

Sequence-Defined Oligomers from Hydroxyproline Building Blocks for Parallel Synthesis Applications

Rosemary L. Kanasty, Arturo J. Vegas, Luke M. Ceo, Martin Maier, Klaus Charisse, Jayaprakash K. Nair, Robert Langer, and Daniel G. Anderson*

Abstract: The functionality of natural biopolymers has inspired significant effort to develop sequence-defined synthetic polymers for applications including molecular recognition, self-assembly, and catalysis. Conjugation of synthetic materials to biomacromolecules has played an increasingly important role in drug delivery and biomaterials. We developed a controlled synthesis of novel oligomers from hydroxyproline-based building blocks and conjugated these materials to siRNA. Hydroxyproline-based monomers enable the incorporation of broad structural diversity into defined polymer chains. Using a perfluorocarbon purification handle, we were able to purify diverse oligomers through a single solid-phase extraction method. The efficiency of synthesis was demonstrated by building 14 unique trimers and 4 hexamers from 6 diverse building blocks. We then adapted this method to the parallel synthesis of hundreds of materials in 96-well plates. This strategy provides a platform for the screening of libraries of modified biomolecules.

Sequence-defined polymers play many roles in biological systems. The enormous structural diversity of biopolymers such as proteins and nucleic acids enables their many functions, including catalysis, self-assembly, molecular recognition, and information storage. The potential of these sequenced biopolymers has inspired interest in developing synthetic polymers with precisely defined monomer sequences for applications in biomaterials as well as for the tuning of bulk material properties.^[1] Strategies for controlling monomer sequence in synthetic polymers include controlled radical polymerizations,^[2–6] DNA-templated synthesis,^[7] and sequential or iterative synthesis.^[8–12] Sequential synthesis, in which each monomer is added to a polymer chain individually, offers

complete control over polymer sequence and enables the incorporation of a variety of diverse properties. Sequential synthesis methods are typically carried out on a solid support and include peptoid synthesis through the submonomer method,^[8,9] aminolysis of thiolactones,^[10] the Passerini^[11] and Biginelli^[13] multicomponent reactions, and the synthesis of triazene-based polymers,^[14] poly(alkoxyamine amide)s,^[15] and poly(amidoamine)s.^[16,17] While solid-supported synthesis allows facile purification, solution-phase synthesis conditions are desired because they provide homogenous reaction kinetics and allow the direct analysis of reaction mixtures without cleavage of the materials from the solid support.^[18,19]

Sequence-defined polymers provide precise control of composition, and thereby chemical and material properties, making them attractive for various applications that can benefit from parallel synthesis and library screening, such as nucleic acid delivery,^[20,21] biomaterials,^[22–25] and drug discovery.^[26–28] The combination of synthetic materials and biologics through bioconjugation has also played an increasingly important role in the development of novel therapeutics.^[29–31] The integration of biomolecular conjugation with library screening may be particularly useful in the development of novel bioconjugates. Given this potential to affect multiple fields, novel and scalable approaches to generating sequence-controlled polymers are needed.

Herein, we present a unique strategy for the synthesis of sequence-defined oligomers from building blocks based on hydroxyproline using only methods that are adaptable to high-throughput synthesis. We hypothesized that hydroxyproline, modified to create diverse monomer building blocks, could be sequentially linked to form novel polyurethanes while minimizing the use of protecting-group chemistry. Our

[*] Dr. R. L. Kanasty, Prof. R. Langer, Prof. D. G. Anderson
Department of Chemical Engineering
Massachusetts Institute of Technology
77 Massachusetts Avenue, Cambridge, MA 02139 (USA)
E-mail: dgander@mit.edu

Dr. R. L. Kanasty, Prof. A. J. Vegas, L. M. Ceo, Prof. R. Langer,
Prof. D. G. Anderson
David H. Koch Institute for Integrative Cancer Research
Massachusetts Institute of Technology
500 Main St., Cambridge, MA 02142 (USA)

Prof. A. J. Vegas, L. M. Ceo, Prof. R. Langer, Prof. D. G. Anderson
Department of Anesthesiology, Boston Children's Hospital
300 Longwood Ave, Boston, MA 02115 (USA)

