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Rapid Synthesis of a Lipocationic Polyester Library via Ring-Opening Polymerization of Functional Valerolactones for Efficacious siRNA Delivery

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Supporting Information

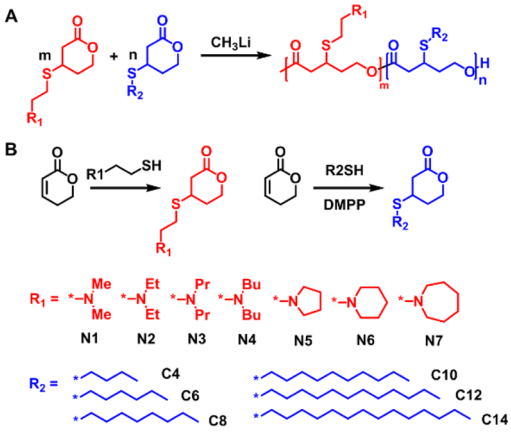
ABSTRACT: The ability to control chemical functionality is an exciting feature of modern polymer science that enables precise design of drug delivery systems. Ring-opening polymerization of functional monomers has emerged as a versatile method to prepare clinically translatable degradable polyesters.[1] A variety of functional groups have been introduced into lactones; however, the direct polymerization of tertiary amine functionalized cyclic esters has remained elusive. We report a strategy that enabled the rapid synthesis of >130 lipocationic polyesters directly from functional monomers without protecting groups. These polymers are highly effective for siRNA delivery at low doses *in vitro* and *in vivo*.

Gene silencing via the RNA Interference (RNAi) mechanism is a promising strategy to treat major diseases including cancer, genetic disorders, and viral infections. However, the success of short interfering RNA (siRNA)-based therapies has been limited by the difficulty of delivering these highly anionic biomacromolecular drugs into cells.[2] Polymers are an important class of materials for drug and nucleic acid delivery due to the versatility in constructing different nanostructures including micelles, polyplexes, dendrimers, and polymer−siRNA conjugates. Yet they currently lag behind in efficacy compared to lipid-based carriers.[2c]

Herein, we report a library of 139 degradable lipocationic polyesters that were directly synthesized from tertiary amine-bearing valerolactone and alkylated valerolactone monomers, thereby overcoming current synthetic limitations in functionality and scalability. Initiation with methyl lithium promoted rapid polymerization with high monomer conversion and decent control over molecular weight. Cationic and hydrophobic moieties were incorporated at precise ratios, which allowed us to fine-tune the material composition and correlate structure with siRNA delivery activity. Formulated polymeric nanoparticles (NPs) exhibited high delivery efficiency, enabling >95% knockdown *in vitro* for the top performing materials using only a 5 nM siRNA dose. Automated, high throughput screening of this library revealed a strong correlation between delivery efficacy and chemical structure. NPs could localize to tumors *in vivo* after intravenous delivery and were able to silence gene expression in tumor-bearing mice. We believe that this report introduces a versatile way to directly synthesize lipocationic polymers for gene delivery and is a promising step toward closing the activity gap between lipids and polymers.

Cationic polymers, such as polyethylenimine and polylysine,[2a] are widely used as nucleic acid carriers; however, application of these materials to *in vivo* disease models is often limited by their cytotoxicity. Since incorporating biodegradable bonds will facilitate elimination of materials used in biomedical applications, the development of degradable polymer-based siRNA delivery systems represents an important goal. Aliphatic polyesters are used in FDA-approved products, but lack the required functional groups to complex and deliver nucleic acids.[3] Numerous studies of lipids and nondegradable polymers have implicated tertiary amines and alkyl chains as key functional groups for effective siRNA delivery.[4] Yet, their potential incompatibility with esters has made direct synthesis of degradable polymers with amino groups challenging. Step-growth polymerization can be used to overcome this issue,[1a,5] but these methods do not offer control over molecular weight and molecular weight distribution.

Scheme 1. [a]



[a](A) A combinatorial library of 139 lipocationic polyesters was synthesized. (B) Unprotected monomers were synthesized in one step from commercially available DPO with aminothiols (N1−N7) and alkylthiols (C4−C14).

