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Scalable Synthetic Oligodeoxynucleotide Purification with Use of a Catching by Polymerization, Washing, and Releasing Approach

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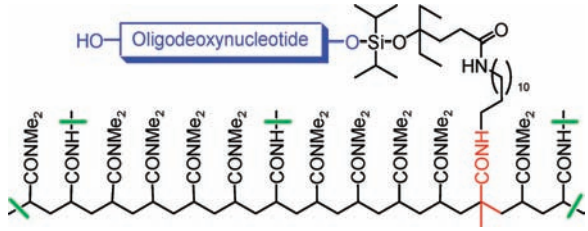
ABSTRACT



Synthetic oligodeoxynucleotides are purified with use of a catching by polymerization, washing, and releasing approach. The method does not require any chromatography, and purification is achieved by simple operations such as shaking, washing, and extraction. It is therefore useful for large-scale purification of synthetic oligonucleotide drugs. In addition to purification of oligonucleotides, this catching by polymerization concept is expected to be equally useful for purification of other synthetic oligomers such as peptides and oligosaccharides.

There is a high expectation on using synthetic oligonucleotides (ONs) as therapeutics to cure human diseases.[1] Currently, one such agent is used clinically and more are on various stages of clinical trials. In addition, numerous projects with the goal of developing ON drugs through selective gene silencing are in progress. As a result, large quantities (kilograms to metric tons) of pure synthetic ONs are needed for preclinical research, clinical trials, and patient use.[2] Due to the advancement of solid phase synthesis technology, large-scale ON synthesis has become possible. However, the crude ON contains various impurities, which must be removed for most applications. Current purification technologies include trityl-on reverse phase (RP) HPLC, anion-exchange HPLC, polyacrylamide gel electrophoresis (PAGE), and various types of affinity chromatography.[3] These methods are either unsuitable or highly expensive for large-scale purification, and ON purification has been considered as a bottleneck in antisense drug development.[2] In this communication, we report the use of an unprecedented concept—catching by polymerization—for the development of a simple

(1) Recent reviews: (a) Caruso, G.; Caffo, M.; Raudino, G.; Alafaci, C.; Salpietro, F. M.; Tomasello, F. *Recent Pat. CNS Drug Discovery.* **2010**, *5*, 53. (b) Hau, P.; Jachimczak, P.; Bogdahn, U. *Expert Rev. Anticancer Ther.* **2009**, *9*, 1663. (c) Chi, K. N.; Gleave, M. E. *Drug Future* **2009**, *34*, 525. (d) Richardt-Pargmann, D.; Vollmer, J. *Oligonucleotide Ther.* **2009**, *1175*, 40. (e) Murao, S.; Fujii, M. *Curr. Org. Chem.* **2009**, *13*, 1366. (f) Seguin, R. M.; Ferrari, N. *Expert Opin. Invest. Drugs* **2009**, *18*, 1505. (g) Akhtar, S. *J. Drug Targeting* **2009**, *17*, 491. (h) Zhao, X. B.; Pan, F.; Holt, C. M.; Lewis, A. L.; Lu, J. R. *Expert Opin. Drug Delivery* **2009**, *6*, 673.

(2) Schulte, M.; Luhring, N.; Keil, A.; Sanghvi, Y. S. *Org. Process Res. Dev.* **2005**, *9*, 212.

(3) Examples: (a) In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D. , Jones, R. A., Eds.; John Wiley & Sons: New York. (b) Fang, S.; Bergstrom, D. E. *Tetrahedron Lett.* **2004**, *45*, 7987. (c) Fang, S.; Bergstrom, D. E. *Nucleic Acids Res.* **2003**, *31*, 708. (d) Fang, S.; Bergstrom, D. E. *Bioconjugate Chem.* **2003**, *14*, 80. (e) Sproat, B. S.; Rupp, T.; Menhardt, N.; Keane, D.; Beijer, B. *Nucleic Acids Res.* **1999**, *27*, 1950. (f) Pearson, W. H.; Berry, D. A.; Stoy, P.; Jung, K. Y.; Sercel, A. D. *J. Org. Chem.* **2005**, *70*, 7114. (g) Beller, C.; Bannwarth, W. *Helv. Chim. Acta* **2005**, *88*, 171.

