Oligo(serine ester) Charge-Altering Releasable Transporters: Organocatalytic Ring-Opening Polymerization and their Use for *in Vitro* and *in Vivo* mRNA Delivery

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Ⓢ *Supporting Information*

ABSTRACT: RNA technology is transforming life science research and medicine, but many applications are limited by the accessibility, cost, efficacy, and tolerability of delivery systems. Here we report the first members of a new class of dynamic RNA delivery vectors, oligo(serine ester)-based charge-altering releasable transporters (Ser-CARTs). Composed of lipid-containing oligocarbonates and cationic oligo(serine esters), Ser-CARTs are readily prepared (one flask) by a mild ring-opening polymerization using thiourea anions and, upon simple mixing with mRNA, readily form complexes that degrade to neutral serine-based products, efficiently releasing their mRNA cargo. mRNA/Ser-CART transfection efficiencies of >95% are achieved *in vitro*. Intramuscular or intravenous (iv) injections of mRNA/Ser-CARTs into living mice result in *in vivo* expression of a luciferase reporter protein, with spleen localization observed after iv injection.

Messenger RNA (mRNA) is advancing fundamental research and medicine through its ability to induce the transient catalytic expression of target proteins *in vitro*, *in vivo*, and *ex vivo*. Applications of mRNA include protein replacement therapy, gene editing, vaccination, and cancer immunotherapy.[1] However, the challenge of developing synthetically accessible, affordable, safe, and effective delivery vectors that extracellularly protect and intracellularly release mRNA have hampered applications, driving demand for improved delivery systems.[2,3] Current delivery strategies focus on mechanical methods and viral and nonviral vectors.[4–6] Mechanical methods that temporarily render the cellular membrane permeable are limited to accessible tissues and *ex vivo* techniques, often suffer from poor cell viability, and encounter scalability challenges.[7] Viral vectors offer broader administration options but are coupled with cost and immunogenicity concerns and cargo size limitations.[8,9] These restrictions have stimulated interest in nonviral vectors, typically lipid nanoparticles and cationic polymers that form electrostatic complexes with polyanionic nucleic acids.[7,10–16] Despite advances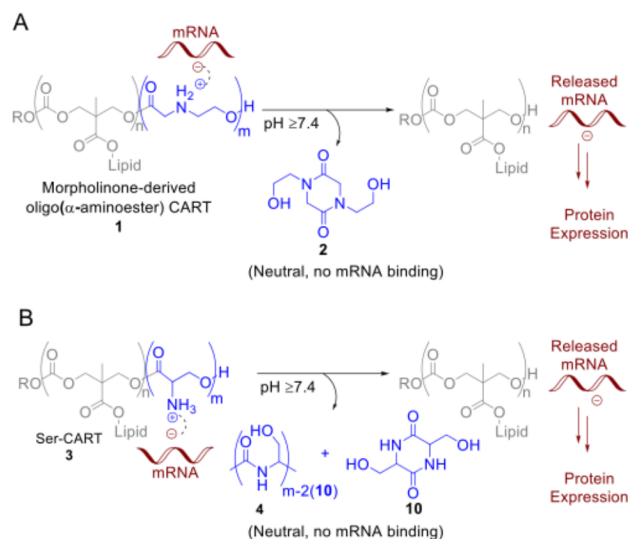 in nonviral vectors, challenges with accessibility, formulation, efficacy, tolerability, and targetability have prompted the search for improved delivery systems, with a particular emphasis on degradable vectors.[17–22]

We recently reported a new class of synthetic biodegradable gene delivery materials, dubbed charge-altering releasable transporters (CARTs) (e.g., **1**).[23] These first-generation CARTs **1** are amphipathic diblock co-oligomers consisting of a lipophilic oligocarbonate sequence followed by a cationic morpholinone-derived α-amino ester backbone. These transporters operate through an unprecedented mechanism in which the cationic oligo(α-amino ester) block electrostatically complexes the anionic nucleic acid cargo and subsequently undergoes an irreversible rearrangement to neutral small molecules (e.g., **2**), resulting in cargo release (Scheme 1A). While morpholinone-based CARTs **1** are effective for mRNA and plasmid delivery in many cell lines, including T-lymphocytes, these first-generation transporters represent only one subclass of a potentially broad and unexplored platform of charge-altering vectors for gene delivery.[23–27]