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Direct synthesis of polymers using ring-opening polymerization (ROP) offers greater control over polymer composition and the ability to make block copolymers.[1] A number of excellent examples of functional polyesters have been reported via direct polymerization of functional lactones and/or postpolymerization modification routes.[6] To date, however, low yields due to multistep synthetic pathways have limited the scale and chemical scope of polymer production.[1b] Moreover, there has been no report on polymerization of amine-bearing lactones. The development of combinatorial polymer libraries is an effective way to discover efficacious nucleic acid carriers.[4b,5a,b] We were therefore motivated to employ a strategy to prepare functional lactone monomers in one step from commercially available starting materials that could be polymerized with high monomer conversion to yield a scalable polymer library. These attributes are essential to be able to synthesize and screen a variety of copolymer compositions and discover optimal delivery materials. To our knowledge, this is the first reported synthesis of lactones bearing tertiary amines and combinatorial ring-opening polymerization.

To develop an efficacious degradable polymer delivery system, we used a unique synthetic strategy to rapidly build a library of lipocationic polyesters via anionic ROP. As previously noted, tertiary amines and alkyl chains are critical functional groups for effective siRNA delivery.[2c,4,7] However, the synthesis of amine-containing lactones (and polyesters) is not straightforward because nucleophilic amines can hydrolyze esters over time. To pursue this goal, we initially explored aza-Michael addition of secondary amines to 5,6-dihydro-2*H*-pyran-2-one (DPO), but the resulting functionalized valerolactones could not be polymerized. Monomers were successfully synthesized, but underwent retro-Michael addition in the presence of Lewis acid catalysts and did not open under basic conditions. Concurrent to these efforts, successful thiol-Michael addition to DPO was reported.[6f] Inspired by that paper, we adapted our protocol to utilize functional thiols. We synthesized seven tertiary amine containing aminothiols via reaction with ethylene sulfide (Figure S1). The resulting amino thiols were reacted with DPO at a 1:1 ratio to give tertiary amine functionalized valerolactone monomers (Scheme 1). Six alkylated valerolactone monomers were also synthesized via a similar strategy, but required addition of dimethylphenylphosphine (DMPP) to catalyze the reaction. In this way, monomers were synthesized through a single step, which enabled functional monomer/polymer synthesis in gram scale (Figure S34). Although we purified most of the monomers reported herein, complete reaction conversion enabled the polymerization to be conducted in one pot from monomer synthesis to polymer synthesis.

To explore structure–activity relationships (SARs), we synthesized random copolymers from all monomers through anionic ROP using methyl lithium[8] as the initiator (Scheme 1). This allowed us to prepare polymers without initiator chain end functionality, so that delivery ability could be better correlated with the polymer composition. Homo- and random (co)-polymerizations were carried out in bulk in a glovebox and reached high monomer conversion (90% on average) (Table S1), which allowed for siRNA delivery screening directly without purification. The library consists of different combinations of the two monomer types at three different mole ratios in the feed (3:1, 2:2, and 1:3). The final monomer incorporation was very close to the feed ratio (Table S2). There was good agreement of molecular weight to theoretical molecular weight based on Gel Permeation Chromatography (GPC) and ^{1}H NMR (Table S1). The mechanism of this reaction involves the nucleophilic attack of the carbonyl carbon on the monomer by a methyl anion in the initiator, which results in scission of the acyl-oxygen bond, and the polymerization propagates via an alcoholate ion (Scheme S3). It is worth noting that polymerization with some conventional ROP catalysts including tin(II) octanoate, alkoxides, and organocatalysts[6f,9] were not successful. However, Grignard reagents were able to initiate the functional valerolactones reported in this communication (Table S4). In this way, polymers with functional groups at the chain ends could be prepared (a full exploration will be published in a separate report). The use of addition reactions and easily obtainable starting materials allowed us to rapidly build a library of 139 functional polyesters in about 1 week.

