ORGANIC LETTERS

2007
Vol. 9, No. 11
2187−2190

Enzyme-Free Interrogation of RNA Sites via Primers and Oligonucleotides 3′-Linked to Gold Surfaces

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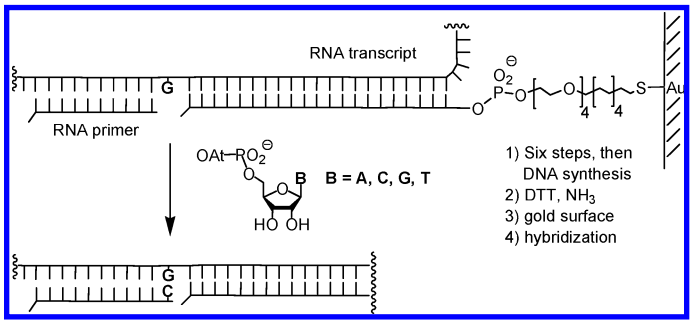
Received March 24, 2007

ABSTRACT



The synthesis of a phosphoramidite is described that was used for the preparation of oligonucleotides with a 3′-terminal thiol, linked to the DNA via a SAM-forming undecyl chain and a nonadsorptive tetraethylene glycol unit. A gold surface featuring oligonucleotide probes allowed for label-free in situ mass spectrometric determination of a nucleotide in subpicomole quantities of an RNA transcript.

Of the two polynucleotides, DNA and RNA, the latter is less well developed in terms of sequencing technology, amplification, and labeling. The range of biological roles is more diverse for RNA than DNA, though. Not only is RNA known to be important as genetic material, e.g., for retroviruses, it is also a key component of cellular machineries, a regulator of gene expression, and the material for ribozymes.[1] New functional RNA is being developed through in vitro selection (SELEX),[2,3] and modified RNA is emerging in therapeutics,[4] making novel techniques for the interrogation of RNA desirable.

We became interested in enzyme-free primer extension as an inexpensive method for determining nucleotides in RNA. Traditionally, enzyme-free extension of RNA has been slow and has led to product mixtures,[5] making it unsuitable for sequence analysis. New leaving groups for the activated monomers and "helper" oligonucleotides accelerate these reactions. Downstream-binding helpers also increase the yield and ensure formation of a single extension product.[6] But, the quantities required for such assays were too large for genotyping applications.

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(1) (a) Cech, T. R. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 759−768. (b) Altman, S. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 749−758.
(2) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−822.
(3) Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. *Annu. Rev. Biochem.* **1995**, *64*, 763−797.
(4) (a) Eaton, B. E.; Gold, L.; Hicke, B. J.; Janjic, N.; Jucker, F. M.; Sebesta, D. P.; Tarasow, T. M.; Willis, M. C.; Zichi, D. A. *Bioorg. Med. Chem.* **1997**, *5*, 1087−1096. (b) Soutschek, J.; Akinc, A.; Bramlage, B.; Charisse, K.; Constien, R.; Donoghue, M.; Elbashir, S.; Geick, A.; Hadwiger, P.; Harborth, J.; John, M.; Kesavan, V.; Lavine, G.; Pandey, R. K.; Racie, T.; Rajeev, K. G.; Rohl, I.; Toudjarska, I.; Wang, G.; Wuschko, S.; Bumcrot, D.; Koteliansky, V.; Limmer, S.; Manoharan, M.; Vornlocher, H. P. *Nature* **2004**, *432*, 173−178.
(5) Kozlov, I. A.; Orgel, L. E. *Mol. Biol.* **2000**, *34*, 781−789.
(6) Vogel, S. V.; Deck, C.; Richert, C. *Chem. Commun.* **2005**, 4922−4924.

10.1021/ol070724g CCC: $37.00
Published on Web 05/05/2007

Mass spectrometry as read-out does not require labeling and produces more specific signals than fluorescence. In situ mass spectrometric detection of short DNA strands hybridized to probe sequences on gold surfaces has recently been shown,[7] including nonenzymatically extended primers.[8] This work was limited to four-strand systems, short DNA templates, and required 3′-amino-2′,3′-dideoxynucleosides at the 3′-terminus of the primer (Figure 1a). We wished to make

