptake [15,44,51,52]. ON immuno-NPs likely accumulate in the cell cytoplasm after endosomal escape, achieving target specificity through a specific cell surface receptor [52–55]. In contrast, Lipofectamine is taken up non-specifically by lipid-mediated endocytosis [21,56]. While the mechanism of endosomal escape by Her-NP delivered siRNAs and AONs is still under investigation, potential explanations include endo-lysosomal escape or lysosomal degradation [42,56]. Poly(D,L-lactide-*co*-glycolide) (PLGA) NPs have a negative charge in physiological pH, which interact with the

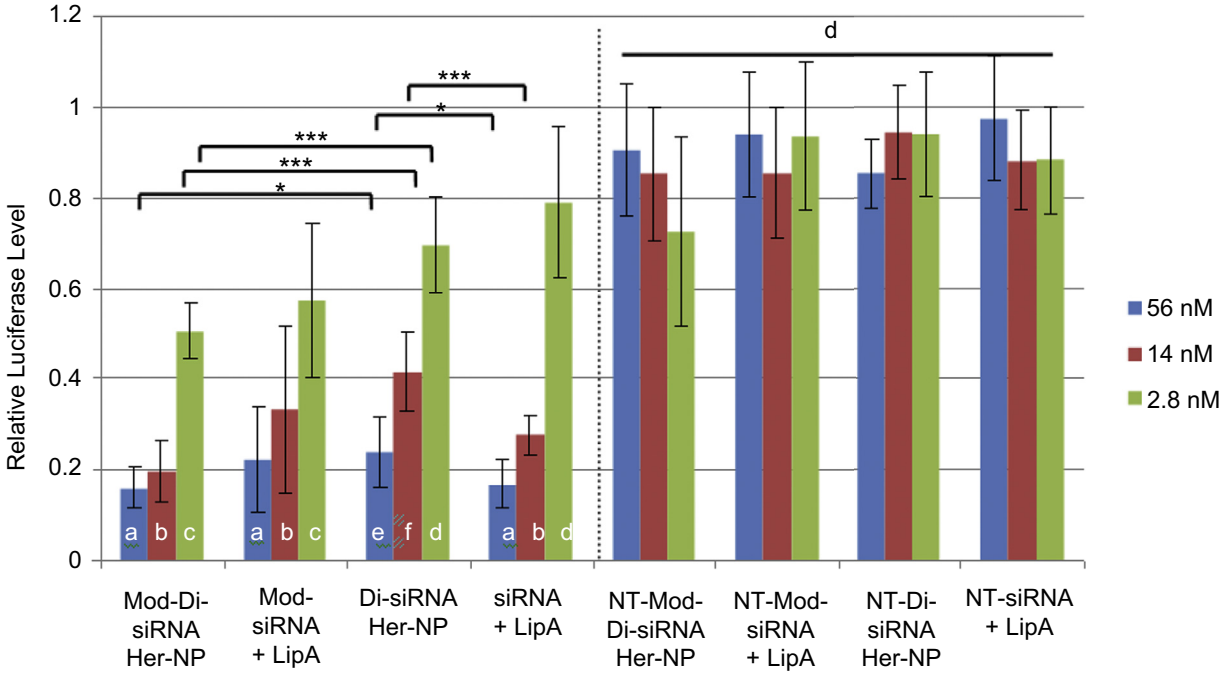

Fig. 2. Luciferase silencing results. Modified and unmodified Di-siRNAs (sequences in Table 1) were delivered to SKOV3-luc cells by Her-NPs, 21mer siRNAs with Lipofectamine (LipA) for 24 h and immediately measured for luciferase activity. Non-targeting (NT) sequences showed no activity. Luciferase levels were normalized to untreated cells; untreated controls had no luciferase knockdown and were set at 1. Treatments were repeated 8 times (mean ± standard deviation plotted). Bars with the same lower case letter denote no statistical significance between pairs ($p > 0.05$). Bars with different lower case letters are statistically different ($p < 0.01$). Statistical significance is denoted by *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

acidic endo-lysosomal membrane to achieve endo-lysosomal escape into the cytosol [56]. Alternatively, the negatively charged polymer and its counterions may create an osmotic imbalance that eventually ruptures the lysosome and releases the polymeric micelles. Her-NPs have a similar backbone comprised primarily of lactide monomers and net negative charge due to the 2-methyl, 2-carboxytrimethylene carbonate repeat units [45], and may follow a similar mechanism of endosomal escape.