Prof. R. Langer, Prof. D. G. Anderson
Division of Health Science Technology
Massachusetts Institute of Technology
77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

and
Institute for Medical Engineering and Science
Massachusetts Institute of Technology
77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

and
Harvard-MIT Division of Health Science and Technology
Massachusetts Institute of Technology
77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Prof. A. J. Vegas, L. M. Ceo
Department of Chemistry, Boston University
590 Commonwealth Avenue, Boston, MA 02215 (USA)

Dr. M. Maier, Dr. K. Charisse, Dr. J. K. Nair
Anlylam Pharmaceuticals, 300 Third Street, Cambridge, MA 02142 (USA)

Supporting information for this article can be found under:
<http://dx.doi.org/10.1002/anie.201602748>.

method enables the efficient incorporation of monomers that span a diverse structural space. Monomer coupling reactions consistently proceeded with nearly quantitative conversion, and oligomers were recovered with excellent purity. In place of a solid support, we employed a fluorous protecting group as a purification handle, which provides the advantage of solution-phase kinetics while allowing facile purification through solid-phase extraction. Fluorous solid-phase extraction (FSPE) exploits the interaction of perfluorocarbon-tagged molecules with a perfluorocarbon-modified silica matrix, allowing facile separation of fluorous-tagged material from untagged materials. This strategy is adaptable to parallel synthesis in a multiwell plate format, since the reaction conditions and purification methods are universal despite varying oligomer structure.

The hydroxyproline-based monomer structure was chosen for its ability to form stable polymer chains while incorporating a variety of side-chain functionalities. Linear monomer backbone structures were also explored, but cyclization of these monomers or of polymer chains was observed to compete with monomer coupling, especially when basic side chains were included (see the Supporting Information). Such cyclization side reactions are observed in other stepwise polymer synthesis methods; for example, diketopiperazines and aspartimides are known to form during peptide synthesis.^[32] The cyclic structure of monomers based on hydroxyproline prevents this uncontrolled cyclization and favors formation of the desired product. The only side reaction observed during the synthesis of hydroxyproline-based polyurethanes was hydrolysis of the active intermediate, which resulted in incomplete coupling.

The monomers were synthesized through amidation of the carboxylic acid on CbzZ-protected hydroxyproline, followed by deprotection of the amine (Figure 1a). Fluorous-CbzZ-protected starting monomers were synthesized by coupling a fluorous-CbzZ-NHS ester to hydroxyproline, followed by amidation of the carboxylic acid. Monomer coupling conditions were chosen to drive complete conversion of the fluorous-tagged monomer or polymer chain. The hydroxyl group of the starting monomer was activated by the addition of excess carbonyldiimidazole (CDI). The free hydroxy group was completely converted into the activated imidazole-*N*-carboxylic ester after one hour as observed by LCMS. Excess reagent was quenched and the intermediate was dried thoroughly to avoid hydrolysis of the imidazole-*N*-carboxylic ester. The second monomer was then added in excess and reacted overnight to yield the carbamate product (Figure 1b).

The fluorous protecting group at the end of each polymer chain allowed rapid purification of the oligomers by solid-phase extraction (SPE) through fluorous silica (Figure 1c). After each monomer addition, the reaction mixture was loaded onto a fluorous silica matrix and washed with an aqueous solvent to remove excess monomer and any impurities that lack a fluorous tag. The fluorous product was then eluted with a fluorophilic solvent such as acetone or tetrahydrofuran. This method afforded efficient separation of oligomers from excess reagents for a wide range of monomer and oligomer properties. Fluorous tags provide the benefit of facile purification afforded by solid-phase synthesis,