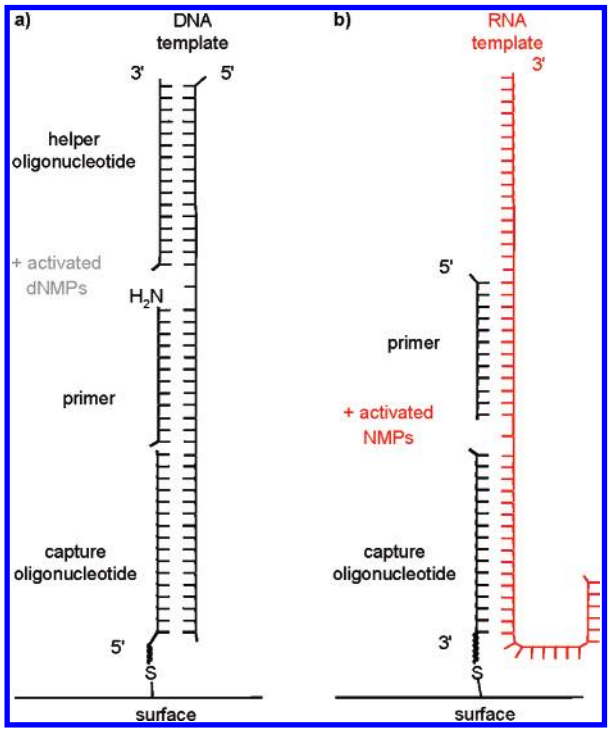


Figure 1. Components of chemical primer extension assays for determining nucleotides in template strands: (a) known system with a short DNA template and 4 strands and (b) three-strand system with a long RNA transcript as the template.

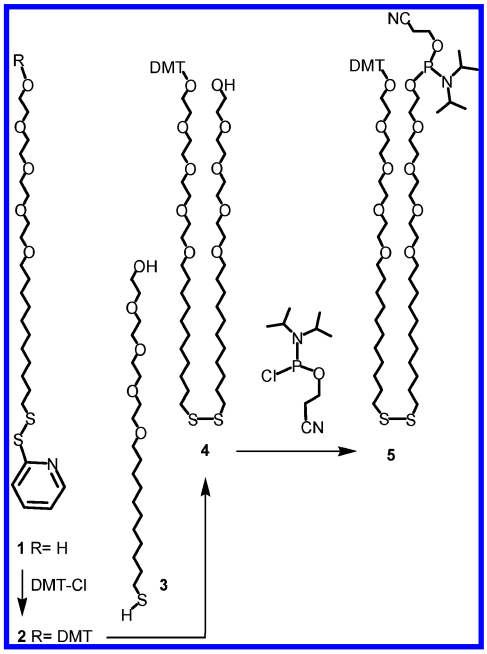
base calls in subpicomole quantities of RNA. RNA is less prone to fragment under MALDI conditions than DNA.[9] We designed a three-strand system with an RNA transcript as template, a primer, and a 3′-linked oligonucleotide that serves as a capture strand *and* as a helper (Figure 1b).

Planar surfaces allow for interrogating templates in arrayed format. Gold readily reacts with thiols or disulfides in organic or aqueous solutions, but few other functional groups.[10] Gold is also suitable as a target surface for MALDI-TOF MS, allowing for sensitive detection of noncovalently bound molecules.[11,7] Further, gold allows for surface plasmon resonance monitoring,[12] and may be obtained in the form of nanoparticles[13] that aggregate upon hybridization of DNA sequences.[14] Complexes between DNA and cationically modified gold clusters release the DNA in glutathione-dependent fashion,[15] and a release can also be induced photochemically.[16]

Unlike common bifunctional linkers for the immobilization of oligonucleotides that are attached to the 5′-terminus, our system required a 3′-appended linker. This linker cannot be readily introduced at the end of conventional DNA syntheses where the 3′-terminus is bonded to the solid support. To the best of our knowledge, no method existed for preparing DNA with a 3′-appended, thiol-terminated linker consisting of a long alkyl chain for formation of a self-assembled monolayer (SAM) and a distal oligoethyleneglycol portion reducing nonspecific adsorption. But, methods for other 3′-thiol-bearing DNAs have appeared in the literature, including one for the synthesis of oligonucleotides with a 3′-terminal thymidine residue featuring an alkylthiol moiety,[17] one for 3′-disulfide functionalized DNA with an amide linkage,[18] and one involving controlled pore glass (cpg) with a disulfide and a butyl spacer.[19] Disulfide-derivatized cpg with propyl or hexyl chains is used for the preparation of DNA attached to gold nanoparticles.[20]