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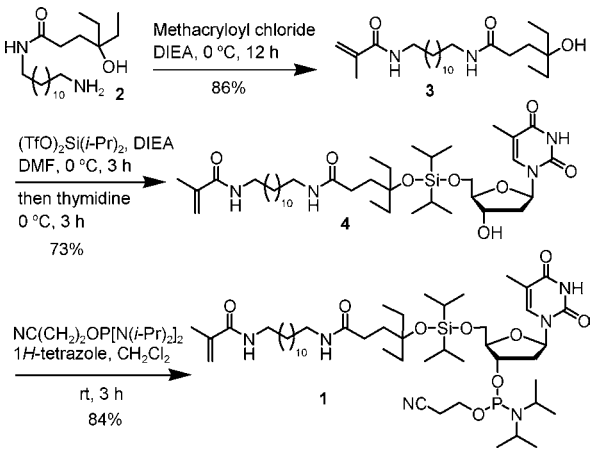
method for ON purification.[4] This method is easy to perform and uses inexpensive materials, and therefore is suitable for large-scale purification.

The major impurities in crude ON are the failure sequences generated in the coupling step in each synthetic cycle. For a typical 20-mer oligodeoxynucleotide (ODN) synthesis, the yield of full-length ODN is 40−70% depending on the scale of the synthesis, and 30−60% of ODNs are failure sequences.[3f] During automated synthesis, the failure sequences are capped with acetic anhydride in each synthetic cycle to prevent them from coupling with phosphoramidite monomers in the next synthetic cycles. We reasoned that if a phosphoramidite that contains a reversibly linked polymerizable function is used in the last synthetic cycle, it will only be coupled to the 5′-end of the full-length ODN. As a result, after synthesis, cleavage, and deprotection, all full-length ODN will contain a polymerizable group while the failure sequences will not. The full-length sequence can then be incorporated into a polymer through a simple copolymerization process, and failure sequences and other impurities can be removed by simple washing. Pure ODN can then be obtained by cleaving from the polymer. If successful, this purification method does not need any chromatography and expensive solid phase affinity extraction materials, and purification can be achieved by simple operations such as shaking, washing, and extraction.

The polymerization reaction we chose for this process was the radical acrylamide polymerization reaction. This reaction uses inexpensive materials, and is widely used by biologists and biochemists for PAGE. The required phosphoramidite **1** that contains the polymerizable function (the methacrylamide) and the cleavable linker (the diisopropyl silyl acetal) was synthesized using the route shown in Scheme 1. The

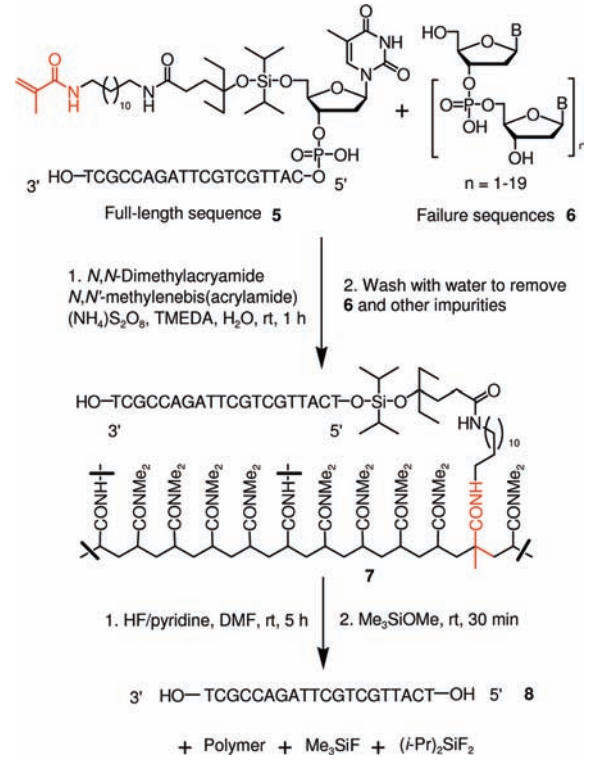
Scheme 1. Synthesis of Phosphoramidite **1**



amino tertiary alcohol **2** was prepared by using a known procedure we reported previously.[5] This compound was acrylated with methacryloyl chloride to give **3**, which was coupled with thymidine by diisopropylsilyl bis(trifluoromethane sulfonate) to give **4**. Phosphinylation of **4** with Beaucage's method provided phosphoramidite **1**.[3a]

To prove the purification concept, the 20-mer ODN **5** (see Scheme 2) was synthesized on a 1 μmol scale with standard

Scheme 2. Purification of ODN with Use of the Catching by Polymerization, Washing, and Releasing Approach



phosphoramidite chemistry on CPG on a synthesizer. To perform the cleavage and deprotection with ammonium hydroxide at room temperature, the UltraMild DNA synthesis conditions were used (see the Supporting Information).[6] In the last synthetic cycle, phosphoramidite **1** was used to incorporate the last nucleotide and the polymerizable methacrylamide group.