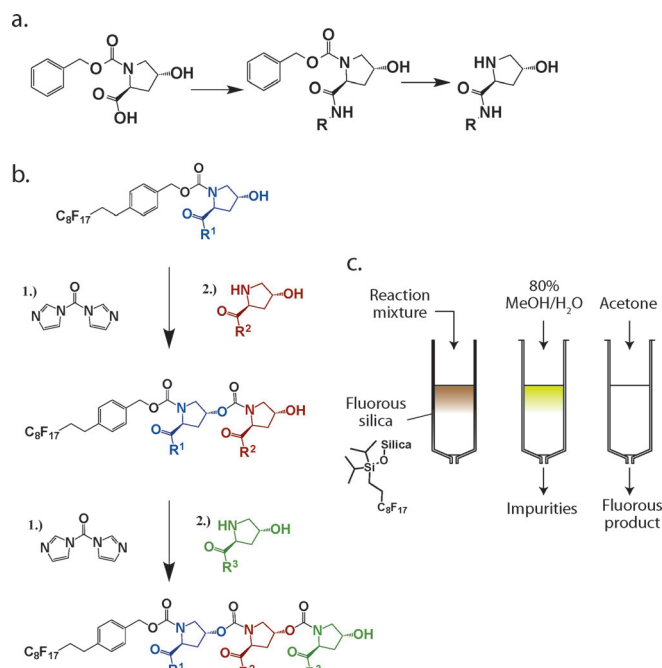


Figure 1. Synthesis and purification of hydroxyproline-based oligocarbamates. a) Monomer synthesis by amidation of hydroxyproline b) Monomer coupling using carbonyldiimidazole (CDI) c) Purification by fluorous solid-phase extraction (FSPE).

but with the advantage of solution-phase reaction kinetics and direct analysis of materials without the need for cleavage of materials from a solid support. This method can be readily adapted for high-throughput purification, since fluorous columns are available in a 96-well plate format.

To test the robustness and versatility of this method, we synthesized a set of 6 diverse monomers (Figure 2) and then combined these into 14 different trimers. We chose a monomer set that encompasses broad chemical space, including side chains of varying hydrophobicity, size, and charge in order to test the ability of this approach to generate structurally diverse materials. The reactions were monitored by LCMS and the monomer coupling consistently proceeded with high conversion (see the Supporting Information).

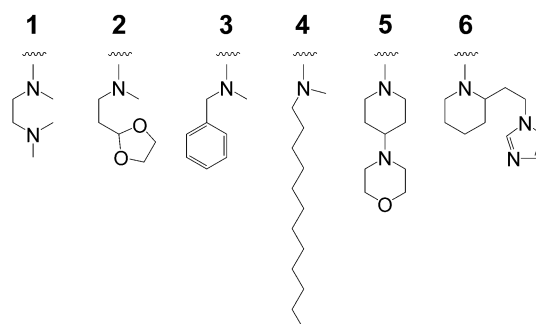


Figure 2. Structures of the monomer side chains. A set of 14 unique trimers was synthesized from six starting monomers. For naming purposes in Tables 1 and 2, the monomer side chains are assigned the number 1–6.

Minimal impurities were observed, and the most prevalent impurity was unreacted starting material (see the Supporting Information). This was due to hydrolysis of the imidazole-*N*-carboxylic ester intermediate, since formation of the intermediate proceeded reliably with complete conversion. This hydrolysis was the only side reaction observed, and hydrolysis was eliminated by thoroughly drying all reagents and controlling the exposure of the intermediate to moisture. Purity was optimal when the intermediate was dried down within 30 minutes of quenching and used within two hours.

For the 14 trimers synthesized, product purity was consistently high (averaging 94 % for dimers and 92 % for trimers), and the recovered yield was variable, averaging 72 % per step (Table 1 and Table 2). We observed reduced yield for the most hydrophilic oligomers, which was due to partial elution of the product in the aqueous wash during fluoruous SPE. Reducing the amount of washing improved the recovery in subsequent steps without reducing the purity. While not attempted here, we hypothesize that the yields with more hydrophilic monomers could be further improved by using purification tags with longer perfluorocarbon chains.

Table 1: Purity and yield of the dimers.^[a]

R1\R2	1	2	3	4	5	6
1		100/48		95/54		
2	90/55				97/76	
3				96/80		80/68
4		100/90		90/94		87/86
5			100/48	97/85		
6			100/68			

[a] Purity [%]/Yield [%].

Table 2: Purity and yield of the trimers.^[a]

R1R2\R3	1	2	3	4	5	6
12		71/36				
14				100/48		89/14
21				73/83	78/34	
25			100/67			
34	100/64					
36					90/54	
42					100/82	
44			100/87			
46					91/58	
53				100/97		
54		99/86				
63		95/60				

[a] Purity [%]/Yield [%].

To evaluate the utility of this method for synthesizing longer chains, we selected a subset of the trimers with varying hydrophilicity and charge and extended these chains to hexamers (Figure 3). As before, monomer coupling proceeded smoothly, often with complete conversion. Incomplete conversion resulting from hydrolysis of the intermediate was again the only side reaction observed (Figure 3). Incompletely reacted oligomers are retained by the fluoruous SPE purification, which results in the loss of a monomer from the sequence as polymerization is continued. To retain purity at higher degrees of polymerization, the moisture exposure of intermediates must be tightly controlled. The yield of recovered product averaged 80 % per step, an improvement over the average yield observed during trimer synthesis. This improvement was due to adjustments made to the purification process: the amount of aqueous washing was reduced by half, which improved the yield without sacrificing purity.