Here we report the synthesis and evaluation of a new class of charge-altering vectors based on oligo(serine esters), denoted as Ser-CARTs (**3**). Differing from CARTs with oligocationic backbones, Ser-CARTs incorporate a charge-altering side-chain amine to complex the mRNA cargo and produce neutral serine-based byproducts upon degradation, resulting in mRNA release (Scheme 1B). In addition to their biocompatibility and expected rearrangement into peptides **4**,[28–30] oligo(serine esters) were selected for study over other degradable amine-functionalized polyesters[18,31] because of their activating α-amino ester motif. Studies suggest that the rapid rearrangement of morpholinone-derived oligo(α-amino esters) is partially due to the activation of a backbone ammonium group positioned α to the ester repeating unit.[23] In this study, we synthesized and characterized the degradation of side-chain ammonium-containing oligo(serine esters), which are structural isomers of oligo(serine amides) **4**.[32] We demonstrate that Ser-CARTs are readily formed (one flask) and efficiently deliver mRNA in cultured cells and live mice.

Scheme 1. mRNA Release Mechanisms of (A) Morpholinone-Derived CARTs 1[23] and (B) Ser-CARTs 3[a]

[a]Both systems utilize activated α-amino esters, with oligo(serine esters) rearranging to neutral serine-based products via O−N acyl shifts.

To study Ser-CARTs, we developed a polymerization method that avoids the control issues and harsh conditions previously reported for oligo(serine ester) synthesis.[30,33−37] Our procedure benefits from the commercial availability of the *N*-trityl-L-serine lactone monomer (serine lactone) and an organocatalytic ring-opening polymerization (OROP)[38−43] strategy, utilizing a thiourea anion catalyst recently developed for the OROP of lactones and cyclic carbonates (Figure 1).[44]
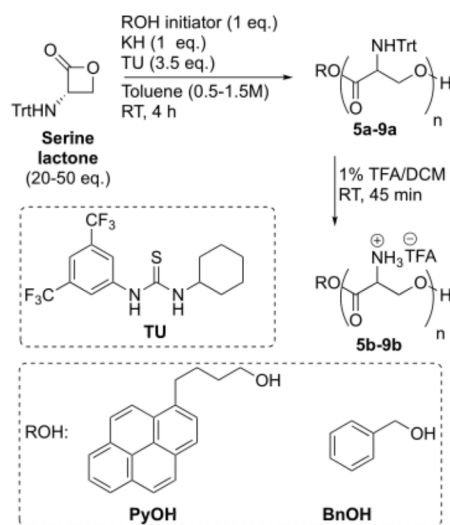

Figure 1. Poly(serine ester) synthesis.

Specifically, we found that the OROP of serine lactone with 1-(3,5-bis(trifluoromethyl)phenyl)-3-cyclohexylthiourea (TU) and potassium hydride (KH) in the presence of an alcohol initiator proceeds at room temperature in hours to generate trityl-protected poly(serine esters) **5a−9a** with predictable molecular weights (M_n = 7−17 kDa) and narrow dispersities (Đ = 1.11−1.24), avoiding the previously reported multimodal

distributions (Tables 1 and S1).[35,36] After polymer isolation, trityl groups are removed using 1% TFA to yield cationic

Table 1. Poly(serine ester) Characterization[a]

	ROH	$[M]$[b]	$[M]/$ $[ROH]$	conv. (%)[c]	DP[d]	M_n (kDa)[e]	Đ
5a	BnOH	1.0	50	>95	62	16.7	1.17
6a	BnOH	0.5	50	89	47	12.6	1.24
7a	BnOH	1.5	50	93	61	15.6	1.20
8a	BnOH	1.0	20	>95	20	7.7	1.21
9a	PyOH	1.0	50	>95	49	15.8	1.21