Interestingly, both modified and unmodified Di-siRNAs maintained their potency when delivered on the corona of the NPs. While one might have expected that this exposure on the external corona (vs. the inner core) would have diminished oligonucleotide potency, the PEG corona itself may partially protect the ONs from nucleases [57]. Importantly, P(LA-*co*-TMCC)-*g*-PEG micelles incubated in biologically relevant solutions have demonstrated kinetic stability. These polymeric

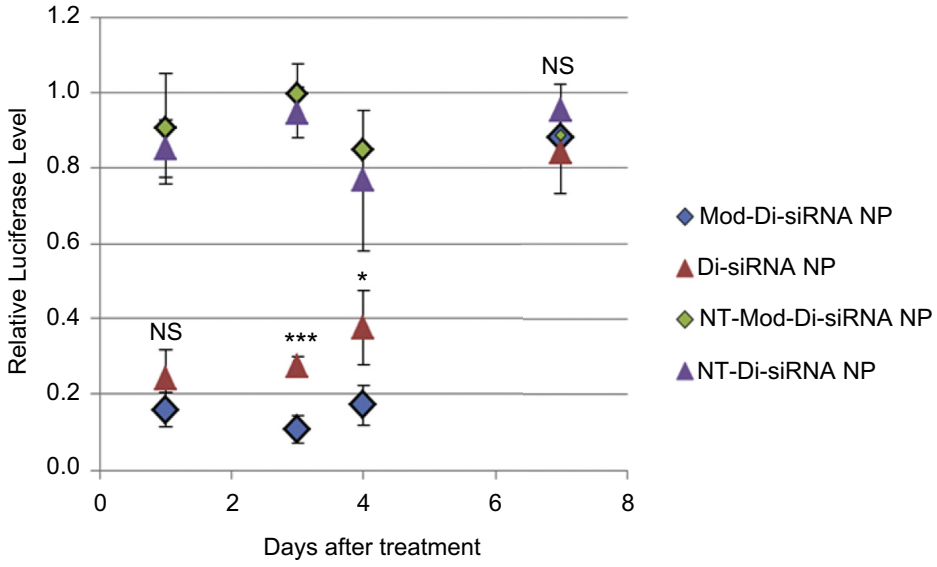

Fig. 3. Persistence study for siRNA NPs at 56 nM. SKOV-3luc cells were treated with either Mod-Di-siRNA-Her-NPs, Di-siRNA-Her-NPs or their non-targeting controls for 5 h. Luciferase levels were measured at 1, 3, 4 and 7-day intervals following treatment. Silencing was persistent for up to 4 days, after which cells reached confluency and were split. By day 7, treatment groups ceased to show knockdown. Each sample was repeated 5 times (mean ± standard deviation shown). Statistical significance between Mod-Di-siRNA-Her-NP and Di-siRNA-Her-NP is denoted by *($p < 0.05$), ***($p < 0.001$). Both Mod-Di-siRNA-HER-NP and Di-siRNA-Her-NP samples are significantly different than the relevant non-targeting controls at all time points except day 7 ($p < 0.001$).