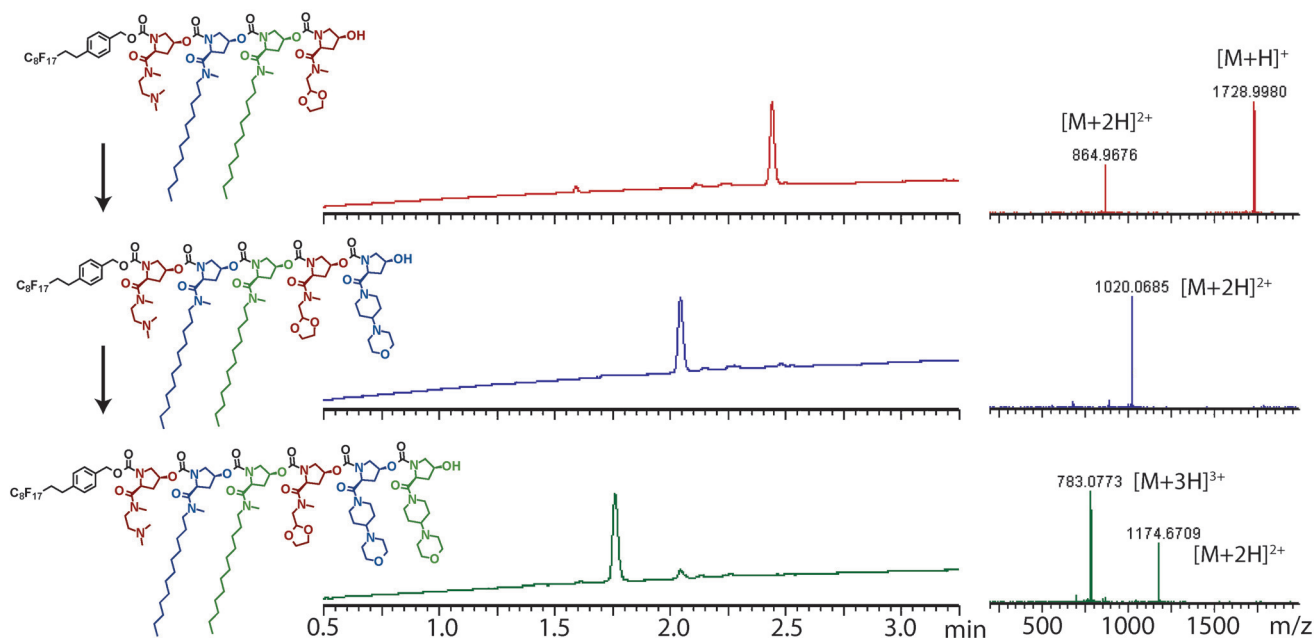


Figure 3. LCMS analysis of oligomer synthesis. LC traces of the tetramers, pentamers, and hexamers show UV absorbance at 214 nm. Mass spectra show ions contributing to the corresponding peak in the MS trace. Calculated masses: 1727.82 (tetramer), 2036.99 (pentamer), 2346.15 (hexamer). The purity of the hexamer is 91 %, with the major observed impurity being unreacted pentamer.

By using these methods, we were able to produce defined polymers with significant chemical diversity. Since the reaction and purification conditions require few steps and are functional with various structures, we believe that this synthetic approach may be appealing for parallel synthesis and screening applications. To evaluate the utility of this method for high-throughput parallel synthesis, we next synthesized and purified a library of 216 unique trimers entirely in a 96-well plate format. An automated liquid handler was programmed to combine solutions of starting monomer and CDI in a dry nitrogen environment. After CDI quenching, solvents were evaporated using a Genevac system. Plates were then returned to the liquid-handling robot, which was programmed to add solutions of the next monomer. Purification was carried out by fluorous solid-phase extraction on 96-well fluorous plates according to the manufacturer's instructions.