[a]Polymerizations were run for 4 h at room temperature in toluene. [b]Serine lactone molar concentration. [c]Determined by NMR spectroscopy. [d]Degree of polymerization, determined by NMR end-group analysis after dialysis. [e]Determined by gel-permeation chromatography.

poly(serine esters) **5b−9b** with no significant decrease in molecular weight, as determined by end-group analysis (Figure S1). This facile controlled polymerization of serine lactone is noteworthy, as prior reports suggested that β-lactone OROP is inefficient.[45−47]

As studies indicate that the ring opening of β-lactones can occur by two mechanisms,[48] we performed the stoichiometric ring opening of serine lactone with 1 equiv of benzyl alcohol using the KH/TU catalyst. Analysis of the resulting product by HMBC NMR indicated that ring opening proceeded through acylation of benzyl alcohol by the lactone to generate the alkoxy-terminated benzyl serine ester rather than by nucleophilic attack at the β-carbon[48] to generate the carboxylate (Figure S2).

Having developed an effective poly(serine ester) synthesis, we next investigated whether poly(serine esters) would rearrange in biologically relevant pH regimes in the absence of an mRNA cargo. While uncomplexed poly(serine ester) **9b** (degree of polymerization (DP) = 47) is stable under its generation conditions, at pH 7.4 it begins to degrade in minutes, producing in hours the known rearrangement product, oligo(serine amide) **4**,[28,29] and also a previously unreported product, dimerized serine diketopiperazine (DKP, **10**), as confirmed by NMR and LC−MS analyses (Figure 2A; also see the Supporting Information). Analysis of the degradation **9b** at pH 7.4 revealed that the DKP yield increased over 24 h, resulting in final yields of 55% DKP and 45% oligo(serine amides) of various lengths (Figure 2B). While prior reports indicated that the aqueous degradation of poly(serine esters) generates poly(serine amides) by a series of O-to-N acyl shifts,[28,29] our studies uncovered DKP as a significant serine-based byproduct.