To date, the most efficacious materials for *in vivo* siRNA delivery have been lipid nanoparticles (LNPs) composed of a cationic or ionizable lipid, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, and lipid poly(ethylene glycol) (PEG).[4a,c,7a] These components reduce aggregation and provide enhanced NP stability at physiological conditions. In order to prepare *in vivo* ready NPs and to mitigate potential toxicity of cationic polymers, we employed a similar strategy in this work, replacing cationic lipids with lipocationic polymers (Figure 1A).

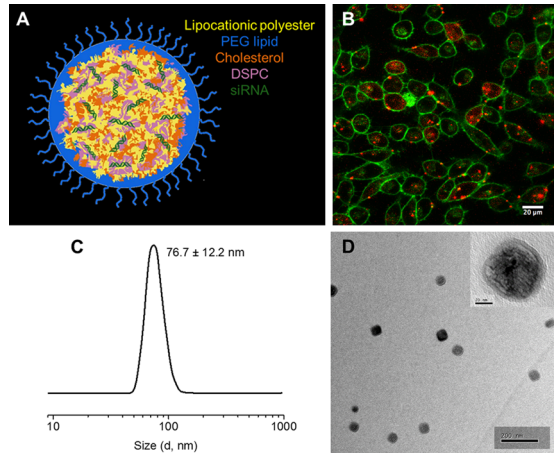

Figure 1. (A) Representative scheme of polymeric nanoparticle composition. (B) Cellular internalization of Cy5.5-siRNA-loaded N1C8 (2:2) NPs (red) after 3 h of incubation in HeLa-Luc cells. The cell membrane was stained with CellMask (green). (C) Particle Size distribution measured by DLS and (D) TEM image obtained for formulated NP N1C8 (2:2).

A NP formulation consisting of polymer/DSPC/cholesterol/PEG lipid = 50:10:35:5 (mole) typically had an average diameter of ~75 nm in PBS by dynamic light scattering (DLS) (Figure 1C). The size could be tuned from 40 to 300 nm by adjusting the mixing conditions and the formulation components. For example, increasing the PEG lipid amount yielded smaller NPs (Figure S41). The morphology of an efficacious polymeric NP N1C8 (2:2) was studied using Transmission Electron Microscopy (TEM) (Figure 1D). An average diameter of 70 nm was observed, which is in agreement with DLS results. The NPs exhibit spherical morphology where a less dense particle shell consists mainly of PEG lipid and a more textured and electron dense core consists of siRNA in aqueous pockets surrounded by DSPC and lipocationic polymers. This is consistent with previous computational modeling reports of nanostructured LNPs.[10] The cellular uptake of various lead polymeric NPs was

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monitored in HeLa-Luc cells using confocal microscopy. NPs were internalized into cells within 3 h (Figures 1B and S42).

The lipocationic polyester library was screened for siRNA delivery efficacy using an *in vitro* luciferase reporter assay in HeLa-Luc cells with the aid of an automated, fluid-handling robot (Figure S38). NPs were formulated by rapidly combining an ethanol solution of lipocationic polymers, DSPC, cholesterol, and lipid PEG with an acidic aqueous buffer containing siRNA at a final molar ratio of 100:1 (polymer/siRNA). After dilution in PBS, the formulated nanoparticles were directly added to growing cells. Luciferase activity and cytotoxicity were measured after 48 h relative to untreated cells. The polymeric NPs were nontoxic to cells at the screening dose (blue dots), with ∼15% of the polymer library enabling more than 80% knockdown efficiency (red bars). Six polymers enabled >90% silencing at a screening dose of 38.4 nM. Delivery using only DSPC, cholesterol, and lipid PEG did not exhibit significant silencing at this dose (Figure S38). Delivery efficiency strongly correlated with chemical structure because cationic and hydrophobic moieties were incorporated at precise ratios (Table S2). A heat map organized by the feeding ratio of the aminothiol monomer vs alkylthiol monomer elucidated trends related to hydrophobicity and pK_a (Figure 2). In the top third of the heat map