The route to our reagent for the preparation of DNA with a 3′-appended thiol linker is shown in Scheme 1. The linker

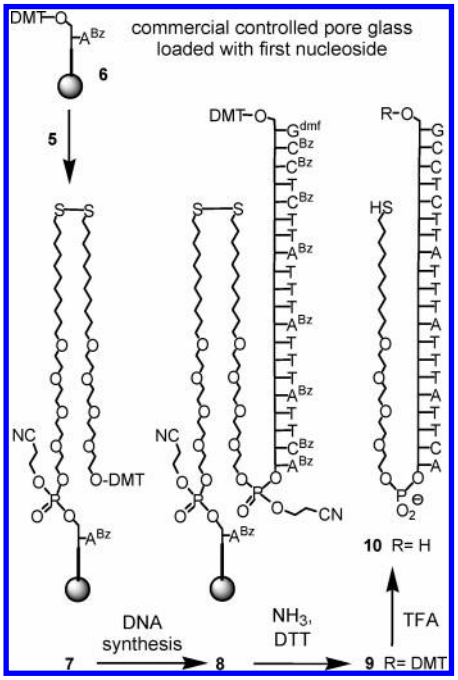
Scheme 1. Synthesis of Phosphoramidite Linker Reagent



is structurally related to those used by Whitesides,[21] Mrksich,[22] and others.[7] Starting material **1** was prepared from 1-bromoundec-10-ene in three steps.[21] Mixed disulfide **1** was

(7) Plutowski, U.; Richert, C. *Angew. Chem., Int. Ed.* **2005**, *44*, 621−625.
(8) Hagenbuch, P.; Kervio, E.; Hochgesand, A.; Plutowski, U.; Richert, C. *Angew. Chem., Int. Ed.* **2005**, *44*, 6588−6592.
(9) Nordhoff, E.; Cramer, R.; Karas, M.; Hillenkamp, F.; Kirpekar, F.; Kristiansen, K.; Roepstorff, P. *Nucleic Acid Res.* **1993**, *21*, 3347−3357.
(10) Whitesides, G. M.; Kriebel, J. K.; Love, J. C. *Sci. Prog.* **2005**, *88*, 17−48.
(11) Su, J.; Mrksich, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4715−4718.
(12) Persson, B.; Stenhag, K.; Nilsson, P.; Larsson, A.; Uhlen, M.; Nygren, P.-A. *Anal. Biochem.* **1997**, *246*, 34−44.
(13) Brust, M.; Walker, M.; Bethell, D.; Schiffrin, D. J.; Whyman, R. J. *J. Chem. Soc., Chem. Commun.* **1994**, 801−802.
(14) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547−1562.