We propose that the degradation of poly(serine esters) to generate DKP **10** follows a charge-altering mechanism (Figure 2A) related to that proposed for the degradation of morpholinone-based poly(α-amino esters) (Figure S3) but now involving a primary side-chain amine.[23,28,29] For pH values at which some of the pendant ammonium groups are deprotonated, nucleophilic attack of the resultant primary amine on an adjacent H-bond-activated ester carbonyl (five-membered O−N acyl shift) would generate an amide and contract the polymer backbone, positioning the proximal amine for a six-membered O−N acyl shift to liberate DKP. Importantly, the formation of the expected oligo(serine amide) **4** and the newly observed DKP **10** provide charge-altering
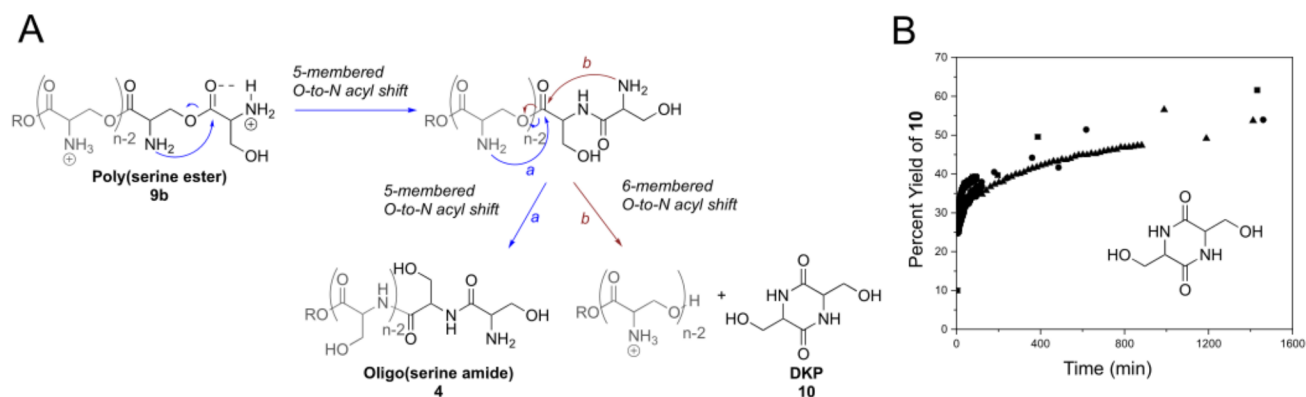
Figure 2. (A) Proposed rearrangement of poly(serine esters). (B) Time-dependent yield of DKP **10** when **9b** was subjected to pH 7.4–buffered D$_2$O at room temperature (trial 1 ■, trial 2 ●, trial 3 ▲).
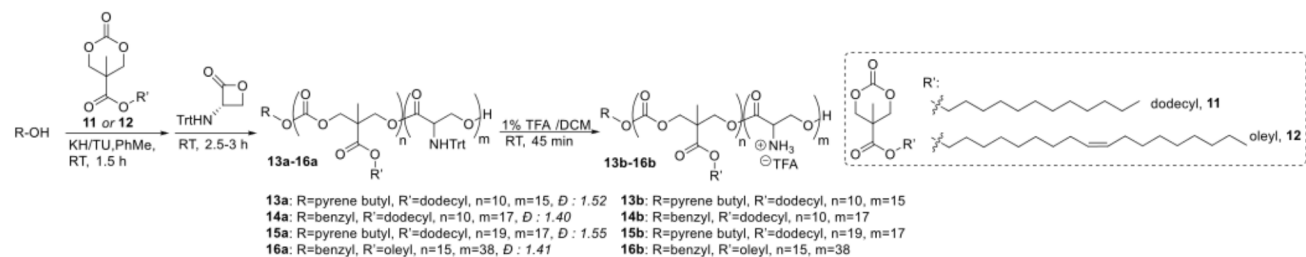

Figure 3. Synthesis of Ser-CARTs **13b−16b**.

transformations from cationic α-amino esters to neutral β-hydroxyamides that are critical for CART-mediated mRNA complexation, delivery, and release.[23]

Having shown that uncomplexed poly(serine esters) degrade to neutral products, we explored the use of Ser-CARTs for polyanion complexation and delivery, focusing on mRNA. As lipid domains are vital in polyanion delivery vehicles,[10,24,49−53] we generated a series of amphiphilic diblock co-oligomers composed of a dodecyl (C12)- or oleyl (C18)-modified oligocarbonate sequence and a cationic oligo(serine ester) sequence from carbonate monomer **11** or **12** and serine lactone, respectively, using our thiourea anion catalyst system. Co-oligomers **13a−16a** (R′ = dodecyl: $n = 10$, $m = 15$; $n = 10$, $m = 17$; $n = 19$, $m = 17$; R′ = oleyl: $n = 15$, $m = 38$) were synthesized by a straightforward three-component, step-economical (one-flask) procedure using alcohol initiators (Figure 3 and Table S2). Deprotection of **13a−16a** with 1% TFA afforded cationic Ser-CARTs **13b−16b**.

To assess the efficacy of Ser-CARTs for mRNA delivery and expression, we investigated the transfection of cultured cells using Ser-CARTs complexed with mRNA encoding green fluorescent protein (EGFP) and analyzed by flow cytometry the total fluorescence and percentage of cells transfected. Notably, mRNA/Ser-CART polyplexes are produced by simple mixing of mRNA with Ser-CARTs. To optimize the in vitro formulation, we screened charge ratios of 5:1 to 100:1 (cation:anion (+/−)) using dodecyl-Ser-CART **13b** formulated with EGFP mRNA for delivery into HeLa cells. The highest fluorescence was observed at a charge ratio of 50:1 (+/−) under serum-free conditions, and the intracellular EGFP expression was confirmed by confocal and fluorescence microscopy (Figures S4−S6). Using this charge ratio, we compared EGFP mRNA delivery using Ser-CART **13b** to that using the commercial transfection reagent Lipofectamine 2000