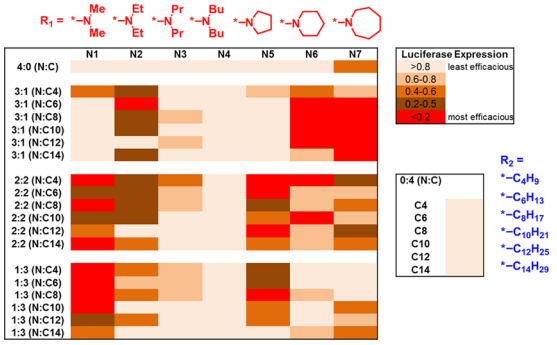

Figure 2. Heat map showing *in vitro* siRNA delivery and structure–activity relationships (SAR) for the formulated polyester library in Hela-Luc cells. The formulation consisted of polymer/DSPC/cholesterol/PEG lipid = 50:10:38:2 (mole). Hydrophobicity generally increases from top to bottom, by increasing the feed ratio of the alkyl monomer (4:0 to 1:3) and by increasing the alkyl length (C4 to C14).

(3:1 amino/alkyl) ratio, the greatest activity is seen with the most hydrophobic amines, piperidine (N6) and azepane (N7). Moreover, N7 was the only homopolymer that showed activity. For polymers containing the dimethylamine group (N1), additional hydrophobic content is required to promote NP stability at pH 7.4 and enable delivery (see bottom left third of heat map). When the hydrophobicity was increased (going from N1 to N3 left to right), less additional hydrophobic content from the alkyl comonomers was required to give a high delivery efficiency. Within a defined series that showed a smooth decrease of activity: N1C8 (2:2) → N2C8 (2:2) → N3C8 (2:2) → N4C8 (2:2), the pK_a decreased from 6.1 to 4.0 (Figure S36). This is in agreement with reported pK_a data for LNPs.[4c] Dibutylamine (N4) polymers were completely inactive, likely due to steric hindrance that reduced binding combined with a decreased pK_a (Figure S36). For monomers containing cyclic amine side chains, N5 displayed a similar trend as N1, where a more hydrophobic comonomer was needed to give better delivery efficiency. N6–N7 were more hydrophobic and, therefore, were most active when copolymerized with less hydrophobic monomers (3:1 ratio) (top third of the heat map). Overall, the 2:2 group showed the largest number of hits because this feed ratio provides the highest degree of balance of lipocationic properties. These data suggest an optimized combination of amino monomers and hydrophobic monomers is necessary to impart delivery activity.

To investigate *in vitro* efficacy at low doses of siRNA, a dose response was conducted for the top 10 performing polymers (Figure 3). Polymers were resynthesized and purified by dialysis to verify activity. NPs were incubated with cells at doses between 2.4 and 38.4 nM siRNA. Dose dependent silencing was observed for all the polymers tested. Five polymers facilitated greater than 80% silencing at a siRNA dosage of 9.6 nM. Also, two polymers enabled >90% silencing at a dosage of only 2.4 nM. In contrast, RNAiMax was less effective in silencing luciferase expression head-to-head at the same doses. To our knowledge, this is among the most potent polymer-based delivery systems reported to date.

Cancer therapy is one of the most promising applications for siRNA delivery. We therefore evaluated the ability of lipocationic polyester NPs to localize and deliver siRNA to tumors. We delivered a single dose of 2.5 mg/kg siRNA (1.25 mg/kg siLuc + 1.25 mg/kg Cy5.5-siLuc) via intravenous (IV) tail vein injection to nude mice bearing MDA-MB-231-Luc xenograft tumors in both flanks. After 2.5 h, remarkably high tumor accumulation of N1C4 (2:2) NPs was measured (Figure 4A). Fluorescence signals from the liver and kidneys were also visualized. *Ex vivo* imaging of harvested organs confirmed effective tumor uptake (Figure S43). Moreover, luciferase activity in the tumors was greatly reduced after intratumoral (IT) injection of 2.5 mg/kg