The ODN synthesis result was analyzed with RP HPLC (Figure 1, trace a). The full-length sequence **5** appeared at 57 min due to the hydrophobic group at its 5′-end. Most impurities have a retention time between 15 and 20 min. For polymerization, the crude ODN (~50 nmol) was dissolved in water. The polymerization monomer *N*,*N*-dimethylacrylamide and a small amount of cross-linking agent *N*,*N*′-methylenebis(acrylamide) were added. The polymerization was initiated by ammonium persulfate and TMEDA (Scheme 2). The reaction is not very sensitive to

(4) Fang, S. U.S. Patent pending US20080081902.
(5) Tang, W.; Fang, S. *Tetrahedron Lett.* **2008**, *49*, 6003.

(6) The diisopropylsilyl acetal linker can also survive normal ODN cleavage and deprotection conditions such as NH_4OH (29%)/H_2NMe (40%) (1:1), 65 °C, 30 min. When these conditions are used, the 2-cyanoethyl phosphoramidites benzoyl-dA, isobuyryl-dG, acetyl-dC, and dT can be used for the synthesis. See ref 3c.

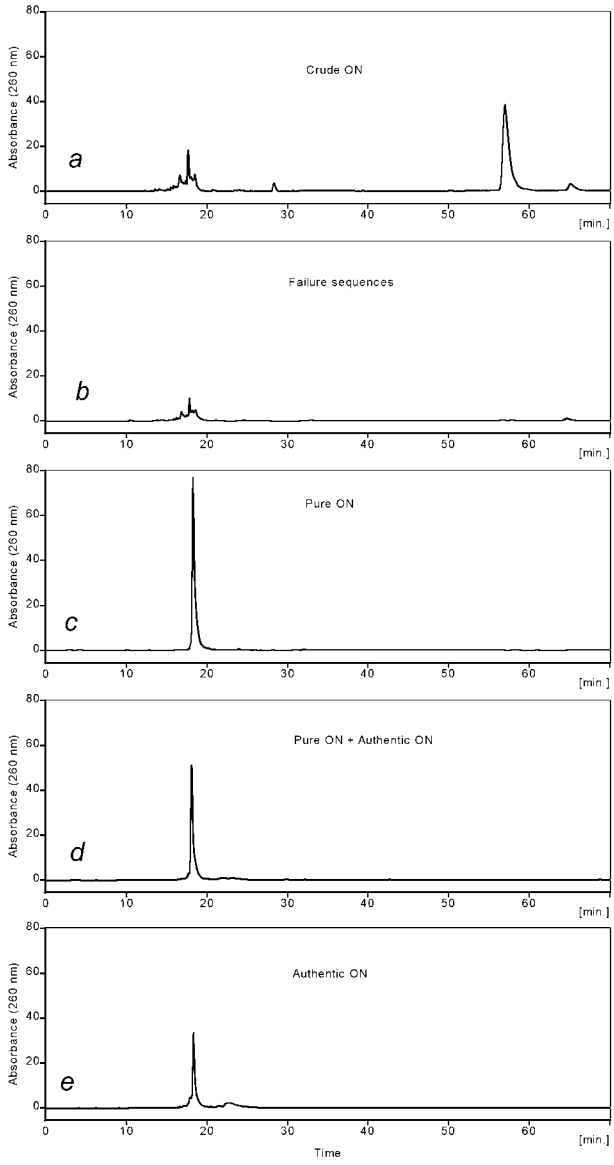


Figure 1. RP HPLC traces of ODNs: (a) crude ODN that contains full-length sequence **5**, failure sequences **6**, and other impurities; (b) failure sequences **6** and other impurities that were removed from the gel after catching the full-length sequence **5** by polymerization; (c) ODN **8** purified by catching by polymerization, washing, and releasing; (d) ODN **8** and authentic sample; and (e) authentic sample.