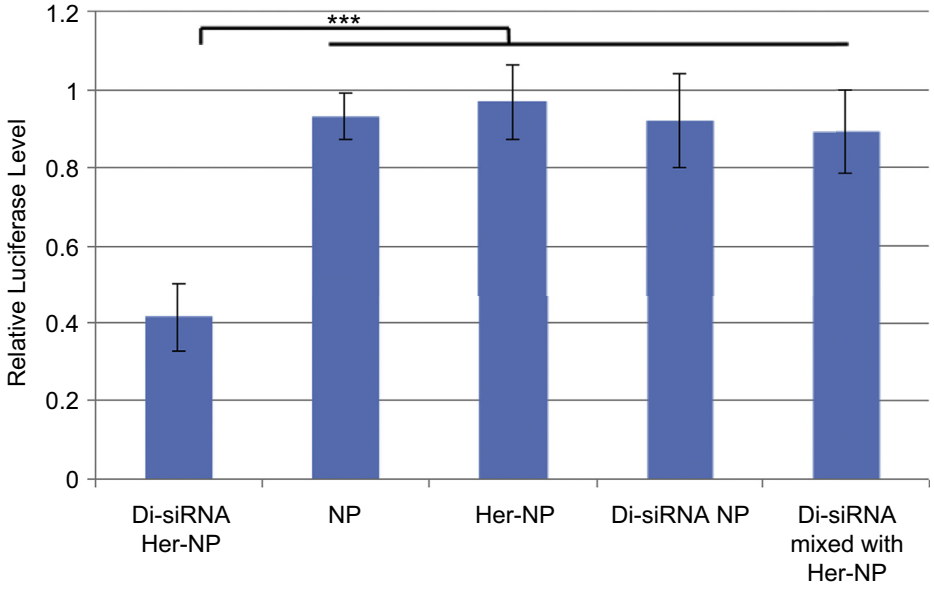

Fig. 4. Targeting effects of Her-NPs against HER2+ SKOV3-luc cells. From left to right, treatments consisted of 24 h exposure with one of: Di-siRNA functionalized Her-NPs, NP alone, Her-NP alone, Di-siRNA functionalized NPs, or Di-siRNA mixed (but not chemically conjugated) with Her-NPs. In all treatments, the polymer concentration was maintained at 1.7 μM and the siRNA concentration was 14 nM. 5 repeats were collected for each group, 8 repeats for the Di-siRNA Her-NP (mean ± standard deviation shown, $^{***}p < 0.001$).

micelles encapsulating Förster resonance energy transfer pairs in complete serum had a half-life of >9 h [58]. SiRNAs modified with 2′F-ANA and 2′F-RNA have been shown to reduce immunostimulation [34], which can be particularly important when using a delivery vehicle [5]. Moreover, modified Di-siRNA provides potent silencing for an extended duration relative to unmodified counterparts.

It is hypothesized that active siRNA is released from the Her-NPs when Dicer processes the dicer-substrate siRNA sequences, as observed in related systems [30,59]. AONs may be released following cleavage of the RNA complement strands covalently attached to the NPs. siRNA and AON release strategies are summarized in Fig. S6. Di-siRNAs feature a 2nt 3′-CA overhang on the antisense strand, which facilitates better Dicer processing versus blunt ended duplexes (although it is also associated with increased degradation in serum) [60]. The 3′ sense strand terminus was selected as the point of attachment to the NPs to facilitate Dicer processing [59]. The method of NP attachment (using labile versus non-labile linkages), and the length of the linker joining the siRNA to the NP have a strong effect on gene silencing activity [61]. Our system provides a labile conjugation approach, with release likely stemming from Dicer processing of the Di-siRNA, rather than cleavage of the linker joining the ONs to the NPs.

5. Conclusions

A new strategy for ON delivery is demonstrated and based on antibody-targeted polymeric nanoparticle micelles carrying chemically modified ONs conjugated on the corona. P(LA-co-TMCC)-g-PEG NPs covalently bound with trastuzumab and modified Di-siRNAs or AONs are efficient delivery vehicles for targeted gene silencing through either RNAi or antisense mechanisms, respectively. The use of Her-NPs as an alternative to cytotoxic cationic transfection reagents for ON delivery demonstrates the benefits achieved through combining three essential components: a biodegradable polymeric nanoparticle, an antibody for active targeting, and chemically modified ONs for gene silencing (2′F-ANA, 2′F-RNA, PS). Targeted delivery and reduced cytotoxicity are achieved without loss of potency.