LCMS analysis of selected wells showed that oligomers were successfully synthesized in the high-throughput format (see the Supporting Information). Fluorous SPE in 96-well plates resulted in separation of the fluorous-tagged oligomers from excess reagents, since only fluorous-tagged products were detected in LCMS analysis of the purified samples (see the Supporting Information). The purity of the trimers synthesized in the high-throughput system was lower than it was for those synthesized individually (average purity = 92 %

for classical synthesis, 71 % for high-throughput synthesis, see the Supporting Information), with the major source of impurities being hydrolysis of the imidazole-*N*-carboxylic ester. This increase in hydrolysis may be due to the increased drying time required to evaporate the larger volume of solvent after CDI quenching (up to 3 h was required for this workup, compared to 1 h in pilot experiments). In prior experiments, we observed increased hydrolysis when the intermediate was handled for more than 2 hours, and we reason that reducing this workup time may improve the purity in high-throughput synthesis. This could be achieved by switching to a more volatile solvent, such as tetrahydrofuran, for the CDI activation steps of the synthesis. The oligomers were weighed after one additional reaction and purification step (addition of azido-PEG as discussed below), and the overall yield of recovered azide-modified trimers averaged 52 %, with a standard deviation of 11.5 % (see Supporting Information). The fluorous purification tag was removed from the library of azide-modified trimers by heating in trifluoroacetic acid (see the Supporting Information).

One potential application for such libraries of sequence-defined oligomers may be in nucleic acid delivery, where combinatorial library screens have played an important role in developing novel materials. We reasoned that hydroxyproline-based oligomers might enable the expansion of these combinatorial studies to include siRNA-conjugated delivery