(L2000) as a positive control and to that using naked EGFP mRNA. Significantly, **13b**-mediated EGFP mRNA delivery resulted in highly efficient (>95%) transfection in multiple cell lines (HeLa, CHO-K1, Raw-Blue), markedly outperforming L2000 (55−71% transfection) (Figure 4A). The transfection efficiency of **13b** is consistent with that of morpholinone-based CARTs **1**, highlighting the importance of the charge-altering block for mRNA delivery (Figure S7). Additionally, greater fluorescence was observed with **13b**-mediated EGFP mRNA delivery than with L2000-mediated delivery in HeLa cells (Figure 4B). Ser-CART **14b** also exhibited >95% transfection for EGFP mRNA delivery in HeLa cells, suggesting that the initiator does not significantly influence transfection, as pyrene butanol was used for **13b** and benzyl alcohol for **14b** (Figure 4C). In contrast to dodecyl-Ser-CARTs **13b** and **14b**, lower transfection levels were observed with more lipid-rich **15b**. Oleyl-Ser-CART **16b** also resulted in >95% transfection. Ser-CARTs retain these high transfection efficiencies when stored at 0 °C under nitrogen (Figure S8). Notably, formulation of EGFP mRNA with poly(serine ester)$_{47}$ **9b** or DKP **10** resulted in negligible fluorescence (Figure S9). Importantly, DKP **10** was found to be nontoxic at concentrations up to 500 μM in HeLa cells (Figure S10).

We next explored the temperature-dependent uptake of Ser-CARTs. HeLa cells treated with Cy5-labeled mRNA/**13b** at 4 °C resulted in a 70% reduction in Cy5 fluorescence relative to cells incubated at 37 °C, indicating mainly endocytic uptake of the polyplexes, as incubation at 4 °C inhibits endocytosis (Figure 4D).[54]

Analysis of the EGFP mRNA/Ser-CART polyplexes by dynamic light scattering indicated hydrodynamic diameters of <190 nm (dodecyl-based **13b**, ~154 nm; oleyl-based **16b**, ~174 nm), which are significantly smaller than those of morpholinone-based CARTs (~250 nm) (Table S3).[23] Zeta
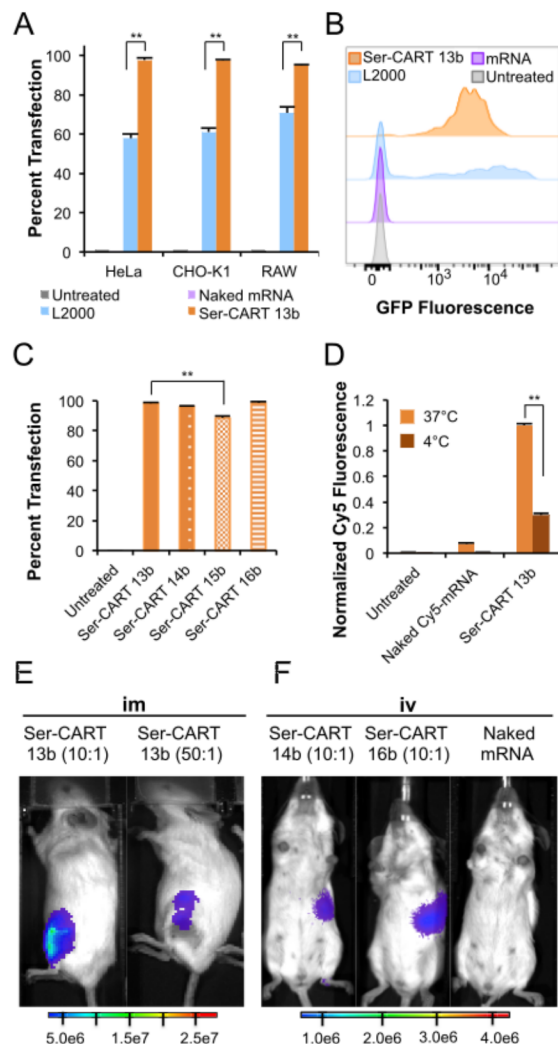
Figure 4. Delivery of mRNA/Ser-CARTs *in vitro* and *in vivo*. mRNA/Ser-CARTs were formulated at a 50:1 (+/−) ratio unless otherwise specified. (A) Percent transfection of HeLa, CHO-K1, or Raw-Blue (RAW) cells using L2000 or EGFP mRNA/**13b**. (B) Histogram of HeLa cells treated with naked EGFP mRNA, L2000, or mRNA/**13b**. (C) Percent transfection of HeLa cells treated with EGFP mRNA/**13b**−**16b**. (D) Cy5-labeled mRNA/**13b** uptake into HeLa cells after 1 h incubation at 4 or 37 °C. (E, F) Representative bioluminescence images of mice 7 h after (E) im injection with fLuc mRNA/**13b** at 10:1 (+/−) (left, *n* = 4) or 50:1 (+/−) (right, *n* = 2) and (F) iv injection with fLuc mRNA/**14b** (left, *n* = 4), fLuc mRNA/**16b** (middle, *n* = 3), both at 10:1 (+/−), or naked fLuc mRNA (right, *n* = 1). Results in (A), (C), and (D) are averages of three or more experiments. Error bars represent ±SD. **, *p* < 0.0004.