Scheme 2. Synthesis of DNA with the 3′-Attached Linker

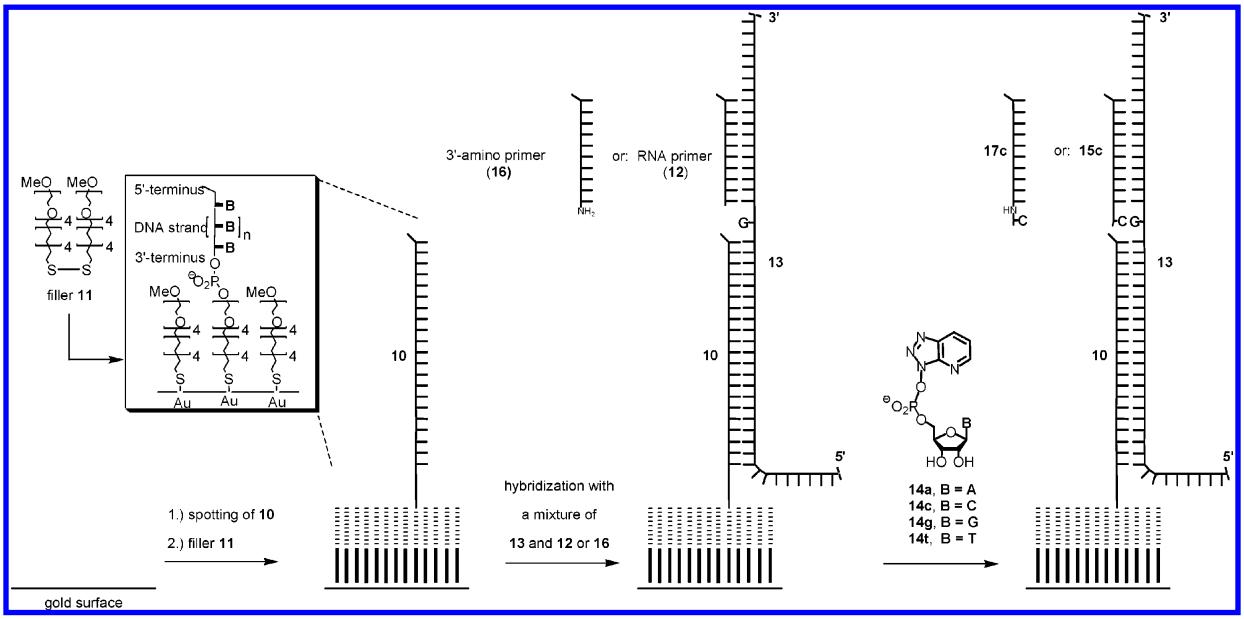


O-protected with the dimethoxytrityl group (DMT) commonly used during DNA chain assembly to give **2** in 78% yield (see the Supporting Information for protocols). The subsequent thiopyridone-releasing disulfide exchange with thiol **3** gave **4** in 88% yield in slow reactions, even under basic conditions. Phosphitylation of **4** gave phosphoramidite **5** in 77% yield. This, like its precursors, was purified by chromatography on silica. Attempts to isolate it by precipitation, a common purification strategy for nucleosidic phosphoramidites, remained unsuccessful.

The synthesis of linker-bearing oligonucleotides (Scheme 2) started from cpg loaded with a nucleoside (**6**), i.e., an inexpensive commercial support for DNA synthesis. A coupling cycle with phosphoramidite **5** yielded **7**, which was subjected to conventional DNA chain assembly to give protected oligonucleotide **8**. Extensive capping of residual unreacted hydroxy groups after the initial coupling step involving **5** prevented the formation of linker-free DNA chains that complicate purification of the final product. Treatment of **8** with aqueous ammonia containing 1,4-D,L-dithiothreitol removed the nucleobase and backbone protection groups, and cleaved the disulfide bridge to give **9**, whose 5′-dimethoxytrityl group facilitated purification. A cartridge step with on-support detritylation gave target oligonucleotide **10** in high purity.

Gold-coated substrates were smoothed in an H_2 flame, and solutions of **10** were applied to individual spots, followed by coating the remaining free surface by treatment with disulfide reagent **11**[7] (Scheme 3). A mixture of RNA primer 5′-CCACAACCCA-3′ (**12**, 800 fmol) and 376 nucleotide-long RNA **13** (100−500 fmol) was then hybridized to the DNA-displaying spots. Template **13** is a partial transcript of the gene encoding transcription factor cdx4 in zebrafish (*Danio rerio*). After a brief wash with NH_4OAc buffer, a solution of the oxyazabenzotriazolides of the four ribonucleoside-5′-monophosphates (A, C, G, and T; **14a**−**t**, 6 mM each)[23] in reaction buffer was added to each spot. Primer extension was allowed to occur at room temperature in a humid chamber with application of fresh monomers after 10 and 23 h. After 27 h, MALDI matrix was applied, and the extended primer was detected spectrometrically (Figure 2). Near-quantitative primer extension with good base selectivity for G as templating base was seen down to 100 fmol of the template (Table 1). Other bases are known to template selectively.[6] Reactions with the same experimental setup, but

Scheme 3. Generating Arrays of Capture Oligonucleotides on SAM-Coated Gold Surfaces and Primer Extension Templated by RNA



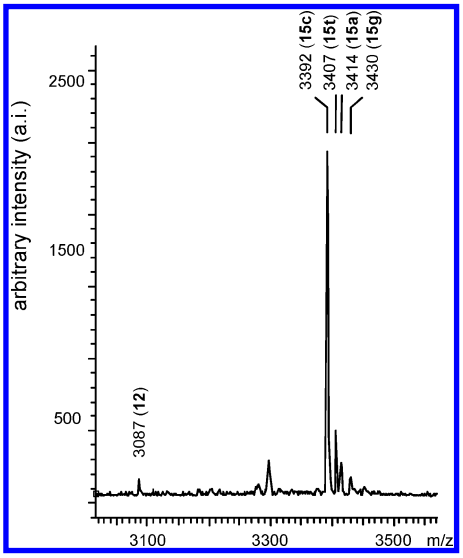


Figure 2. MALDI mass spectrum of surface-bound RNA primer **12** extended to **15**, in an enzyme-free reaction templated by RNA transcript **13** on a gold slide with **10**; 500 fmol of template and 800 fmol primer were hybridized to the surface.