Acknowledgements

The authors gratefully acknowledge financial support from the Canadian Institutes of Health Research (to MSS and MJD), the Natural Sciences and Engineering Research Council (to MSS and PGSM to DC) and the Ontario Graduate Scholarship (to DC) and the Vanier Canada Graduate Scholarship (to GFD).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.07.019

References

[1] Kim WJ, Kim SW. Efficient siRNA delivery with non-viral polymeric vehicles. Pharm Res 2009;26(3):657–66.
[2] Rayburn ER, Zhang RW. Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? Drug Discov Today 2008;13(11–12):513–21.
[3] Deleavey GF, Damha MJ. Designing chemically modified oligonucleotides for targeted gene silencing. Chem Biol 2012;19(8):937–54.
[4] Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, et al. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA 2006;12(7):1197–205.
[5] Whitehead KA, Dahlman JE, Langer RS, Anderson DG. Silencing or stimulation? siRNA delivery and the immune system. Annu Rev Chem Biomol Eng 2011;2:77–96.
[6] Watts JK, Deleavey GF, Damha MJ. Chemically modified siRNA: tools and applications. Drug Discov Today 2008;13(19–20):842–55.
[7] Kalota A, Karabon L, Swider CR, Viazovkina E, Elzagheid M, Damha MJ, et al. 2′-Deoxy-2′-fluoro-beta-D-arabinonucleic acid (2′ F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. Nucleic Acids Res 2006;34(2):451–61.
[8] Yokoyama M. Polymeric micelles as a new drug carrier system and their required considerations for clinical trials. Expert Opin Drug Deliv 2010;7(2):145–58.
[9] Saif MW, Podoltsev NA, Rubin MS, Figueroa JA, Lee MY, Kwon J, et al. Phase II clinical trial of paclitaxel loaded polymeric micelle in patients with advanced pancreatic cancer. Cancer Invest 2010;28(2):186–94.
[10] Lee SY, Kim SY, Lee KY, Jeon Y, Lee K, Kim Y, et al. A randomized clinical trial of paclitaxel loaded polymeric micelle and cisplatin versus paclitaxel and cisplatin in advanced non-small cell lung cancer (NSCLC). Ann Oncol 2010;21:136–7.
[11] Kim S, Park K, editors. Polymer micelles for drug delivery. CRC Press-Taylor & Francis Group; 2010.