potential measurements were used to study the time-dependent surface charge. When added to RNase-free water, mRNA/Ser-CARTs were initially positive (**13b**, 37 ± 6 mV; **16b**, 52 ± 10 mV) but over 1 h became negative (**13b**, −20 ± 7 mV; **16b**, −15 ± 5 mV), consistent with rearrangement of the cationic oligo(serine ester) block to neutral products (Figure S11).

Encouraged by these *in vitro* studies, we explored the *in vivo* utility of Ser-CARTs for mRNA delivery using two different

modes of administration in female BALB/c mice. Luciferase (fLuc)-coding mRNA was chosen as a model reporter gene since luciferase expression can be quantitatively monitored in real time in living mice.[55,56] After fLuc mRNA delivery *in vitro* was confirmed (Figure S12), fLuc mRNA/**13b** polyplexes were administered via intramuscular (im) injection into mice at a 10:1 or 50:1 (+/−) ratio, and expression was visualized after 7 h by bioluminescence imaging (Figure 4E).[25] Both conditions resulted in protein expression, but mice treated at the lower 10:1 (+/−) ratio resulted in enhanced luciferase expression (Figure S13). At the 10:1 (+/−) ratio, fLuc mRNA/**14b** or **16b** polyplexes administered to mice via intravenous (iv) tail vein injection resulted in luciferase expression localized in the spleen, a target organ for several therapeutic indications (Figures 4F and S14). Importantly, mRNA/Ser-CARTs formulated at the 10:1 (+/−) ratio resulted in improved cell viability (78−87% relative to untreated HeLa cells) compared with 50:1 (+/−) *in vitro* (Figure S15).

In conclusion, mRNA delivery with the readily synthesized (one flask) Ser-CARTs results in efficient transfection and high protein expression *in vitro* and *in vivo*. Further benefits of Ser-CARTs over first-generation CARTs include the degradation of the oligo(serine ester) block at biological pH into serine peptides (oligo(serine amides) and DKP), the commercial availability of the monomer, and the smaller size of the polyplexes. The accessibility, tunability, effectiveness, and organ selectivity of mRNA/Ser-CART polyplexes bode well for their use in biomedical research and therapeutic applications. Furthermore, this study establishes the generality of charge-altering architectures for polyanion delivery. We are currently exploring Ser-CARTs for targeting, cotransfections, and clinical indications with an initial emphasis on vaccination and immunotherapy.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b03154.

> Experimental details, including representative spectra and biological assays (PDF)

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Notes
The authors declare no competing financial interest.

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