DNA-primer **16** with a 3′-terminal 3′-amino-2′,3′-dideoxyadenosine residue and the activated ribonucleotides occurred faster, with full primer conversion in good base fidelity in <4 h.

In conclusion, we report gold surfaces coated with self-assembled monolayers displaying 3′-linked oligonucleotides suitable for direct genotyping of individual nucleotides mass spectrometrically in RNA transcripts. An RNA transcript with several hundred nucleotides was shown not to interfere with MALDI-TOF MS. Our method requires only 3 strands and has high sensitivity, which may be improved further by miniaturization. The methodology may become useful for genotyping tumors or viruses[24] mass spectrometrically,[25] particularly if RNA used for quantitative expression analysis or identification of viruses via microarrays[26] is simultaneously genotyped, providing key information on geno- and haplotypes. Approximately 80% of all known virus are retroviruses, such as HIV, Ebola virus, influenza virus, including the H5N1 strain, arbovirus-B, and the hepatitis viruses. Cancer diagnosis will benefit from expression analysis of oncogenes efficiently combined with SNPs detection.[27]

Table 1. Results from Primer Extension with **12** and **16**

primer	quantity of template (fmol)	sequence selectivity[a] +C/+T/+A/+G
RNA (**12**)	100	>3:1:1:1[b]
RNA (**12**)	500	76:13:7:4[b]
amino-DNA (**16**)	500	>99:1:1:1[c]

[a] Product distribution for extended primer. [b] After 27 h. [c] After 4 h.

Acknowledgment. The authors thank Dr. E. Kervio (U. Karlsruhe) for helpful discussions. This work was supported by DFG (grant No. 1063/1-4 and FOR 434).

Supporting Information Available: Syntheses, template sequence, protocols for gold substrate and its use, and NMR and MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Han, G.; Chari, N. S.; Verma, A.; Hong, R.; Martin, C. T.; Rotello, V. M. *Bioconj. Chem.* **2005**, *16*, 1356−1359.
(16) Han, G.; You, C.-C.; Kim, B.; Turingan, R. S.; Forbes, N. S.; Martin, C. T.; Rotello, V. M. *Angew. Chem., Int. Ed.* **2006**, *45*, 3165−3169.
(17) Zuckermann, R.; Corey, D.; Schultz, P. *Nucleic Acid Res.* **1987**, *15*, 5305−5321.
(18) Gupta, G. C.; Sharma, P.; Sathyanarayana, S.; Kumar, P. *Tetrahedron Lett.* **1990**, *31*, 2471−2474.
(19) Bonfils, E.; Thuong, N. T. *Tetrahedron Lett.* **1991**, *32*, 3053−3056.
(20) Storhoff, J. J.; Elghanian, R.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1998**, *120*, 1959−1964.
(21) Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 12−20.
(22) Lee, K.-B.; Park, S.-J.; Mirkin, C. A.; Smith, J. C.; Mrksich, M. *Science* **2002**, *295*, 1702−1705.
(23) The active ester of ribothymidine 5′-monophosphate was used, rather than that of uridine-5′-monophosphate, because its mass is not as similar to that of cytidine 5′-monophosphate.
(24) Bartholomeusz, A.; Schaefer, S. *Rev. Med. Virol.* **2004**, *14*, 3−16.
(25) Stanssens, P.; Zabeau, M.; Meersseman, G.; Remes, G.; Gansemans, Y.; Storm, N.; Hartmer, R.; Honisch, C.; Rodi, C. P.; Böcker, S.; van den Boom, D. *Genome Res.* **2004**, *14*, 126−133.
(26) Lockhart, D. J.; Dong, H.; Byrne, M. C.; Follettie, M. T.; Gallo, M. V.; Chee, M. S.; Mittmann, M.; Wang, C.; Kobayashi, M.; Horton, H.; Brown, E. L. *Nat. Biotechnol.* **1996**, *14*, 1675−1680.
(27) Walker, B. A.; Leone, P. E.; Jenner, M. W.; Li, C.; Gonzalez, D.; Johnson, D. C.; Ross, F. M.; Davies, F. E.; Morgan, G. J. *Blood* **2006**, *108*, 1733−1743.