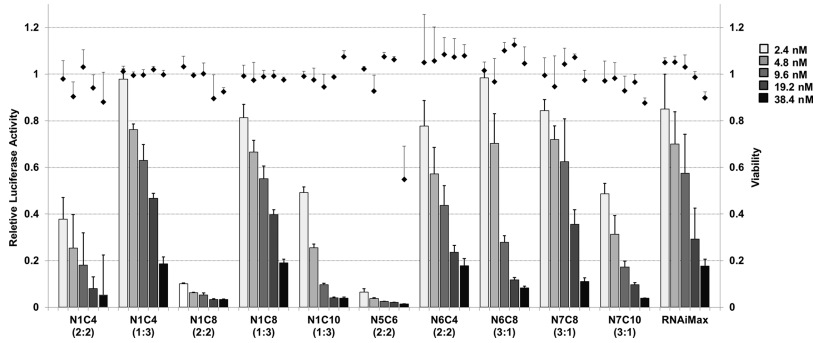

Figure 3. Dose–response of silencing in Hela-Luc cells for selection of the top performing polymers. The dose scale is 6.25 ng (2.4 nM), 12.5 ng (4.8 nM), 25 ng (9.6 nM), 50 ng (19.2 nM), and 100 ng (38.4 nM) going from left to right. Bars represent relative luciferase activity, while dots represent cell viability. Results were normalized to untreated cells (n = 4). N1C8 (2:2) vs RNAiMax: ****P < 0.0001.

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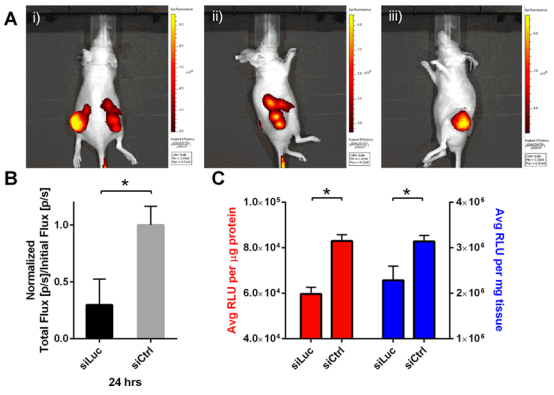

Figure 4. (A) N1C4 (2:2) NPs provided effective accumulation in tumor xenografts after IV injection. A representative mouse is shown from three angles. Luciferase silencing was measured in tumors 24 h after injection by (B) bioluminescence imaging or (C) in tissue lysates normalized against total protein level or total tissue amount ($n = 4$; $^*P < 0.05$).

siLuc. Luciferase was quantified by bioluminescence (Figure 4B) and by tissue homogenization on total protein and tissue levels (Figure 4C).

In the spectrum of delivery systems, polymers have many advantages including tunable structural composition, degradability, and biocompatibility. Yet, they are currently less effective than lipid-based delivery vehicles. To overcome this challenge, we have incorporated key ionizable amines and hydrophobic alkyl chains into polyesters. We synthesized a library of lipocationic polyesters directly from functional monomers in high yield, fast time (~2 min), and gram scale. This was accomplished with precise monomer incorporation ratios to enable tunable hydrophobicity and pK_a. Formulated NPs enabled siRNA mediated silencing *in vitro* and *in vivo* at low dose. Notably, NPs could localize to tumors *in vivo* after IV delivery and were able to silence gene expression in tumor-bearing mice. This new class of lipocationic polyesters is a promising step toward closing the activity gap between lipids and polymers. Finally, we envision that the versatility of the chemical methods may allow preparation of functional polyesters for a variety of applications (not only for gene delivery) because nearly any thiol can be used to synthesize functional monomers.

■ ASSOCIATED CONTENT

Supporting Information

Includes detailed Synthetic Procedures for all aminothiols, monomers, and polymers (including characterization by ^{1}H and ^{13}C NMR, LC-MS, and GPC) (Figures S1−S37), Materials, Instrumentation, and Biological Studies (Figures S38−S44). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b03429.

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Notes

The authors declare no competing financial interest.

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