air but it was performed under a nitrogen atmosphere. The polyacrylamide gel **7** was formed within 30 min at room temperature. To ensure completion of the reaction, the gel was allowed to stand for another 30 min. At this stage, the full-length ODN **5** was incorporated into the polymer **7** while impurities including failure sequences **6** remained in solution. Water was added to the gel to extract the impurities. The extract was analyzed with RP HPLC. As shown in trace b, the full-length ODN **5** was completely incorporated into the gel. After drying the gel under vacuum thoroughly, the full-length unmodified ODN **8** was cleaved with HF-pyridine in DMF at room temperature. Excess HF was quenched with Me_3SiOMe (Scheme 2). The supernatant was removed, and the gel was washed with water. The supernatant and water were combined. Volatiles, which included Me_3SiF, $(i\text{-}Pr)_2SiF_2$, MeOH, pyridine, DMF, and water, were evaporated, and the ODN was analyzed with RP HPLC. As shown in trace c, failure sequences (**6**) and other impurities were completely removed, and ODN **8** was pure. Due to its lower lipophilicity than **5**, **8** has a retention time of 19 min. The recovery yield for the purification process was estimated to be 72% by comparing the area of the peak in trace c at 19 min with that in trace a at 57 min. This yield is higher than those typically obtained with trityl-on and fluorous phase purification methods. We anticipated that the yield of this new technology could be further increased when the process is used for larger scale ODN purification. By comparing traces a, b, and c, we can also conclude that the diisopropyl silyl acetal linker is stable during ODN synthesis, cleave, deprotection, acrylamide gel formation, and extraction of failure sequences.

The identity of ODN **8** was established by comparing with an authentic sample synthesized under standard conditions and purified with RP HPLC by a company (Figure 1). As shown in trace d, a coinjection of **8** purified by using our technology with an authentic sample gave a single peak. The HPLC profile for the authentic sample is shown in trace e. MALDI-TOF analysis of **8** also gave correct molecular weight. Because damaged ODNs resulting from nucleobase modifications under radical polymerization conditions may not be able to be detected by HPLC and MALDI-TOF, the four nucleosides, adenosine, thymidine, guanosine, and cytidine, were subjected to the polymerization conditions, and then recovered from the polyacrylamide gel by extraction. HPLC analysis showed that these compounds are completely stable under the polymerization conditions (see the Supporting Information).

This new ON purification technology has significant advantages over known ones. The most widely used method for ON purification is trityl-on RP HPLC. Compared with this method and other methods that use more hydrophobic tags than DMTr,[3e] our technique does not need any capital expenses including those for the highly expensive and consumable preparative columns. In addition, our technique only needs a minimum amount of organic solvents. The polymerization, removing failure sequences, and extraction of full-length ON are all carried out with water as the solvent. Furthermore, our method is expected to give better results for purification of ONs that are prone to adopt secondary structures and phosphorothioates that contain different diastereoisomers. These materials usually gave broad peaks in chromatography.[7] In the literature, ONs have also been purified with fluorous affinity chromatography[3f,g] and biotin−avidin enabled affinity extraction.[3b−d] Compared with these methods, our technique does not need any expensive affinity materials such as fluorous affinity column and avidin-coated beads.

(7) Sanghvi, Y. S.; Schulte, M. *Curr. Opin. Drug Discovery Dev.* **2004**, *7*, 765.

ON purification methods involving capping failure sequences with reagents that contain reactive groups such as a diene were also proposed.[8] Using these methods, failure sequences could be removed by extraction with dienophile functionalized microspheres through a Diels−Alder reaction. Compared with these methods, our technology does not need any reactive group functionalized microspheres, which could be highly expensive. In addition, due to the intrinsically lower reaction rate of heterogeneous reactions than homogeneous reactions, and the fact that only groups on the surface of solids can participate in reactions and the majority of groups within the solids cannot, to efficiently remove failure sequeces, the previously proposed methods require a large excess of solid phase extraction materials, which will prevent them from being used for large-scale ON purification. In contrast, our technology does not rely on any heterogeneous reactions for purification. In principle, the ratio of the polymerization monomer over ON can be kept to a minimum as long as the steric bulkness of ON does not significantly slow down the polymerization.

In conclusion, we have developed a new method for the purification of synthetic ON. This method is based on an unprecedented concept, which is catching by polymerization.[4] With use of this method, purification was achieved by simple operations such as shaking, washing, and extraction; and the method does not require any expensive equipment and materials. As a result, it is suitable for large-scale purification. Application of this new concept for the purification of other synthetic oligomers such as RNA,[3b] modified ON, peptides,[9] and oligosaccharides[10] is in progress.

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Supporting Information Available: Experimental details, images of NMR spectra of all new compounds and MALDI-TOF mass spectrum of ODN **8**, and images of original HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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