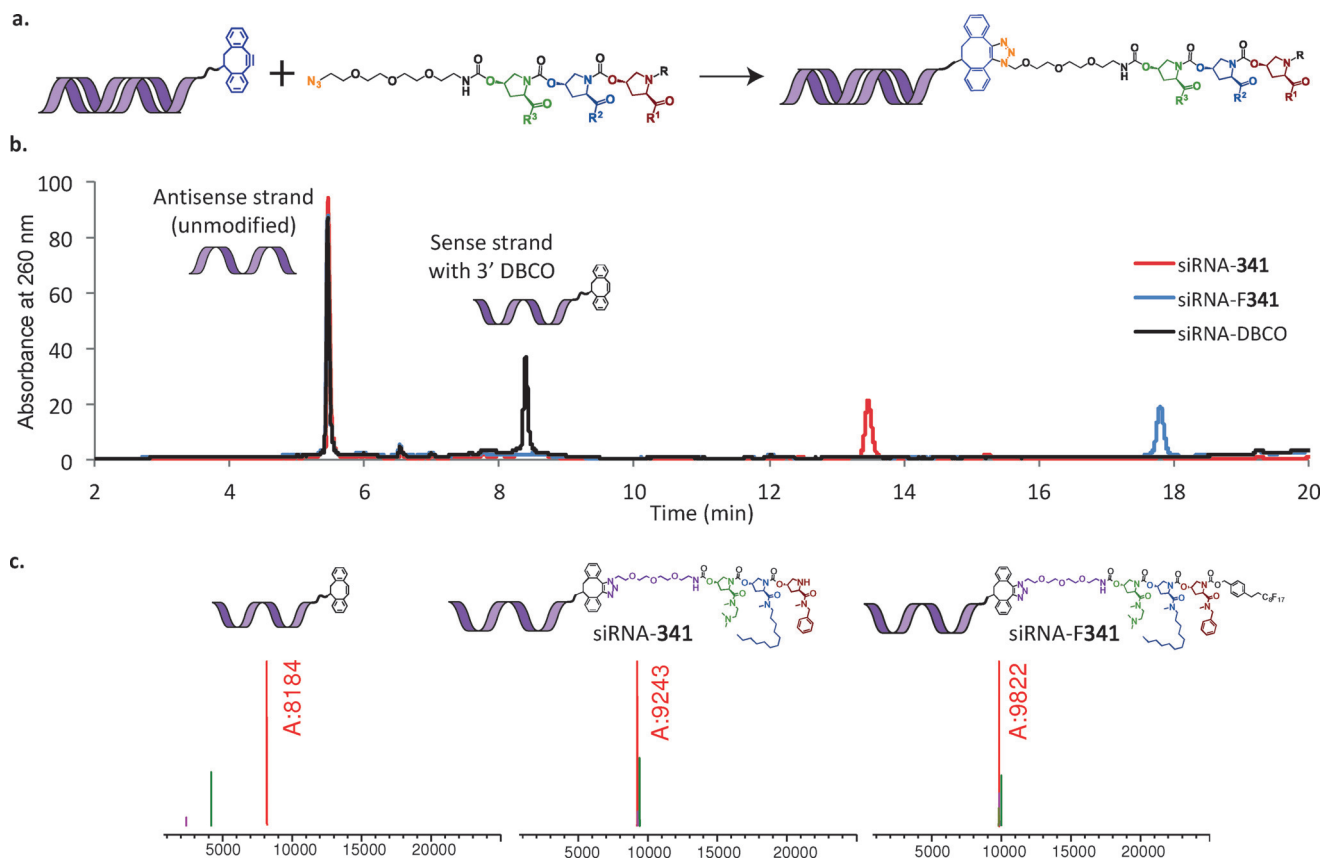


Figure 4. Conjugation of hydroxyproline-based oligomers to siRNA. a) Azide-modified trimers, with and without fluorous tags, were conjugated to dibenzocyclooctyne-modified siRNA and the resulting conjugates were purified by ethanol precipitation. b) LCMS analysis of siRNA conjugates: LC trace. siRNA-341 = DBCO-modified siRNA conjugated to trimer 341 (structure shown in Figure 4c). siRNA-F341 = siRNA conjugated to fluorous-protected trimer 341 (structure shown in Figure 4c). siRNA-DBC0 = unmodified siRNA. c) Observed masses corresponding to single-stranded siRNA conjugates shown. Calculated masses: 9243 (siRNA-341), 9823 (siRNA-F341).

materials. For this purpose, we next explored whether our materials could be conjugated to siRNA for library screening applications. By using the same CDI chemistry, we attached a short polyethylene glycol (PEG) chain with an azide functionality to a subset of five selected trimers (see the Supporting Information). After purification by FSPE, the fluororous protecting group was removed from each of the azide-modified trimers by heating in trifluoroacetic acid (see the Supporting Information).

The azide-modified trimers were conjugated to siRNA bearing a dibenzocyclooctyne (DBCO) group the 3' end the sense strand, which enables facile "click" coupling through 1,3-dipolar cycloaddition without the need for a copper catalyst.^[33] Both fluororous-protected trimers and deprotected trimers conjugated efficiently to the siRNA when using a solvent system of 30% acetonitrile in phosphate-buffered saline (PBS). Excess azide was removed from the mixture by ethanol precipitation of the nucleic acid (see the Supporting Information). Conjugation efficiency was analyzed by LCMS (Figure 4). Conjugation consistently proceeded essentially to completion, based on the shift in elution time in the LC trace. The average recovery of siRNA-conjugated material after ethanol precipitation was 95.5% (see the Supporting Information).

In conclusion, we have shown that sequence-controlled oligomers can be efficiently synthesized from hydroxyproline building blocks using only methods that are readily adaptable to parallel synthesis in multiwell plates. We demonstrate that these materials are able to incorporate a wide variety of monomer properties with precise control and excellent purity. We further demonstrate that these materials can be conjugated to biomacromolecules in a manner amenable to high-throughput screening applications. Our method for the synthesis of novel hydroxyproline-based materials affords precise control over polymer structure using simple chemistry and mild reaction conditions. These materials may benefit applications that require precise control over polymer structure, such as self-assembly and molecular recognition, as well as applications that require the exploration of a vast structural space, such as library screening. We anticipate that these materials may find utility in applications such as protein modification, the delivery of biologics, and other biomaterial applications.

Acknowledgements

This work was supported by the National Institutes of Health (grant EB000244), the Koch Institute Support (core grant P30-CA14051) from the National Cancer Institute, and Alnylam Pharmaceuticals. We thank Ramesh Indrakanti of Alnylam for his assistance in analysis of the siRNA conjugates.