[12] Chen Z. Small-molecule delivery by nanoparticles for anticancer therapy. Trends Mol Med 2010;16(12):594–602.
[13] Belting M. Macromolecular drug delivery: methods and protocols. New York, NY: Humana Press; 2009. p. 1–10.
[14] Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther 2007;83(5):761–9.
[15] Chan DPY, Owen SC, Shoichet MS. Double click: dual functionalized polymeric micelles with antibodies and peptides. Bioconjug Chem 2013;24(1):105–13.
[16] Juliano RL, Ming X, Nakagawa O. The chemistry and biology of oligonucleotide conjugates. Acc Chem Res 2012;45(7):1067–76.
[17] Rettig GR, Behlke MA. Progress toward in vivo use of siRNAs-II. Mol Ther 2012;20(3):483–512.
[18] Juliano R, Alam MR, Dixit V, Kang H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res 2008;36(12):4158–71.
[19] Yuan XD, Naguib S, Wu ZQ. Recent advances of siRNA delivery by nanoparticles. Expert Opin Drug Deliv 2011;8(4):521–36.
[20] Zhang CL, Tang N, Liu XJ, Liang W, Xu W, Torchilin VP. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J Control Release 2006;112(2):229–39.
[21] Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, et al. Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods 2004;33(2):95–103.
[22] Kim HK, Davaa E, Myung CS, Park JS. Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid. Int J Pharm 2010;392(1–2):141–7.
[23] Guo JF, Fisher KA, Darcy R, Cryan JF, O'Driscoll C. Therapeutic targeting in the silent era: advances in non-viral siRNA delivery. Mol Biosyst 2010;6(7):1143–61.
[24] Ozpolat B, Sood AK, Lopez-Berestein G. Nanomedicine based approaches for the delivery of siRNA in cancer. J Intern Med 2010;267(1):44–53.
[25] Howard KA, Rahbek UL, Liu XD, Damgaard CK, Glud SZ, Andersen MO, et al. RNA interference in vitro and in vivo using a chitosan/siRNA nanoparticle system. Mol Ther 2006;14(4):476–84.
[26] Miyata K, Nishiyama N, Kataoka K. Rational design of smart supramolecular assemblies for gene delivery: chemical challenges in the creation of artificial viruses. Chem Soc Rev 2012;41(7):2562–74.
[27] Cutler JI, Zhang K, Zheng D, Auyeung E, Prigodich AE, Mirkin CA. Polyvalent nucleic acid nanostructures. J Am Chem Soc 2011;133(24):9254–7.
[28] Felber AE, Castagner B, Elsabahy M, Deleavey GF, Damha MJ, Leroux JC. siRNA nanocarriers based on methacrylic acid copolymers. J Control Release 2011;152(1):159–67.
[29] Elsabahy M, Wazen N, Bayo-Puxan N, Deleavey G, Servant M, Damha MJ, et al. Delivery of nucleic acids through the controlled disassembly of multifunctional nanocomplexes. Adv Funct Mater 2009;19(24):3862–7.
[30] Patel PC, Hao LL, Yeung WSA, Mirkin CA. Duplex end breathing determines serum stability and intracellular potency of siRNA-Au NPs. Mol Pharm 2011;8(4):1285–91.
[31] Lee H, Lytton-Jean AKR, Chen Y, Love KT, Park AI, Karagiannis ED, et al. Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery. Nat Nanotechnol 2012;7(6):389–93.
[32] Jin HL, Lovell JF, Chen J, Lin QY, Ding L, Ng KK, et al. Mechanistic insights into LDL nanoparticle-mediated siRNA delivery. Bioconjug Chem 2012;23(1):33–41.
[33] Dowler T, Bergeron D, Tedeschi AL, Paquet L, Ferrari N, Damha MJ. Improvements in siRNA properties mediated by 2′-deoxy-2′-fluoro-beta-D-arabinonucleic acid (FANA). Nucleic Acids Res 2006;34(6):1669–75.
[34] Deleavey GF, Watts JK, Alain T, Robert F, Kalota A, Aishwarya V, et al. Synergistic effects between analogs of DNA and RNA improve the potency of siRNA-mediated gene silencing. Nucleic Acids Res 2010;38(13):4547–57.
[35] Min KL, Viazovkina E, Galarneau A, Parniak MA, Damha MJ. Oligonucleotides comprised of alternating 2′-deoxy-2′-fluoro-beta-D-arabinonucleosides and D-2′-deoxyribonucleosides (2′ F-ANA/DNA 'altimers') induce efficient RNA cleavage mediated by RNase H. Bioorg Med Chem Lett 2002;12(18):2651–4.
[36] Watts JK, Damha MJ. 2′ F-arabinonucleic acids (2′ F-ANA) – history, properties, and new frontiers. Can J Chem revue Canadienne De Chim 2008;86(7):641–56.