Keywords: bioconjugates · fluororous synthesis · oligomers · sequence-defined polymers · siRNA

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 9529–9533

Angew. Chem. **2016**, *128*, 9681–9685

- [1] J. F. Lutz, M. Ouchi, D. R. Liu, M. Sawamoto, *Science* **2013**, *341*, 6146.
- [2] S. Pfeifer, J. F. Lutz, *J. Am. Chem. Soc.* **2007**, *129*, 9542.
- [3] S. Ida, T. Terashima, M. Ouchi, M. Sawamoto, *J. Am. Chem. Soc.* **2009**, *131*, 10808.
- [4] K. Satoh, M. Matsuda, K. Nagai, M. Kamigaito, *J. Am. Chem. Soc.* **2010**, *132*, 10003.
- [5] Y. Hibi, M. Ouchi, M. Sawamoto, *Angew. Chem. Int. Ed.* **2011**, *50*, 7434; *Angew. Chem.* **2011**, *123*, 7572.
- [6] D. Chan-Seng, M. Zamfir, J. F. Lutz, *Angew. Chem. Int. Ed.* **2012**, *51*, 12254; *Angew. Chem.* **2012**, *124*, 12420.
- [7] J. Niu, R. Hili, D. R. Liu, *Nat. Chem.* **2013**, *5*, 282.
- [8] R. N. Zuckermann, J. M. Kerr, S. B. H. Kent, W. H. Moos, *J. Am. Chem. Soc.* **1992**, *114*, 10646.
- [9] H. J. Olivos, P. G. Alluri, M. M. Reddy, D. Salony, T. Kodadek, *Org. Lett.* **2002**, *4*, 4057.
- [10] P. Espeel et al., *Angew. Chem. Int. Ed.* **2013**, *52*, 13261; *Angew. Chem.* **2013**, *125*, 13503.
- [11] S. C. Solleder, M. Meier, *Angew. Chem. Int. Ed.* **2014**, *53*, 711; *Angew. Chem.* **2014**, *126*, 729.
- [12] M. Porel, C. Alabi, *J. Am. Chem. Soc.* **2014**, *136*, 13162.
- [13] A. C. Boukis, A. Llevot, M. A. R. Meier, *Macromol. Rapid Commun.* **2016**, *37*, 643.
- [14] J. W. Grate, K. Mo, M. D. Daily, *Angew. Chem. Int. Ed.* **2016**, *55*, 3925–3930; *Angew. Chem.* **2016**, *128*, 3993–3998.
- [15] R. K. Roy et al., *Nat. Commun.* **2015**, *6*, 7237.
- [16] L. Hartmann, E. Krause, M. Antonietti, H. G. Börner, *Biomacromolecules* **2006**, *7*, 1239.
- [17] L. Hartmann, S. Häfele, R. Peschka-Süss, M. Antonietti, H. G. Börner, *Chem. Eur. J.* **2008**, *14*, 2025.
- [18] C. C. Tzschucke et al., *Angew. Chem. Int. Ed.* **2002**, *41*, 3964; *Angew. Chem.* **2002**, *114*, 4136.
- [19] S. Pfeifer, Z. Zarafshani, N. Badi, J. F. Lutz, *J. Am. Chem. Soc.* **2009**, *131*, 9195.
- [20] A. Akinc et al., *Nat. Biotechnol.* **2008**, *26*, 561.
- [21] K. Whitehead et al., *Nat. Commun.* **2014**, *5*, 4277.
- [22] M. S. Algahtani et al., *J. Controlled Release* **2014**, *190*, 115–126.
- [23] M. Gu et al., *Biomaterials* **2013**, *34*, 6133.
- [24] F. Yang, Y. Mei, R. Langer, D. G. Anderson, *Comb. Chem. High Throughput Screening* **2009**, *12*, 554.
- [25] A. L. Hook et al., *Adv. Mater.* **2013**, *25*, 2542.
- [26] P. Timmerman et al., *J. Biol. Chem.* **2009**, *284*, 34126.
- [27] W. Lian, P. Upadhyaya, C. Rhodes, Y. Liu, D. Pei, *J. Am. Chem. Soc.* **2013**, *135*, 11990.
- [28] D. Wang, Y. Li, Y. Zhang, Y. Liu, G. Shi, *Comb. Chem. High Throughput Screening* **2012**, *15*, 232.
- [29] R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat. Mater.* **2013**, *12*, 967.
- [30] M. Leal et al., *Ann. N. Y. Acad. Sci.* **2014**, *1321*, 41.
- [31] R. Duncan, M. J. Vicent, *Adv. Drug Delivery Rev.* **2013**, *65*, 60.
- [32] M. D'Hondt et al., *J. Pharm. Biomed. Anal.* **2014**, *101*, 2.
- [33] K. N. Jayaprakash et al. *Org. Lett.* **2010**, *12*, 10.

Received: March 18, 2016

Revised: May 4, 2016

Published online: July 1, 2016