[37] Eckstein F. Nucleoside phophorothioates. J Am Chem Soc 1966;88(18):4292–4.
[38] Eckstein FA. Dinucleoside phosphorothioate. Tetrahedron Lett 1967;(13):1157–60.
[39] Eckstein F. Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? Antisense Nucleic Acid Drug Dev 2000;10(2):117–21.
[40] Watts JK, Katolik A, Viladoms J, Damha MJ. Studies on the hydrolytic stability of 2′-fluoroarabinonucleic acid (2′ F-ANA). Org Biomol Chem 2009;7(9):1904–10.
[41] Damha MJ, Wilds CJ, Noronha A, Brukner I, Borkow G, Arion D, et al. Hybrids of RNA and arabinonucleic acids (ANA and 2′ F-ANA) are substrates of ribonuclease H. J Am Chem Soc 1998;120(49):12976–7.
[42] Souleimanian N, Deleavey GF, Soifer H, Wang S, Tiemann K, Damha MJ, et al. Antisense 2[prime]-deoxy, 2[prime]-fluoroarabino nucleic acid (2[prime] F-ANA) oligonucleotides:In vitro gymnotic silencers of gene expression whose potency is enhanced by fatty acids. Mol Ther Nucleic Acids 2012;1:e43.
[43] Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Edit 2001;40(11):2004–21.
[44] Shi M, Wosnick JH, Ho K, Keating A, Shoichet MS. Immuno-polymeric nanoparticles by Diels-Alder chemistry. Angew Chem Int Edit 2007;46(32):6126–31.
[45] Lu J, Shoichet MS. Self-assembled polymeric nanoparticles of organocatalytic copolymerizated D, L-lactide and 2-methyl 2-carboxytrimethylene carbonate. Macromolecules 2010;43(11):4943–53.
[46] Lu J, Shi M, Shoichet MS. Click chemistry functionalized polymeric nanoparticles target corneal epithelial cells through RGD-cell surface receptors. Bioconjug Chem 2009;20(1):87–94.
[47] Shi M, Shoichet MS. Furan-functionalized co-polymers for targeted drug delivery: characterization, self-assembly and drug encapsulation. J Biomat Sci-polym E 2008;19(9):1143–57.
[48] Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 2005;23(2):222–6.
[49] Meldal M, Tornoe CW. Cu-catalyzed azide-alkyne cycloaddition. Chem Rev 2008;108(8):2952–3015.
[50] Foster DJ, Barros S, Duncan R, Shaikh S, Cantley W, Dell A, et al. Comprehensive evaluation of canonical versus Dicer-substrate siRNA in vitro and in vivo. RNA 2012;18(3):557–68.
[51] Ho K, Lapitsky Y, Shi M, Shoichet MS. Tunable immunonanoparticle binding to cancer cells: thermodynamic analysis of targeted drug delivery vehicles. Soft Matter 2009;5(5):1074–80.
[52] Shi M, Ho K, Keating A, Shoichet MS. Doxorubicin-conjugated immunonanoparticles for intracellular anticancer drug delivery. Adv Funct Mater 2009;19(11):1689–96.
[53] Yoo HS, Park TG. Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate. J Control Release 2004;100(2):247–56.
[54] Park JW, Hong K, Carter P, Asgari H, Guo LY, Keller GA, et al. Development of anti-P185(Her2) immunoliposomes for cancer-therapy. P Natl Acad Sci USA 1995;92(5):1327–31.
[55] Alam MR, Dixit V, Kang HM, Li ZB, Chen XY, Trejo J, et al. Intracellular delivery of an anionic antisense oligonucleotide via receptor-mediated endocytosis. Nucleic Acids Res 2008;36(8):2764–76.
[56] Panyam J, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J 2002;16(10).
[57] Tang Y, Li YB, Wang B, Lin RY, van Dongen M, Zurcher DM, et al. Efficient in vitro siRNA delivery and intramuscular gene silencing using PEG-modified PAMAM dendrimers. Mol Pharmaceut 2012;9(6):1812–21.
[58] Lu J, Owen SC, Shoichet MS. Stability of self-assembled polymeric micelles in serum. Macromolecules 2011;44(15):6002–8.
[59] Kow SC, McCarroll J, Valade D, Boyer C, Dwarte T, Davis TP, et al. Dicer-labile PEG conjugates for siRNA delivery. Biomacromolecules 2011;12(12):4301–10.
[60] Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall WS, Karpilow J, et al. The contributions of dsRNA structure to dicer specificity and efficiency. RNA 2005;11(5):674–82.
[61] Singh N, Agrawal A, Leung AKL, Sharp PA, Bhatia SN. Effect of nanoparticle conjugation on gene silencing by RNA interference. J Am Chem Soc 2010;132(24):8241–3.